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Regulation of *Caenorhabditis elegans* Male Mate Searching Behavior by the Nuclear Receptor DAF-12

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ABSTRACT

Coordination of animal behavior with reproductive status is often achieved through elaboration of hormones by the gonad. In the nematode *Caenorhabditis elegans*, adult males explore their environment to locate mates. Mate searching is regulated by presence of mates, nutritional status, and a signal from the gonad. Here we show that the gonadal signal acts via the nuclear receptor DAF-12, a protein known to regulate several *C. elegans* life-history traits. DAF-12 has both activational and organizational functions to stimulate exploratory behavior and acts downstream of the gonadal signal, outside of the gonad. DAF-12 acts upstream of sensory input from mating partners and physiological signals indicating nutritional status. Mate searching was rescued in germ-line ablated animals, but not if both germ line and somatic gonad were ablated, by a precursor of the DAF-12 ligand, dafachronic acid (DA). The results are interpreted to suggest that the germ line produces a DA precursor that is converted to DA outside of the germ line, possibly in the somatic gonad. As it does in other pathways in which it functions, in regulation of male mate searching behavior DAF-12 acts at a choice point between alternatives favoring reproduction (mate searching) *vs.* survival (remaining on food).

TRANSCRIPTION factors of the nuclear receptor (NR) family serve to coordinate biological pathways in diverse tissues through their responses to lipophilic ligands that circulate through the body. Their roles encompass multiple aspects of organismal biology, including physiology, growth, differentiation, metabolism, and behavior (Mangelsdorf *et al.* 1995). They are widely expressed in the mammalian brain, but their role in development or function of the nervous system is not well understood (Gofflot *et al.* 2007). Through elaboration of hormones by the gonad, NRs play an important role in coordinating reproductive behavior with sexual phenotype, both by promoting development of sex-specific neuronal structures and circuits (organizational effects) and by activating expression of behavioral pathways in the mature nervous system (activational effects) (Meisel and Sachs 1994; Pfaus 1999; Nef and Parada 2000; Baum 2002; Carter 2002; Morris *et al.* 2004).

Caenorhabditis elegans, with its small nervous system composed of identifiable neurons, provides a powerful model system in which to study regulation of animal behavior, including sexual behavior (White *et al.* 1986; Chalfie and White 1988; De Bono and Maricq 2005). In *C. elegans*, male-specific sexual behavior consists of copulation and exploratory behavior (Lipton *et al.* 2004; Barr and Garcia 2006; Emmons 2006). Exploratory behavior serves to bring males into the vicinity of hermaphrodite mates, where they respond to short-range secreted cues (Simon and Sternberg 2002; Chasnov *et al.* 2007; White *et al.* 2007). During copulation, the male slides its tail along the hermaphrodite body to find the vulva, inserts its spicules, and transfers sperm. In support of these sex-specific behaviors, the male nervous system contains a complement of 89 male-specific neurons, along with 294 neurons that make up the core nervous system shared with the hermaphrodite (Sulston *et al.* 1980; Portman 2007).

C. elegans male-specific exploratory behavior has been studied as a model of a motivated sexual behavior (Lipton *et al.* 2004). If mating partners are not present, males explore their environment and will leave a source of food to do so (Lipton *et al.* 2004). Once mating partners are located on a food source, males remain there. Since exploratory behavior ceases when a mating partner on food is located, their exploratory behavior causes males to accumulate with hermaphrodites on food and therefore functions as a mate-searching strategy.

We have exploited the *C. elegans* male's exploratory behavior to study regulation of a male-specific reproductive behavior. Immature males remain on food while mature males lacking germ cells have a diminished tendency for exploration, showing that exploratory

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behavior is regulated both by developmental stage and by presence of the germ line (Lipton *et al.* 2004). In view of the role in other animals of hormones in coordinating sexual behavior with maturity and gonadal status, we wondered whether control of behavior by the gonad in *C. elegans* also involved regulation by a hormone. As the NR gene *daf-12* had previously been shown to mediate gonadal effects in regulation of aging (Hsin and Kenyon 1999), the DAF-12 NR was a candidate for a steroid receptor to mediate a hormonal effect of the gonad on mate-searching behavior.

daf-12, which encodes a NR of the vitamin D family, is ubiquitously expressed in *C. elegans* tissues and has effects on diverse life-history traits, including choice of a reproductive *vs.* a survival (dauer diapause) pathway during larval development, developmental timing of cell lineages, and aging (Hsin and Kenyon 1999; Antebi *et al.* 2000; Broue *et al.* 2007; Gerisch *et al.* 2007). Two related steroid derivatives termed dafachronic acids (DA) act as DAF-12 ligands (Motola *et al.* 2006). A cytochrome P450, product of the *daf-9* gene, is proposed to catalyze final oxidative steps of the DA biosynthetic pathway, while a Rieske-like oxygenase, product of the *daf-36* gene, acts on cholesterol at an early biosynthetic step (Motola *et al.* 2006; Rottiers *et al.* 2006).

Like other NRs, DAF-12 acts as a switch in conjunction with a corepressor protein, DIN-1. In the absence of ligand binding, a DAF-12/DIN-1 complex promotes life-history traits associated with enhanced survival, including longevity, diapause, and fat storage. In the presence of ligand, DIN-1 is displaced and DAF-12 with bound ligand promotes reproductive development and a normal life span (Ludewig *et al.* 2004; Gerisch *et al.* 2007).

Here we show that the *daf-12* pathway has both organizational and activational effects on the *C. elegans* male nervous system and regulates male-specific exploratory behavior. As in the other pathways in which it acts, the liganded form of DAF-12 acts both during development and in adulthood to promote a behavior that favors reproduction—male exploration for mates—while the unliganded form, in conjunction with DIN-1, promotes an alternative strategy—remaining on food in the absence of mates. We show that the *daf-12* pathway acts downstream of the gonad to regulate behavior and provide evidence that the germ line may be one source of a DA precursor.

MATERIALS AND METHODS

The leaving assay: We quantitatively measure male exploratory behavior by a behavioral assay termed the *leaving assay*, which exploits the male's tendency to leave a food source lacking mating partners (Lipton *et al.* 2004). In the leaving assay, the fraction of animals (of 20 in a typical assay) that have not yet traveled a certain distance (3 cm) away from a food source (a lawn of *Escherichia coli*) is determined at various time points. From these data the population average rate of leaving is determined. We have shown previously that the probability of leaving food per hour, P_L, is a constant, characteristic of a particular genotype under given conditions (Lipton *et al.* 2004). In the leaving assay under the conditions used here, wild-type adult males leave a food patch with a probability $P_L = 0.097 \pm 0.002$ (SEM) (Figure 1A; Table 1). For hermaphrodites and juvenile males, P_L is essentially 0. In the context of this assay, male exploratory behavior is sometimes referred to as leaving behavior.

Statistical analysis of leaving assays: Male leaving behavior was modeled with the exponential model $N(t)/N(0) = \exp(-\lambda t)$. $N(0)$ is the number of worms at time zero, and $N(t)$ is the number of worms at time t (in hours). λ is the P_L value or the probability that each individual will become a "leaver" per hour. To estimate the parameters of the exponential model, including mean P_L value, SEM, and the 95% confidence intervals, data were pooled across experiments and right censored after 24 hr. The R survival package (http://cran.r-project.org/web/packages/survival/index.html) was then used to fit the censored data to an exponential parametric survival model, using maximum likelihood. A constant hazard rate (λ) was estimated using the data and used as the P_L value. P_L values are represented as straight lines through the survival curves in the figures shown.

Strains: Strains used include the following: CB4088 *him-5(e1490)V*; SS149 *mes-1(bn7)X*; EM318 *unc-51(e369)him-5(e1490)V*; EM408 *daf-12(m20)X; him-5(e1490)V*; EM918 *daf-12(rh193)X; bxIs14, him-5(e1490)V*; EM920 *daf-12(m25)X; bxIs14, him-5(e1490)V*; EM923 *daf-12(m421)X; bxIs14, him-5(e1490)V*; EM924 *daf-12(m420)X; him-5(e1490)V*; EM926 *daf-12(m422)X; bxIs14, him-5(e1490)V*; EM927 *daf-12(rh61)X; him-5(e1490)V*; EM930 *daf-9(m540)X; bxIs14, him-5(e1490)V*; EM932 *mgEx661; dpy-7(sc27), daf-9(e1406)X; him-5(e1490)V*; EM933 *daf-12(rh84)X; bxIs14, him-5(e1490)V*; EM934 *daf-12(rh257)X; bxIs14, him-5(e1490)V*; EM935 *daf-12(rh285)X; bxIs14, him-5(e1490)V*; EM936 *daf-12(m583)X; him-5(e1490)V; him-5(e1490)V*; EM956 *daf-9(e1406), daf-12(m20)X; him-5(e1490)V*; EM964 Ex[*sdf-9p::gfp*]; *him-5(e1490)V*; EM1038 *daf-12(m419)X; him-5V*; AA317 *daf-12(dh115, rh61)X*; and DR2207 *daf-9(e1406), daf-12(m20)X*. Worm strains *daf-12(dh115, rh61)X* from A. Antebi, pKOG9 *[sdf-9p::gfp]* from I. Katsura, and DR2207 *daf-9(e1406), daf-12(m20)X* from D. Riddle were generously provided by these individuals. The remaining strains were ordered from the Caenorhabditis Genetics Center or constructed in this laboratory.

The *daf-9*::GFP strain (EM932) was derived by crossing *him-5(e1490)* into mgEx661 [genotype *[daf-9p::daf-9::GFP] daf-9(e1406), dpy-7(sc27)X*] (Mak and Ruvkun 2004).

Strain construction and worm culture: In most cases *him-5* was added using standard crossing methods. However, many of the *daf-12* class 3 alleles do not have a visible phenotype. So we made *daf-12; him-5* doubles with class 3 alleles by taking advantage of the fact that *daf-12* is on the X chromosome. Males are X/O while hermaphrodites are X/X. *him-5(e1490)V* males were crossed with *daf-12* hermaphrodites. The resulting hemizygous (X/O) males were then crossed back to the parent strain to produce homozygous *daf-12* hermaphrodites. The presence of *daf-12* was verified by testing their inability to form dauers on crowded starved plates at 25°. A number of the lines used in this article carried the integrated transgenic construct *bxIs14*, which is a *pkd-2::GFP* fusion construct integrated into chromosome V (Jia and Emmons 2006), which allowed both ray axon guidance and leaving behavior to be scored. Neither *bxIs14* (L. Jia, personal communication) nor *him-5* (J. Lipton, personal communication) affects male leaving behavior.

Worms were cultured according to standard *C. elegans* propagation methods (Brenner 1974; Sulston and Hodgkin 1988), on nematode growth media (NGM) agar and the OP50 strain of *E. coli* as a food source. Most strains contained the *him-*

5(e1490) mutation, which spontaneously produces a high percentage of males (Sulston and Hodgkin 1988). Wild type refers to *him-5(e1490)* mutants (on the N2 background) unless mentioned otherwise. In the cases of *daf-12(rh61, dh115)X* and *mes-1(bn7)X*, an alternative method was used to generate males. Since the gene of interest was on the X chromosome, hemizygous (XO) mutant males were generated and tested after the first-generation cross with wild-type *him-5* fathers and homozygous mutant mothers.

Starvation assays: Starvation assays were performed as before (Lipton *et al.* 2004). Briefly, newly matured adult males were washed three times in M9, placed on a clean agar plate (no bacteria), and allowed to starve for 12 hr prior to the initiation of the leaving assay. Because males often left the plate and died on the walls while being starved, sterile Sephadex beads in M9 were pipetted onto the center of the plate. Male worms were often found swimming over and around the beads and the presence of the beads decreases the number of males that died on the wall of the starvation plate.

Male retention by hermaphrodites: The retention assays were performed as in Lipton *et al.* (2004). Briefly, newly matured adult males were placed on a leaving assay plate with four mature paralyzed (*unc-51, him-5V*) hermaphrodites and the leaving assay was performed as usual.

Ablations: Gonad and germ-line ablations were performed using genetic methods and laser ablation. For the genetic approach, *mes-1(bn7)X* was used since it has a variable defect in the germ line such that animals with and without germ lines grow and can be seen among siblings. The penetrance of the defect varies according to rearing temperature (Capowski *et al.* 1991); we reared animals at 20°, producing ~50% defective males. When viewed under the compound microscope (Zeiss Axioimager A1, AX10) the germ lines of *mes-1(bn7)X* males were either full of sperm or empty. At the conclusion of leaving assays worms were anesthetized (25 μm levamisole) and mounted on 5% agarose pads and classified as germ-line defective or germ-line containing.

Germ-line and gonad laser ablations were performed using the method of Bargmann and Horvitz (1991). Briefly, in the larval stage 1 (L1) the worm gonad consists of the four-cell gonad primordium. Whole gonad ablation was conducted by killing all four cells (Z1–Z4) of the gonad precursor, and selective germ-line ablation was conducted by killing only the two germ-line precursors (Z2 and Z3). Mock animals were mounted and anesthetized (10 μm levamisole) alongside ablated animals. Successful germ-line and gonad ablation was confirmed after leaving assays were complete by mounting and viewing the gonad under the compound microscope (Zeiss Axioimager A1, AX10).

XXX cell ablations were performed using the same method, except a fluorescent reporter was used to recognize XXX cells and verify their loss at the conclusion of experiments. To track XXX cells we used a *him-5(e1490)* line containing a plasmid with *sdf-9p* (3.5 kb) fused to GFP (a generous gift of Isao Katsura). This plasmid drives GFP expression in XXX cells (Ohkura *et al.* 2003).

Steroid rescue experiments: Worms were treated with 250 nm Δ^4-dafachronic acid (3-keto-4-cholestenoic 25S-26 acid, the generous gift of D. Mangelsdorf), 25 μm of dafachronic acid precursor (4-cholesten-3-one, steraloids reference no. 135620, catalog no. C6250-000; http://www.steraloids.com), or solvent (ethanol) without hormone. Final steroid concentrations were calculated with respect to the volume of agar on which it was plated (Motola *et al.* 2006). The appropriate steroid/ethanol solution was then spread on an agar plate together with a 5× concentrate of an overnight bacterial culture (*E. coli* OP50) at a ratio of 9:1 (v/v) bacteria to steroid. The lid of the plate was left ajar and solutions were allowed to soak into the culture plate for at least 2 hr prior to plating the worms. For exposure during development and adulthood, hermaphrodite worms were allowed to lay eggs on steroid or control plates and the progeny were reared from egg to L4 on the treatment plate. The L4 progeny were then separated to fresh steroid or control plates and tested in a leaving assay after 12 hr exposure to the steroid as adults. For adult exposure, worms were transferred to steroid or control plates on the day that they matured for 12 hr and then tested for mate searching behavior. During the assay steroids were not present. To test the ability of steroid to rescue the behavior of laser ablated animals, each worm was tested twice, first with no steroid exposure then with steroid exposure. To reduce the difference in age of worms between the first and the second experiment, leaving assays were run for 12 hr instead of 24 and worms were treated with steroids in the 12 hr between the two experiments.

RESULTS

The DAF-12 NR pathway regulates male exploratory behavior: To examine the role of the *daf-12* pathway in the regulation of male exploratory behavior, we tested the effect of altering known components of this pathway. First, we examined exploratory behavior in males homozygous for the mutation *daf-12(m583)*, which encodes a severely truncated form of the DAF-12 protein. We measured exploratory behavior with the *leaving assay*, which determines the rate at which isolated males leave a source of food (materials and methods). *daf-12(m583)* males left food significantly slower than wild-type males [wild type: *him-5(e1490)*] (Figure 1A; Table 1). Therefore wild-type *daf-12* is required for normal male leaving behavior. *daf-12(m583)* hermaphrodites remained on food similar to wild type. *daf-12(m583)* males were able to copulate and produce progeny (data not shown).

Four DAF-12 protein isoforms have been reported (Antebi *et al.* 2000; Snow and Larsen 2000). Two (A1 and A3) encompass all 17 DAF-12 exons and differ by 16 amino acids in exon 12. These two isoforms are truncated within exon 3 before the Zn-finger DNA-binding domain (DBD) by the *daf-12(m583)* mutation and are unlikely to have activity in this mutant. A third reported isoform (A2), which includes all of the DAF-12 protein sequence, including the DBD, Hinge, and the ligand-binding domain (LBD), but excluding the N-terminal hypervariable region, begins within exon 3 and is unaffected by the *m583* mutation (Snow and Larsen 2000). Since *daf-12(m583)* is dauer defective as well as male leaving defective, the A2 isoform alone apparently cannot supply *daf-12* activity for either of these phenotypes. A fourth isoform (B) begins with exon 13 and includes only the LBD; it is of unknown function. Considering the mutations used in this work, *m583*, *m422*, and *m20* affect A isoforms only, while *m419*, *rh61dh115*, *rh61*, *rh84*, and *rh285* affect both A and B isoforms.

To determine whether it was the liganded or the unliganded form of DAF-12 that stimulated exploratory

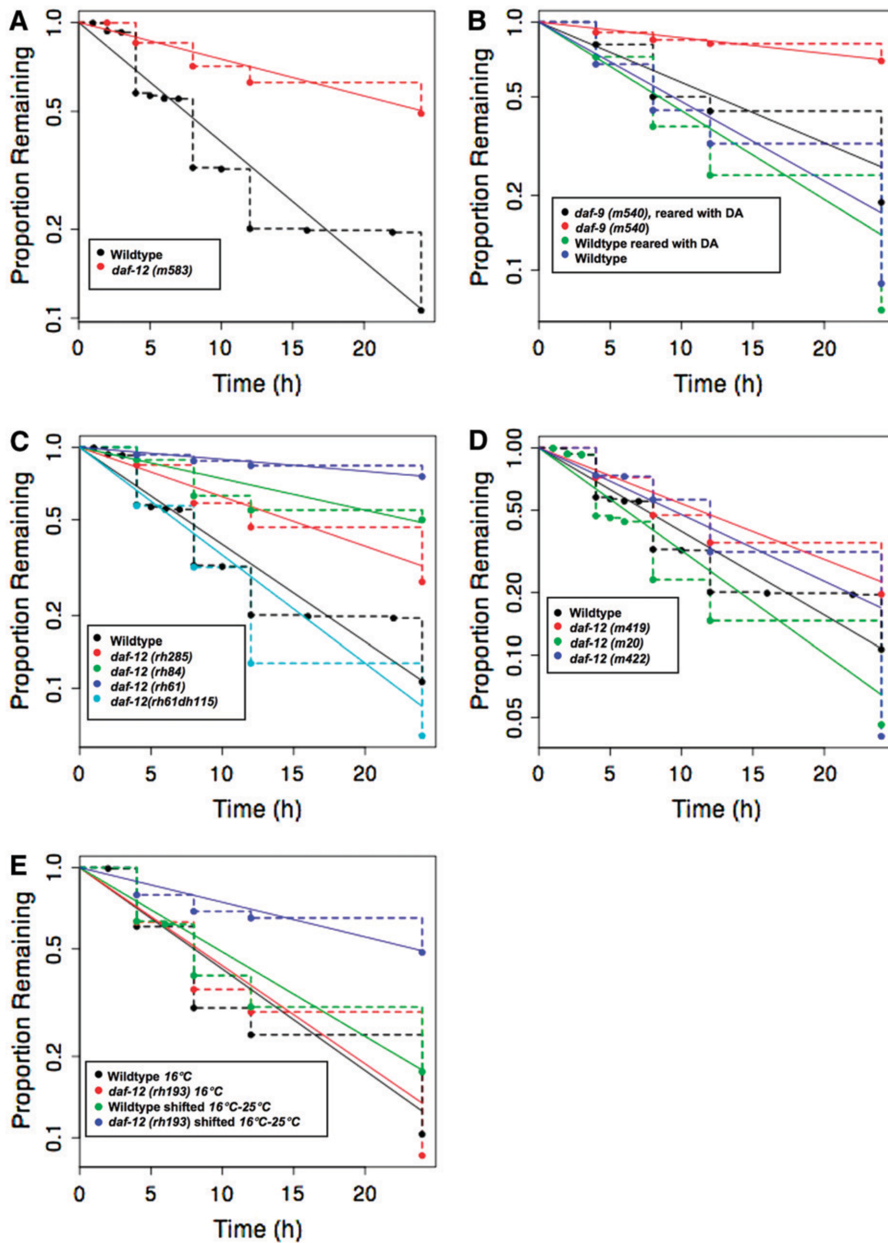

FIGURE 1.—The DAF-12 pathway regulates male exploratory behavior. The rate of male exploratory behavior is determined by the leaving assay. For various genotypes and treatments, the fraction of (usually 20) males that have not yet traveled 3 cm away from a 1-cm patch of *E. coli* food (nonleavers) is scored at various times after the start of the assay. The points on the graphs show the individual observations. The leaving rate, taken as a measure of the tendency for exploratory behavior, is calculated as the probability of leaving per hour, P_L. The data are analyzed using the R survival package (http://cran.r-project.org/web/packages/survival/index.html) to fit the censored data to an exponential parametric survival model, using maximum likelihood. The slopes of the straight lines through the data illustrate the constant hazard rate (λ), taken here as P_L. The results are compiled in Table 1 and summarized in Figure 2 for the various *daf-12* alleles. (A) *daf-12* gene function is required for a wild-type rate of exploratory behavior. Males homozygous for a *daf-12* strong loss-of-function mutation leave food more slowly than wild-type males. (B) The DAF-12 ligand DA stimulates exploratory behavior. Males homozygous for *daf-9(m540)*, a hypomorphic allele of the *daf-9* biosynthetic gene required for synthesis of DA, leave food more slowly than wild-type males. Leaving rate is restored by treatment of *daf-9* animals with DA. (C) Evidence a DAF-12/DIN-1 corepressor complex inhibits leaving. The structures of the *daf-12* alleles shown are illustrated in Figure 2. Successive deletion of the DAF-12 C-terminal LBD results in decreased leaving rate. Introduction of a point mutation that prevents DIN-1 binding [*daf-12(rh61dh115)*] restores wild-type leaving rate. Wild-type data are the same as in A. (D) The DAF-12 N-terminal DBD stimulates exploratory behavior. The structure of the *daf-12* alleles shown is illustrated in Figure 2. Alleles of *daf-12* with deletions covering the entire LBD including the DIN-1 binding region have a high rate of leaving. Wild-type data are the same as in A. (E) *daf-12* has an adult function to stimulate exploratory behavior. Males homozygous for a temperature-sensitive allele of *daf-12* have reduced leaving rate when shifted to nonpermissive temperature as adults. Quantitative analysis of the data in A–D is given in Table 1. Data for the series of *daf-12* alleles shown in A, C, and D were compared to pooled data for wild type.

behavior, we examined males mutant for the ligand biosynthetic gene *daf-9*. Males homozygous for the hypomorphic allele *daf-9(m540)* had a slow rate of leaving like the *daf-12* loss-of-function phenotype (Figure 1B; Table 1). Since a reduced level of ligand slowed the rate of leaving, it appeared to be the liganded form of *daf-12* that stimulated exploratory behavior. Animals null for *daf-9* could not be tested because they constitutively form dauers or sterile adults (GERISCH *et al.* 2001; JIA *et al.* 2002). This result therefore left open the question of the phenotype in the complete absence of ligand.

We rescued the effect of the *daf-9* mutation by exogenously supplying DA, consistent with the conclusion that DA is the steroid hormone in the *daf-12* pathway for male behavior as it is in other pathways (MOTOLA *et al.* 2006). *daf-9(m540)* animals were raised to adulthood from eggs laid on agar containing 250 nM Δ^4-dafachronic acid, one of the two dafachronic acids identified in worms (we refer to this compound as DA). Upon maturing, they were cultured on DA for a further 12 hr and then tested in the leaving assay in the absence of hormone. Growth on DA-supplemented medium restored

TABLE 1

Involvement of the gonad and the dafachronic acid pathway in the modulation of exploratory behavior in *C. elegans* males

Strain	Leaving		*P*-value[a]			Reps (*n*)
	P_L	95% C.I.	*vs.* wild type	*vs. daf-12(rh61)*	*vs. daf-12(m583)*	
daf-12 mutants						
Wild type	0.097	0.092–0.102	NA			72 (1461)
daf-12(m583)X	0.029	0.026–0.032	<0.0001	<0.0001	NA	35 (619)
daf-12(rh285)X	0.047	0.035–0.064	<0.0001	<0.0001	<0.01	4 (58)
daf-12(rh84)X	0.030	0.021–0.043	<0.0001	<0.001	0.8	4 (62)
daf-12(rh61)X	0.011	0.008–0.016	<0.0001	NA	<0.0001	6 (131)
daf-12(rh61dh115)X	0.10	0.08–0.13	0.38	<0.0001	<0.0001	2 (63)
daf-12(m419)X	0.062	0.055–0.070	<0.0001	<0.0001	<0.0001	9 (296)
daf-12(m20)X	0.114	0.106–0.122	<0.0001	<0.0001	<0.0001	47 (889)
daf-12(m422)X	0.074	0.062–0.089	<0.01	<0.0001	<0.0001	6 (121)
daf-9(m540)X	0.053	0.042–0.067	<0.0001			6 (114)
				P-value		
				vs. daf-9	*vs. daf-12(m20)*	
daf-12(m20), daf-9(e1406)X	0.24	0.20–0.29	<0.0001	<0.0001	<0.015	7 (118)
			vs. wild type, same temperature			
daf-12 temperature shift						
Wild type at permissive temperature	0.086	0.072–0.104				7 (129)
daf-12(rh193ts)X at permissive temperature	0.084	0.068–0.103	0.081			6 (113)
Wild type shifted as adult	0.072	0.059–0.087				7 (128)
daf-12(rh193ts)X shifted as adult	0.030	0.023–0.039	<0.0001			6 (106)
				P-value		
			vs. wild type	*vs. daf-9*		
DA rescue experiments						
Wild type	0.08	0.06–0.09	NA	<0.0001		4 (73)
Wild type + DA from egg	0.08	0.06–0.12	0.69			2 (29)
Wild type + DA as adult	0.08	0.06–0.11	0.51			2 (41)
daf-9(m540)X	0.03	0.02–0.04	<0.0001	NA		4 (69)
daf-9(m540)X + DA from egg	0.06	0.04–0.08	0.31	<0.05		2 (32)
daf-9(m540)X + DA as adult	0.04	0.02–0.06	<0.01	0.38		2 (37)
			vs. alone, same strain			
With hermaphrodites						
Wild-type male alone	0.10	0.08–0.13				5 (80)
Wild type + hermaphrodites	0.002	0.001–0.007		<0.0001		4 (60)
daf-12(m20)X male alone	0.136	0.089–0.209				1 (21)
daf-12(m20)X + hermaphrodites	0.009	0.003–0.024		<0.0001		1 (21)

(*continued*)

TABLE 1

(Continued)

Strain	Leaving		*P*-value[a]			Reps (n)
	P_L	95% C.I.		*vs.* fed, same strain		
Starved animals (censored after 8 hr)						
Wild type	0.059	0.041–0.086				4 (70)
Wild type, starved	0.015	0.007–0.032		<0.0005		4 (59)
daf-12(m20)X	0.103	0.067–0.160				2 (31)
daf-12(m20)X, starved	0.021	0.009–0.050		<0.0005		2 (31)
			P-value			
			vs. wild-type mock	*vs.* intact, same strain	*vs. daf-12 (m583)* gonad (−)	
daf-12 and gonad ablation						
Wild-type mock	0.11	0.08–0.16				5 (30)
Wild-type gonad ablated	0.023	0.01–0.04		<0.0001		6 (26)
daf-12(m583)X mock	0.041	0.03–0.06	<0.001			4 (31)
daf-12(m583)X gonad ablated	0.033	0.02–0.06	<0.0005	0.57		4 (26)
daf-12(m20)X mock	0.12	0.09–0.16	0.72			3 (47)
daf-12(m20)X gonad ablated	0.076	0.05–0.12	0.19	0.07	<0.05	2 (25)
			P-value			
			vs. wild type, no steroid	*vs.* no preDA, same ablation		
Steroid replacement of gonad and germ line						
Wild-type mock, no steroid	0.09	0.07–0.11				11 (80)
Germ-line ablated, no steroid	0.04	0.02–0.08	<0.05			4 (17)
Gonad ablated, no steroid	0.03	0.01–0.67	<0.01			5 (19)
Germ-line ablated with DA precursor	0.09	0.05–0.15	0.9	0.1		4 (17)
Gonad ablated with DA precursor	0.04	0.02–0.09	<0.05	0.41		5 (19)
			P-value			
			vs. mes-1 sperm (+)	*vs.* sperm (−) no preDA		
mes-1(bn7)X, sperm (+), no steroid	0.11	0.08–0.16				1 (39)
mes-1(bn7)X, sperm (−), no steroid	0.06	0.04–0.10	<0.05			1 (27)
mes-1(bn7)X, sperm (−) with preDA	0.15	0.10–0.23	0.35	<0.01		1 (25)
			P-value			
			vs. wild type	*vs.* no preDA, same strain		
DA precursor addition						
Wild type	0.11	0.08–0.15				2 (43)
daf-9(m540)X	0.030	0.02–0.05	<0.0001			2 (33)
daf-12(m583)X	0.035	0.02–0.05	<0.0001			2 (46)
daf-12(m583)X + preDA as adult	0.054	0.04–0.08	<0.005	0.1		2 (42)
daf-9(m540)X + preDA from egg	0.042	0.02–0.08	<0.0005	0.74		1 (15)
daf-9(m540)X + preDA as adult	0.034	0.02–0.06	<0.005	0.41		1 (20)

preDA refers to the dafachronic acid precursor 4-cholesten-3-one. Reps indicates the number of independent leaving assays. n refers to total number of animals examined across all trials.

[a] Unless otherwise specified, the first *P*-value is for a comparison to the control group, which is in the first row of each section. *P*-values for second and third comparisons are listed when appropriate. The specific comparison is indicated above each section.

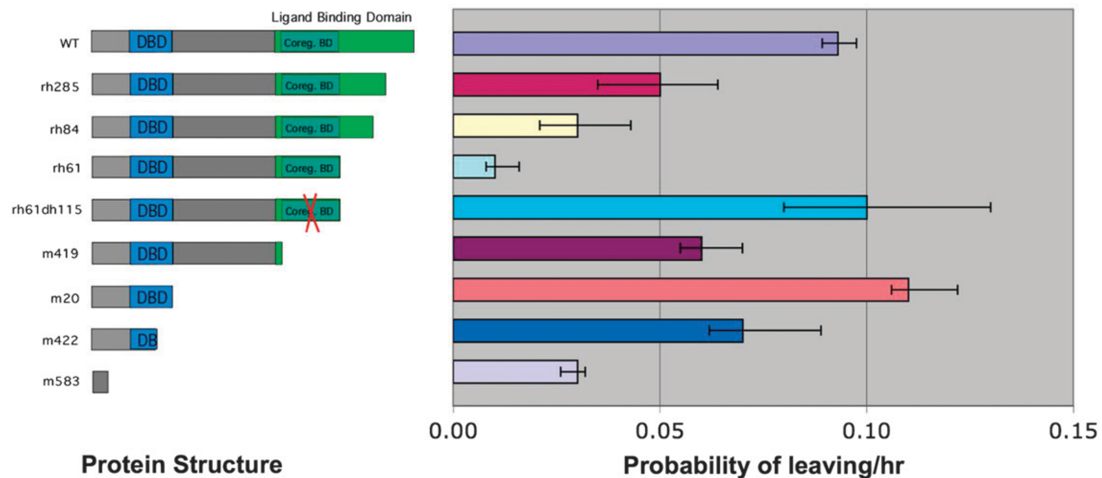

Figure 2.—Correspondence of DAF-12 protein structure with exploratory behavior for a series of C-terminal deletion alleles. Deletion of the LBD excluding the corepressor binding region decreases exploratory behavior. Introduction of a point mutation that abolishes DIN-1 binding (red "x") restores exploratory behavior, as does deletion of the LBD together with the DIN-1 binding region. Error bars are 95% confidence intervals.

the leaving rate of adult males to the wild-type level (Figure 1B; Table 1). The effect was specific to males, since adult hermaphrodites raised on DA remained on food like untreated hermaphrodites (data not shown). However, when worms were first matured on plates without drug and then cultured only as adults on drug for 12 hr and tested for leaving without drug, leaving by *daf-9(m540)* males was the same as without drug treatment (Table 1). Thus there appeared to be a requirement for ligand-stimulated *daf-12* function during male development for rescue of exploratory behavior.

To further examine whether the DAF-12 protein had a function in the absence of ligand binding, we examined animals carrying a series of mutations that deleted increasing segments of the protein beginning at the C terminus and successively deleting the LBD, the DIN-1 corepressor binding domain, and the DBD (Figure 1, C and D). The measured rates are given in Table 1 and summarized in Figure 2. Deletion of increasing amounts of the LBD [*daf-12(rh285)* and *daf-12(rh84)*] gradually decreased the rate of exploratory behavior. When the LBD was deleted up to the DIN-1 binding region, but leaving the DIN-1 binding region intact [*daf-12(rh61)*], the rate of leaving was slower than in the presumptive null background. This form of the protein therefore appeared to inhibit exploratory behavior. This inhibition appeared to require DIN-1 binding since introduction of a point mutation into the DIN-1 binding domain that abolishes DIN-1 binding [*daf-12(rh61dh115)*] (Ludewig *et al.* 2004) restored a wild-type rate of leaving. A similar high rate of leaving was obtained when the entire DIN-1 binding domain was deleted but the DBD remained intact [*daf-12(m419)* and (*daf-12(m20)*]. Thus it appears that the DAF-12 N-terminal region containing the DBD is capable of activating exploratory behavior. This effect was specific to males, since *daf-12(m419)* and *daf-12(m20)* hermaphrodites ($P_L = 0.011$ and 0.010, respectively) remained on food like wild-type hermaphrodites ($P_L = 0.007$).

As expected for a ligand-independent form of the protein, *daf-12(m20)* suppressed the slow leaving rate of ligand-defective *daf-9* males. The double mutant *daf-12(m20);daf-9(e1406)* left food with a rate as high as or even higher than either *daf-12(+)* or *daf-12(m20)* (Table 1). *daf-9(e1406)* is a null allele of *daf-9* that can be used in double combination with *daf-12(m20)* because *daf-12(m20)* suppresses the dauer-constitutive phenotype of *daf-9(e1406)*. A higher rate of leaving in the double mutant over that in *daf-12(m20)* could be because of upregulation of *daf-12* expression in the absence of ligand (Gerisch and Antebi 2004; Mak and Ruvkun 2004). Restoration of a high leaving rate to *daf-9(e1406)* by *daf-12(m20)* indicated that DAF-9, and by implication DA, acts solely through the DAF-12 receptor to affect behavior.

***daf-12* functions in the adult as well as during development to stimulate exploratory behavior:** Rescue of exploratory behavior to *daf-9* males by administering DA throughout postembryonic larval development and for 12 hr during adulthood, but not for 12 hr during adulthood alone, indicated that liganded DAF-12 had an organizational function in development of a male nervous system capable of supporting exploratory behavior. To determine whether *daf-12* also had an activational function to stimulate exploratory behavior, we carried out a temperature-shift experiment with a temperature-sensitive allele of *daf-12*. Males homozygous for the temperature-sensitive mutation *daf-12(rh193ts)*, when raised at permissive temperature and then shifted to restrictive temperature as adults, had a lower rate of exploratory behavior than similarly treated wild-type males (Figure 1E). Hence *daf-12* has an adult function

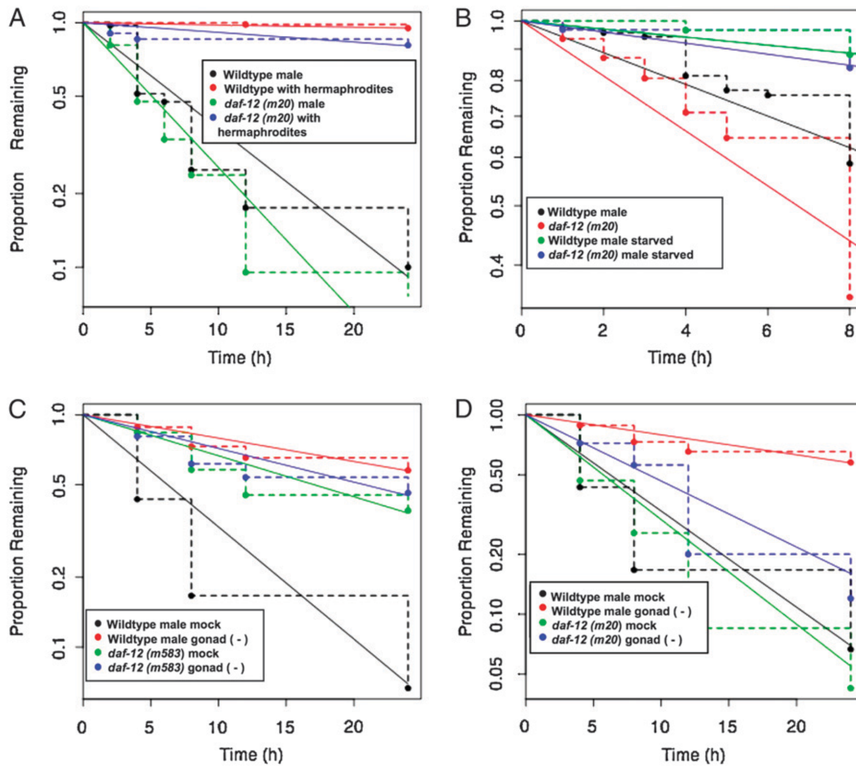

Figure 3.—Relationship between stimulation of exploratory behavior by *daf-12* and that of other signaling pathways that regulate male exploratory behavior. (A) Hermaphrodites inhibit exploratory behavior of males homozygous for ligand-independent *daf-12(m20)*. The effect of five paralyzed (*unc-51*) hermaphrodites on mate searching is shown. (B) A signal indicating nutritional status inhibits exploratory behavior of males homozygous for ligand-independent *daf-12(m20)*. The effect of 12-hr food deprivation on wild-type *vs.* *daf-12(m20)* males is shown. Data are given for the first 8 hr of the leaving assay and censored thereafter since after this time the leaving rate returns to the fed rate. (C) The gonad requires *daf-12* function to stimulate exploratory behavior. In males homozygous for the *daf-12* presumptive null allele *m583*, ablation of the gonad has little or no effect on leaving rate. (D) Ligand-independent *daf-12(m20)* stimulates exploratory behavior independently of the gonad. Wild-type mock ablated and gonad ablated data are the same in C and D.

in stimulating exploratory behavior as well as a role during development.

***daf-12* is required for differentiation of male-specific neurons:** One possible organizational function of *daf-12* is in development of male-specific neural structures. Incompletely penetrant defects in male development in *daf-12* mutants have been reported previously, including delayed cell division during L3 lineages leading to sex-specific ray neurons and incomplete tail spike retraction and fan morphogenesis during L4 (Antebi *et al.* 1998). The 18 bilateral pairs of ray sensory neurons stimulate exploratory behavior when mates are absent and detect mates when they are present (Barrios and Emmons 2008). We therefore examined development of ray neurons in *daf-12* mutants and observed abnormalities in axonal outgrowth. Ray neurons are born and differentiate during the L4 larval stage, at which time they send out axonal processes through commissures from the lumbar ganglia into the pre-anal ganglion (Sulston *et al.* 1980; Jia and Emmons 2006). In 37% of *daf-12(m20)* and 21% of *daf-12(m583)* mutants ray neuron axons showed guidance defects while following commissures to the pre-anal ganglion (data not shown). Thus *daf-12* plays a role in differentiation of the male nervous system. However, since alleles of *daf-12* having opposite effects on exploratory behavior had a similar proportion of abnormal ray axons, the extent to which ray neuron defects contribute to the behavioral abnormality in *daf-12* mutants remains unclear.

Regulation of exploratory behavior by starvation and hermaphrodite signals is independent of *daf-12*: In addition to maturity and presence of the gonad, male exploratory behavior is regulated by presence of mates and nutritional status (Lipton *et al.* 2004). To determine the relationship of these pathways to *daf-12* signaling, we studied the regulation of mate searching in *daf-12* mutants. Homozygous males were fully retained by hermaphrodites for all *daf-12* alleles tested, including an allele missing the C-terminal portion of the LBD, *daf-12(rh84)*, an allele missing the entire LBD including the DIN-1 binding region, *daf-12(m20)*, and the presumptive null allele *daf-12(m583)* (Figure 3A and data not shown). Retention of ligand-independent *daf-12(m20)* males indicated that hermaphrodite signals blocked male exploratory activity downstream of *daf-12* activity. Likewise, retention of the *daf-12(m583)* presumptive null mutant males indicated that the hermaphrodite signal did not block exploratory behavior via the *daf-12* inhibitory activity.

After a period of food deprivation, the leaving rate of *daf-12(m20)* males was decreased in a manner similar to wild type (Figure 3B). Thus, like the hermaphrodite signal, the signal indicating starvation also appears to act downstream of *daf-12* activity.

***daf-12* acts downstream of the gonad to stimulate exploratory behavior:** To determine the relationship between the gonad signal that stimulates mate searching and *daf-12* activity, we examined the leaving behavior of *daf-12* mutants after ablation of the gonad (germ line plus somatic gonad). Stimulation of mate searching by *daf-12* and the gonad might be because both signals acted in a single regulatory pathway. Alternatively, each

might act in a separate, parallel pathway. We examined the effect of gonad ablation in a *daf-12* null background and found that this caused no further decrease in leaving rate over that in the unoperated *daf-12* null (Figure 3C). Since the effects of ablation and mutation were not additive, this suggested that both signals acted in the same pathway.

To order the actions of gonad signaling and *daf-12* activity in this pathway, we examined the effect of gonad ablation on males carrying the ligand-independent *daf-12(m20)* allele. If *daf-12(m20)* acted upstream, possibly during gonad maturation, or to stimulate signaling by the mature gonad, then gonad-ablated *daf-12(m20)* males were expected to have a slow leaving rate like gonad-ablated wild-type males. However, if *daf-12(m20)* acted downstream of gonad signaling, then gonad-ablated *daf-12(m20)* males should leave at a high rate similar to unoperated *daf-12(m20)* males. We found that, unlike wild-type males, the leaving rate of *daf-12(m20)* males was not decreased by ablation of the gonad (Figure 3D). We conclude that *daf-12* is expressed outside of the gonad to stimulate mate searching and this expression is unaffected by gonad ablation. Further, the targets of *daf-12* signaling to stimulate exploratory behavior are outside the gonad and function independently of the gonad signal.

A DA precursor rescues the effect of germ-line but not whole gonad ablation: To determine whether the gonad signal that stimulated exploratory behavior could be DA, we examined the ability of the DA precursor 4-cholestene-3-one to rescue exploratory behavior after germ-line or whole gonad ablation. DA itself was not available to us in sufficient quantity to directly test its ability to rescue gonad-ablated males. However, 4-cholestene-3-one is converted to DA by DAF-9 (Motola *et al.* 2006; Rottiers *et al.* 2006). Hence, a tissue that secretes DA is expected to express DAF-9. We considered two possibilities: either the gonad expressed *daf-9* and synthesized DA or the gonad signal acted by stimulating another tissue to secrete DA, by stimulating synthesis of the DA precursor, by inducing expression of *daf-9*, or both. If the gonad was the site of *daf-9* expression required for synthesis of DA, then it would not be possible to rescue gonad-ablated animals with the DAF-9 substrate. However, if the gonad signal acted via another tissue, then gonad-ablated animals might be rescued.

We found that 4-cholestene-3-one could rescue exploratory behavior of germ-line ablated males. Ablated adult males were tested for leaving behavior and then were placed on plates containing 25 μM 4-cholestene-3-one for 12 hr and retested on plates without drug. Leaving rate was reduced by ablation of the germ line and restored to that of unoperated animals after treatment (Figure 4A). We also tested the germ-line defective strain *mes-1(bn7)*, in which the germ line fails to proliferate in ~50% of animals (Capowski *et al.* 1991). Leaving rate was reduced in those adult *mes-1(bn7)* males that lacked germ cells and was restored by 12 hr treatment with 4-cholestene-3-one (Figure 4B). Exploratory behavior of males mutant for *daf-12* or *daf-9* was not rescued by 12 hr 4-cholestene-3-one treatment, consistent with the conclusion that 4-cholestene-3-one did not act through a *daf-12*-independent pathway (Figure 4, D and E). The behavior of wild-type adult hermaphrodites was also not affected by 12 hr 4-cholestene-3-one treatment (data not shown). Thus it appears that the effect of 4-cholestene-3-one is specific to the *daf-12* pathway in males. Since germ-line ablated or defective males were rescued by 12 hr treatment with 4-cholestene-3-one, it appears that in germ-line defective animals 4-cholestene-3-one is rate limiting for exploratory behavior. Since the presumptive conversion of 4-cholestene-3-one to DA does not require the germ line, the germ line is not the site of DAF-9 activity and hence the germ line is unlikely to be the normal source of DA that stimulates exploratory behavior. However, it remained possible that 4-cholestene-3-one treatment acted by providing an ectopic source of DA not normally involved in regulating exploratory behavior.

A different result was obtained when the entire gonad, germ line plus somatic cells, was ablated. Treatment of whole gonad ablated adult males with 4-cholestene-3-one as described above did not restore the normal rate of exploratory behavior (Figure 4C). We conclude that the somatic gonad may be necessary for expression of *daf-9* in the adult, either because *daf-9* is expressed within this tissue itself or because expression of *daf-9* elsewhere is stimulated by a signal from the somatic gonad. Because rescue by 4-cholestene-3-one treatment requires the somatic gonad, activation of an ectopic, gonad-independent source of DA by 4-cholestene-3-one treatment appeared unlikely. Alternatively, the somatic gonad might be necessary during larval development to promote the organizational effects of the *daf-12* pathway, such that the adult is no longer responsive to DA.

A pair of putative neuroendocrine cells in the head, known as XXX cells, express a *daf-9*::GFP reporter gene (Gerisch and Antebi 2004). We ablated the XXX cells in adult males and in L3 males and observed no effect on leaving behavior (data not shown). Expression of *daf-9*::GFP has also been reported in the hermaphrodite spermatheca (Gerisch and Antebi 2004). We examined the *daf-9*::GFP expression pattern in males and observed expression within the pharynx, gut, and hypodermis, as well as in the XXX cells, but did not observe expression in the male gonad. Thus whether *daf-9* is expressed in the somatic gonad of the male or elsewhere to stimulate exploratory behavior remains unresolved.

DISCUSSION

Coordination of animal behavior with the needs of the organism and its physiology is essential for survival and reproduction. Synchronization of reproductive

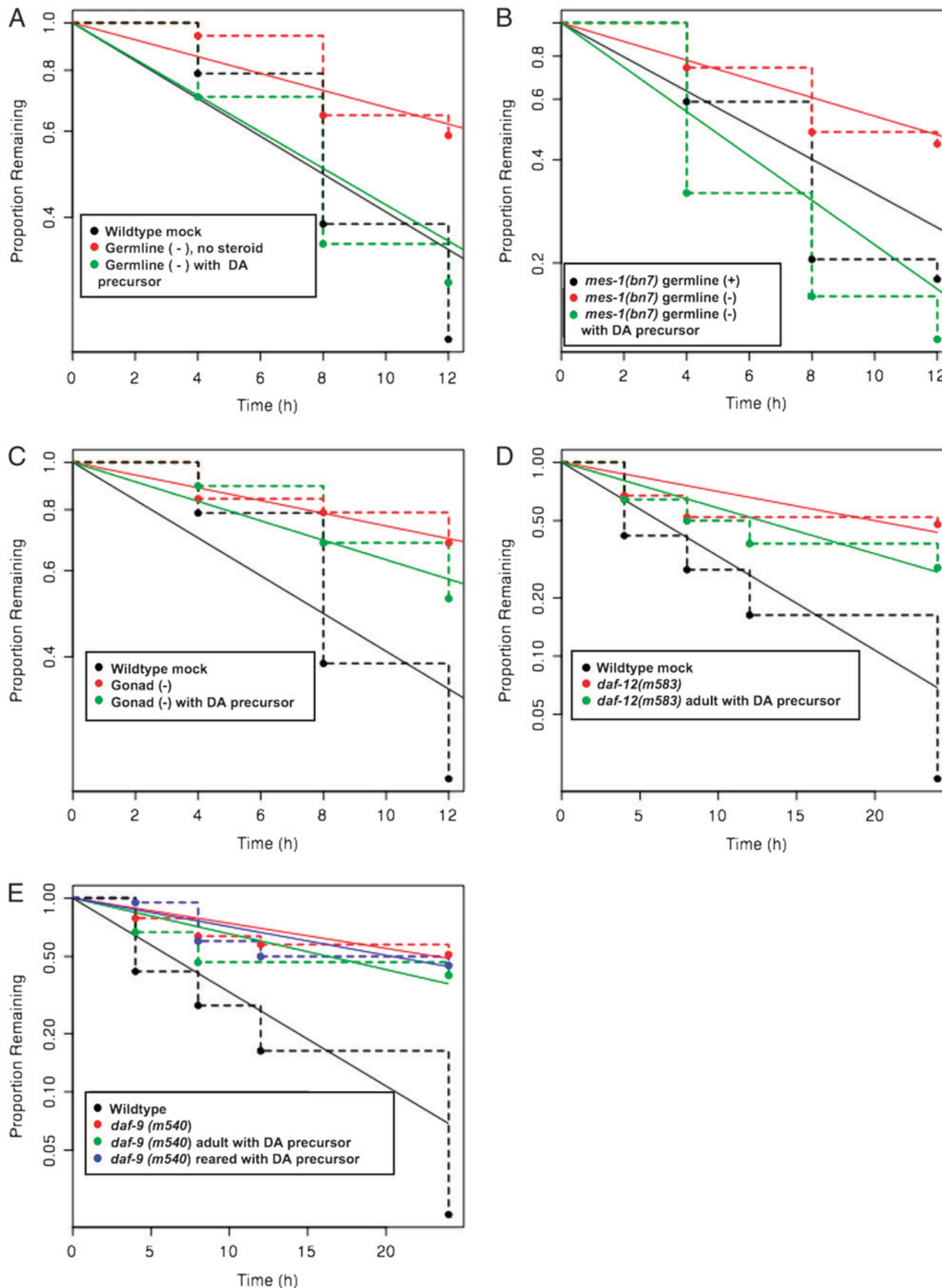

Figure 4.—Stimulation of male exploratory behavior by the DA precursor after germ-line but not whole gonad ablation. (A) The DA precursor can rescue germ-line ablated adult males. (B) The DA precursor can rescue germ-line defective *mes-1(bn7)* males. (C) In gonad ablated males the DA precursor cannot rescue the mate searching defect. Wild-type mock ablated data are the same as in A. (D) The DA precursor fails to rescue mate searching in the *daf-12* null animals. (E) The DA precursor fails to rescue *daf-9(m540)* adult males. Wild-type data are the same as in D.

behavior with gonadal status is achieved in many cases through elaboration of hormones by the gonad. Here we show that in *C. elegans*, a gonadal signal functions through the *daf-12* NR to stimulate a male-specific exploratory behavior that serves to bring males together with mates. *daf-12* functions in nongonadal tissues for full expression of exploratory behavior, while the gonad is required for generation of the DAF-12 ligand, DA, either directly, by synthesizing DA itself, or indirectly through secondary signals.

Among the 284 predicted NR genes in the *C. elegans* genome, *daf-12* has been the most extensively studied (Antebi 2006). Initially identified by its function at a larval choice point between a rapid, reproductive developmental pathway and a diapause (dauer) pathway favoring survival and dispersal, *daf-12* is expressed widely in the tissues and acts to coordinate diverse life-history traits, including developmental timing and adult longevity (Larsen *et al.* 1995; Riddle and Albert 1997; Antebi *et al.* 1998; Gems *et al.* 1998). Like other NRs, both liganded and unliganded forms of DAF-12 have biological function. In general, the liganded form of DAF-12 promotes the reproductive choice and its proper execution, while the unliganded form in conjunction with the corepressor protein DIN-1 functions to promote the survival pathway. However, ligand-defective mutants form abnormal dauer larvae, so this distinction is not absolute (Gerisch *et al.* 2001; Jia *et al.* 2002). Our

results demonstrate for the first time an effect of *daf-12* on behavior and are consistent with the generalization that liganded DAF-12 promotes reproduction and unliganded DAF-12 promotes survival. With respect to behavior, DAF-12 in the presence of ligand promotes the reproductive choice—male exploration for mates—while DAF-12 in the absence of ligand binding, together with DIN-1, promotes survival behavior—remaining on food.

While *daf-12* function is necessary for full expression of exploratory behavior by males, it is not sufficient to induce this behavior in hermaphrodites. Whereas males homozygous for a ligand-independent mutant form of DAF-12, DAF-12(m20), expressed a wild-type rate of exploratory behavior, hermaphrodites homozygous for this allele did not express exploratory behavior, nor did they after treatment throughout development with DA. Thus expression of the DAF-12 ligand only in males does not appear to be a sufficient explanation to account for a male-specific behavioral pattern. Additional sexual dimorphism in the nervous system not requiring function of the *daf-12* pathway presumably accounts for the difference in response of the two sexes (Portman 2007).

daf-12 functions independently of the gonad somewhere in nongonadal tissues. Ultimately, the activity of *daf-12* must converge on the nervous system to influence behavior. However, *daf-12* is expressed broadly and is important in the coordination of major life-history events across anatomical loci (Antebi *et al.* 2000). For example, *daf-12* mutants uncouple developmental timing events across diverse tissues such as the hypodermis, the intestine, and the gonad (Antebi *et al.* 1998, 2000). While the site of *daf-12* action in the aging pathway is not yet known, *daf-12* appears to act directly or indirectly through the intestine to effect changes in longevity after germ-cell ablation (Hsin and Kenyon 1999; Libina *et al.* 2003; Berman and Kenyon 2006). Since the intestine is an important endocrine tissue (Libina *et al.* 2003), *daf-12* might act through the intestine to affect changes in male exploratory behavior by changing downstream endocrine signals that interact with the nervous system.

Likewise, the source or sources of DA required for either the organizational or the activational effects of *daf-12* on behavior are unknown. In view of the possibility raised by our results that DA is generated by the somatic cells of the gonad, expression of a *daf-9*::GFP reporter in the spermatheca of the hermaphrodite is suggestive. Possibly lack of observed expression of this reporter in the male gonad is a reporter gene artifact. However, a source of DA outside the gonad is not excluded by our data. We tested two likely candidates, the paired XXX putative neuroendocrine cells in the head, and found they seemed not to be required. The source of DA that stimulates reproductive growth has not been established and could be distributed over multiple tissues (Rottiers *et al.* 2006).

Our data suggest the germ line and somatic portions of the gonad produce different signals affecting behavior. The germ-line signal is unlikely to be DA itself, since a precursor to DA, 4-cholestene-3-one, requiring the function of DAF-9 for conversion to DA, could rescue exploratory behavior in germ-line defective males. Thus, *daf-9* must be expressed outside of the germ line. Since the effect of whole gonad ablation could not be similarly rescued, the signal from the somatic cells must be different from the germ-line signal. Similarly, the somatic gonad and the germ line have been shown to function in different ways to regulate the rate of aging (Hsin and Kenyon 1999; Alcedo and Kenyon 2004; Yamawaki *et al.* 2008). The simplest hypothesis to account for our observations is that the germ cells generate 4-cholestene-3-one, which is converted to DA by *daf-9* expressed in the gonadal somatic cells. However, it is also possible that the germ-line and somatic gonad signals are unrelated molecules that stimulate DA production by other tissues. Resolution of these issues awaits mosaic analysis of *daf-9* gene function.

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