A *Caenorhabditis elegans* Pheromone Antagonizes Volatile Anesthetic Action Through a Go-Coupled Pathway

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ABSTRACT

Volatile anesthetics (VAs) disrupt nervous system function by an ill-defined mechanism with no known specific antagonists. During the course of characterizing the response of the nematode *C. elegans* to VAs, we discovered that a *C. elegans* pheromone antagonizes the VA halothane. Acute exposure to pheromone rendered wild-type *C. elegans* resistant to clinical concentrations of halothane, increasing the EC_{50} from 0.43 ± 0.03 to 0.90 ± 0.02. *C. elegans* mutants that disrupt the function of sensory neurons required for the action of the previously characterized dauer pheromone blocked pheromone-induced resistance (Pir) to halothane. Pheromone preparations from loss-of-function mutants of *daf-22*, a gene required for dauer pheromone production, lacked the halothane-resistance activity, suggesting that dauer and Pir pheromone are identical. However, the pathways for pheromone's effects on dauer formation and VA action were not identical. Not all mutations that alter dauer formation affected the Pir phenotype. Further, mutations in genes not known to be involved in dauer formation completely blocked Pir, including those altering signaling through the G proteins Goα and Gqα. A model in which sensory neurons transduce the pheromone activity through antagonistic Go and Gq pathways, modulating VA action against neurotransmitter release machinery, is proposed.

THROUGH an unknown mechanism, volatile anesthetics (VAs) disrupt the behavior of all metazoans. In humans, VAs block memory formation, consciousness, and volitional movement, thereby forming the basis for most surgical anesthesia. Identifying VA targets and the mechanism whereby they alter nervous system function has been a longstanding and difficult effort. A major limitation in anesthetic mechanism research has been the lack of genetic or pharmacologic inhibitors of anesthetic potency *in vivo*. Mutations or drugs that produce high-level resistance to VAs have not to our knowledge been described in vertebrates. Screens in Drosophila have isolated mutant stains that are modestly VA resistant to some anesthetic endpoints (Krishnan and Nash 1990; Tinklenberg *et al.* 1991; Leibovitch *et al.* 1995; Gamo *et al.* 1998); however, highly resistant mutants have not thus far been uncovered. In *Caenorhabditis elegans*, several mutants have been found to be markedly resistant to clinical concentrations of VAs, and the implicated genes have been placed in a pathway that regulates neurotransmitter release in *C. elegans* and in higher organisms (van Swinderen *et al.* 1999, 2001). At this point it is unclear if the molecular mechanisms being defined genetically in Drosophila and *C. elegans* will overlap. However, at least at a cellular level VA mechanisms in the two organisms may be similar, given that two of the halothane-resistant Drosophila mutants have been shown to partially antagonize the effects of halothane on glutamate release at the Drosophila larval neuromuscular junction (Nishikawa and Kidokoro 1999).

The fact that in *C. elegans* single gene mutations can confer high-level resistance to clinical concentrations of VAs indicates that one major mechanism is acting at these concentrations in *C. elegans*. Thus, pharmacological antagonism of VA potency would be biologically possible in *C. elegans* if the proper drug were identified. In this study, we describe the serendipitous discovery of a *C. elegans* pheromone that is capable of antagonizing VAs in *C. elegans*. Importantly, the pheromone is not a stimulant of the behavior that VAs disrupt. In other words, the pheromone alters the effects of the drug on behavior, not the behavior itself. This pheromone antagonizes VA action through mechanisms previously implicated genetically to regulate dauer formation and VA sensitivity in the absence of pheromone in *C. elegans*. Our results confirm the importance of these gene products in VA mechanisms and demonstrate that pharmacologic antagonism of general anesthetics is possible *in vivo*. Further, they show that a pheromone can modulate nervous system function in adult *C. elegans* and that the pheromone is likely to be the same pheromone that controls dauer formation in *C. elegans*.

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MATERIALS AND METHODS

Nematode strains and conditions: *C. elegans* mutant strains were obtained from the Caenorhabditis Genetics Center and from several laboratories whose research is referenced in this work. All assays were performed on well-fed young adult animals (1 day post-L4 stage) at room temperature (22°–24°). Strains were grown on uncrowded conditions (100–300 animals per plate) as described previously (Brenner 1974) on nematode growth media (NGM) agar plates seeded with OP50 bacteria.

Behavioral assays: VAs were delivered to *C. elegans* as described previously (Crowder *et al.* 1996). Halothane or isoflurane were injected as liquids onto the tops of sealed glass chambers containing the assay plates. Gas-phase VA concentrations were measured by gas chromatography.

The effect of VAs on locomotion was quantified by the dispersal assay (Crowder *et al.* 1996). Briefly, animals were washed off NGM plates into 1.5-ml polypropylene tubes, rinsed twice with S-basal, rinsed once with water, and then resuspended in 100 μl of water. Ten-microliter aliquots containing 50–100 worms were placed onto the center of dispersal assay plates (10-cm NGM plates seeded with a narrow ring of OP50 *Escherichia coli* along the edge of the plate). The plates were immediately placed into glass chambers, to which anesthetic was added. As soon as the worm-filled water drop dried (usually 2–5 min), the chambers were briefly shaken until the nematodes were induced to unclump and begin dispersing. After 45 min, the fraction of animals reaching the bacterial ring divided by the total number of worms was scored as the dispersal index. Incubation-induced resistance to VAs was measured by the dispersal assay with one difference. Instead of aliquoting the nematodes to dispersal plates immediately after the washing steps, the nematodes were allowed to remain in the 100 μl distilled water for 30 min prior to aliquoting onto dispersal plates. Sensitivity to VA-induced chemotaxis defects was measured as described previously (Crowder *et al.* 1996). The behavioral assay is similar to the dispersal assay except that after the final wash animals are placed on chemotaxis plates, which are agar plates spotted with a chemoattractant, near the edge of the plate. The chemotaxis index was defined as the number of animals present after 2 hr within 0.5 cm of the attractant—the number of animals at an opposing control spot divided by the total number of animals on the plate. Sensitivity to VA-induced mating defects was measured as described previously (Crowder *et al.* 1996). Ten young adult males and two *dpy-11(e224)* hermaphrodites were placed on each of five 3-cm plates seeded with a small spot of bacteria. The five plates were placed into a chamber along with a given concentration of VA for 24 hr after which the males were removed. The mating index for a given chamber was calculated as the fraction of plates with cross-progeny. Concentration/response data were fit by nonlinear regression to the VA EC_{50} (the VA concentration where the effect is half maximal), which is used as the measure of VA sensitivity. Significant difference between EC_{50}'s was determined by simultaneous curve fitting as described previously (van Swinderen *et al.* 1997).

Pheromone extract and assays: A crude *C. elegans* pheromone extract was prepared as described for dauer pheromone (Golden and Riddle 1982). The substance was resuspended in distilled water, producing an oily yellow liquid, and stored at −20°. Dauer studies have calibrated the potency of the extract by measuring the dose required to induce 100% dauer larvae formation in the wild-type strain N2 at 20° (Golden and Riddle 1984). However, even at 10% concentration our pheromone preparations induced only ~50% dauer formation, suggesting that it may have been dilute.

Pheromone-induced resistance (Pir) to VAs was measured by the dispersal and chemotaxis assays as follows. Ten microliters of pheromone extract was diluted in each 990 μl of wash (three S-Basal and one distilled water), thus producing a 1% pheromone extract during the standard nematode washing steps prior to the dispersal assay. Following the washes, the nematodes were resuspended in a 50- to 100-μl aliquot of the same 1% pheromone extract in distilled water and immediately aliquoted to dispersal or chemotaxis plates, and subsequently the respective assays were as described above. For Pir in the mating assay, pheromone was added to OP50 bacteria to produce a 5% pheromone concentration, and the mating assay plates were seeded with a thin lawn of pheromone-containing bacteria 1 day prior to the assay.

RESULTS

Incubation-induced resistance to halothane: During the course of developing a high-throughput assay to quantitate VA-induced locomotion defects in *C. elegans*, we found significant variability in the VA sensitivity of the wild-type *C. elegans* strain N2. The assay, called the dispersal assay, measures the ability of a population of animals to disperse from the center of an agar plate to the edge. Dispersal, like other behaviors requiring coordinated locomotion, is particularly sensitive to VAs and is abolished at concentrations similar to those that anesthetize humans (Crowder *et al.* 1996). The variability in VA sensitivity as measured by the dispersal assay was not random; rather, it was systematic. That is, for a particular dispersal assay, the sensitivity of all animals was consistent and well fit by a single sigmoidal concentration/response curve. However, between assays VA EC_{50}'s varied as much as 50%, and the distribution of the EC_{50}'s was bimodal. Ultimately, we hypothesized that the variability in VA sensitivities was due to the length of time the animals were allowed to remain in liquid prior to being aliquotted onto assay plates. To test this hypothesis, we compared the VA sensitivity of animals allowed to remain in liquid for 30 min prior to aliquotting to those immediately aliquotted (Figure 1). The EC_{50} of incubated animals for the VA halothane was 0.79 ± 0.02 vol%, nearly double that of the nonincubated controls (0.42 ± 0.03 vol%).

We hypothesized that the VA resistance was conferred by a soluble product secreted by *C. elegans* into the water during the 30-min incubation step. To test this possibility, we added the supernatant of animals incubated for 4 hr to a nonincubated population of worms. This conditioned supernatant added to freshly washed animals produced a small but insignificant level of halothane resistance (Figure 1B). We also did the complementary experiment of replacing (refreshing) the supernatant of animals incubating for 4 hr with a final wash of fresh water. A 4-hr incubation conferred resistance to halothane, and when the 4-hr incubated animals were refreshed with their final wash, their halothane sensitivity returned to normal nonincubation levels. These experiments were consistent with the hypothesis that the incubation-induced resistance resulted from a secreted substance. Alternative explanations in-

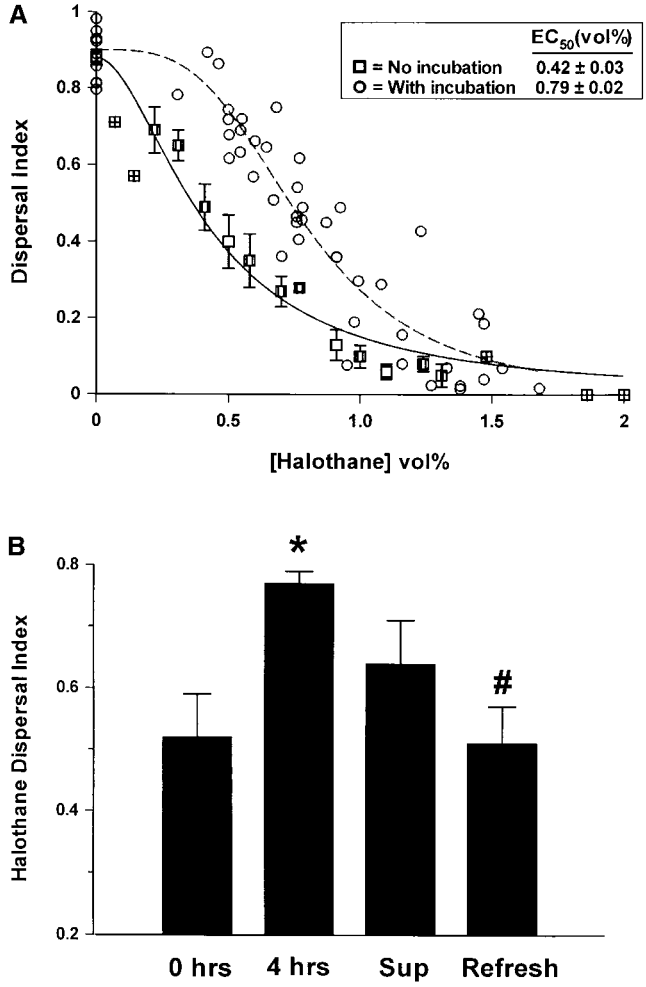

FIGURE 1.—Incubation-induced resistance to halothane. (A) Incubation of wild-type N2 animals conferred resistance to halothane by the dispersal assay. This effect was significant by both simultaneous curve-fitting algorithms (WAUD 1972; DELEAN *et al.* 1978) and analysis of variance for 10 separate EC_{50} estimates for each condition ($P < 0.05$). Because of a large number of points, data for nonincubated N2 are pooled and averaged (±SEM). (B) Dispersal indices in the presence of equal concentrations of halothane (EC_{50} for no incubation condition) were scored under four conditions: (1) 0 hr—spotting the worms immediately after resuspension in water; (2) 4 hr—after incubation of the worms for 4 hr in water; (3) Sup—immediate spotting of worms resuspended in conditioned supernatant of other worms incubated for 4 hr; (4) Refresh—removing the supernatant of worms incubated for 4 hr and replacing with fresh water prior to spotting the animals on the dispersal plates. Shown are mean ± SEM dispersal indices ($n = 3$ experiments). *, significant resistance compared to the "0 hr" condition by ANOVA ($P < 0.05$); #, significant reversal of resistance compared to the "4 hrs" condition by ANOVA ($P < 0.05$).

cluded starvation or hypoxia-induced VA resistance. The normalization of the VA sensitivity of 4-hr-incubated animals by replacement of the supernatant was inconsistent with starvation as the cause. However, these experiments did not rule out hypoxia.

A pheromone extract confers resistance to halothane: To directly test the hypothesis that a secreted pheromone antagonizes VAs in *C. elegans*, we tested the effects of a pheromone extract on VA sensitivity. We prepared the pheromone extract according to the previously described protocol for isolation of dauer pheromone, which promotes larval diapause in *C. elegans* (GOLDEN and RIDDLE 1982). For testing of its effects on VA sensitivity, the pheromone was diluted to a 1% concentration into the dispersal assay wash buffers. This pheromone concentration was initially chosen on the basis of the published potency of dauer pheromone for inducing dauer formation and proved to be maximally effective at antagonizing VA sensitivity (Figure 2C). The dispersal assays were performed exactly like the no-incubation assays except they used the 1% pheromone buffers for the washing steps prior to transferring the animals onto the assay plates. The pheromone induced significant halothane resistance, increasing the dispersal EC_{50} from 0.42 ± 0.03 to 0.95 ± 0.02 vol% (Figure 2A). The pheromone had no effect on locomotion in the absence of anesthetic (Figure 2B), indicating that the resistance was not merely secondary to making the animals hyperactive. The pheromone-induced resistance was dose dependent with a maximal effect achieved at a 1% concentration (Figure 2C). A similar extract from media containing only bacteria but no *C. elegans* did not induce halothane resistance (data not shown). Thus, the resistance activity requires and presumably is secreted by the worms. We refer to this phenotype as Pir (pheromone-induced resistance to volatile anesthetics).

Pheromone extract also conferred resistance to halothane's effects on male mating behavior (Figure 2D). This anesthetic assay involves a completely different behavior and set of neurons and is disrupted by halothane with an EC_{50} = 0.52 ± 0.02 vol% for the N2 strain (Figure 2D). Male mating plates seeded with an *E. coli* (OP50) solution mixed with pheromone extract significantly increased the halothane EC_{50} to 0.91 ± 0.06 vol% against male mating. However, chemotaxis, a third behavior abolished by clinical concentrations of halothane (CROWDER *et al.* 1996), was unaffected by pheromone extract even though the chemotaxis assay involves washing procedures identical to those used for the dispersal assay. We have previously concluded, on the basis of the odorant dependence of the sensitivity of chemotaxis to anesthetics, that VAs do not disrupt chemotaxis through their effects on locomotion (CROWDER *et al.* 1996). The lack of effect of pheromone on VA-induced chemotaxis defects is further evidence that VA mechanisms acting on locomotion and chemotaxis behaviors are distinct.

Dauer pheromone pathway: To define the mechanism underlying the pheromone's antagonism of halothane, we first tested strains carrying mutations in genes in the dauer formation pathway. Dauers are an alternative larval form whose formation is promoted by heat, starvation, and dauer pheromone (RIDDLE and ALBERT 1997). We speculated that the dauer pheromone and its transduction pathway were responsible for pheromone-induced halothane resistance. The most upstream com-

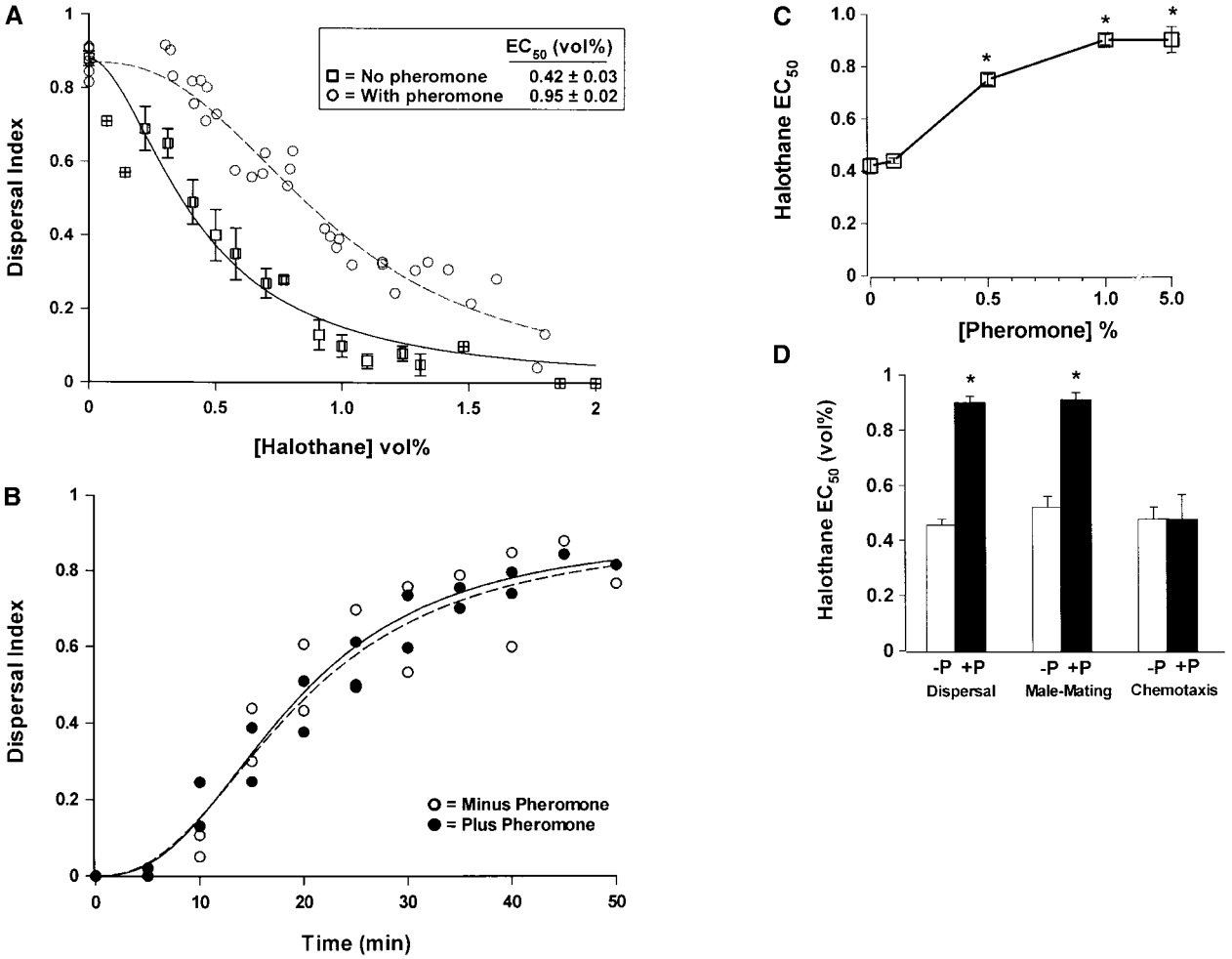

FIGURE 2.—Pheromone-induced resistance to halothane. (A) N2 animals were exposed to 1% *C. elegans* pheromone extract immediately prior to placement onto dispersal assay plates (see MATERIALS AND METHODS). The raw data for pheromone-treated N2 animals are shown in comparison to averaged data for untreated N2. Resistance was significant by curve-fitting algorithms (WAUD 1972; DELEAN *et al.* 1978) and ANOVA of EC_{50}'s ($P < 0.05$). (B) Pheromone extract did not produce significant differences in N2 dispersal in the absence of halothane. Dispersal index was plotted against time for the length of the dispersal assay (45 min). Two separate experiments for each treatment are combined. A best-fit curve of the data showed that it takes ~20 min for animals to perform half-maximally for both treatments. The curves were not significantly different. (C) N2 wild-type animals were exposed to different doses of pheromone. The data shown here are EC_{50}'s ± SE for the dispersal endpoint. The 0.5, 1.0, and 5.0% pheromone concentrations produced significant halothane resistance relative to the no pheromone control. (D) The pheromone extract induced significant resistance to halothane action against male mating behavior but not chemotaxis toward a volatile odorant. For male mating, 50 μl of pheromone extract was diluted into 1 ml of OP50 bacteria. This 5% OP50 solution was seeded onto male mating assay plates; control plates were seeded with straight OP50. The fraction of plates with successful mating after a 24-hr period was scored as the mating efficiency and plotted against halothane concentration to determine halothane EC_{50}'s. In the chemotaxis assay, the method for exposure to pheromone was identical to that for the dispersal assay. The fraction of animals at the odorant spot minus the fraction at the control was scored as the chemotaxis index and plotted against halothane concentration to determine EC_{50}'s. +P, pheromone treatment; −P, no pheromone control.

ponent of the dauer pathway (Figure 3A) is *daf-22*, which is required for the biosynthesis of functional dauer pheromone (GOLDEN and RIDDLE 1985). If *daf-22* is also required for the pheromone regulating VA sensitivity, then a pheromone preparation from *daf-22* loss-of-function mutants should lack Pir activity. Indeed, *daf-22 (m130lf)* pheromone does not induce halothane resistance (Figure 3B). As expected for a gene thought to be involved in the biosynthesis of the pheromone, *daf-22(m130lf)* is not insensitive to wild-type pheromone-induced halothane resistance (Table 1). Thus, *daf-22* is required for the activity but not response to both the dauer and Pir pheromones, consistent with these two pheromones being synthesized by a DAF-22-dependent mechanism and being the same pheromone.

Dauer pheromone is detected by a set of sensory organs called amphids, which contain specialized ciliated sensory neurons exposed to the environment through a pore in the cuticle (ALBERT *et al.* 1981; BARGMANN and HORVITZ 1991). We tested mutants in genes re-

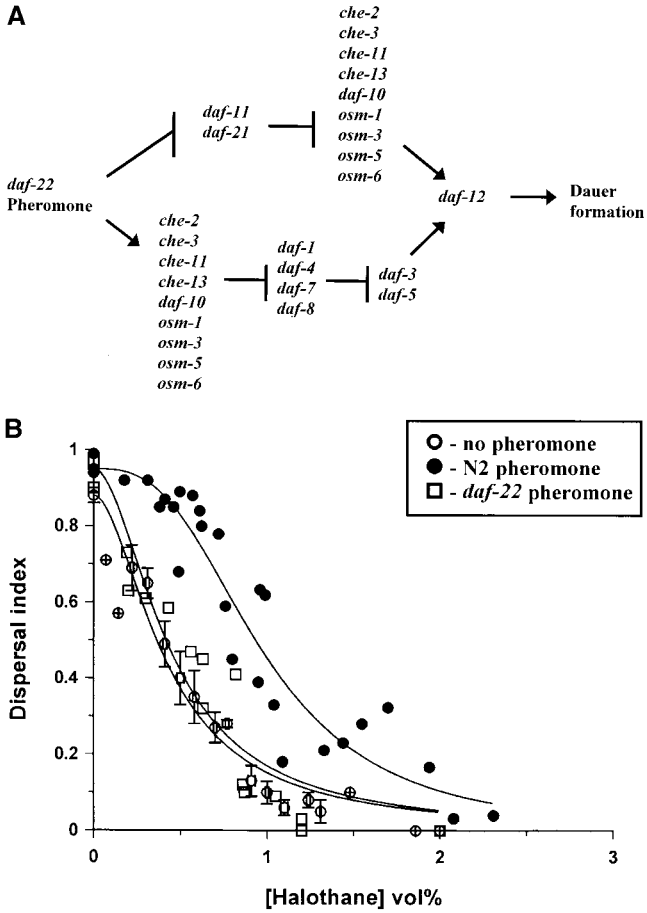

FIGURE 3.—Effect of dauer-formation mutants on Pir. (A) The genetic pathway for control of dauer formation (THOMAS *et al.* 1993). The *daf-2* branch of the pathway is not shown because it was not tested for effects on Pir. (B) Comparison of the effects of no pheromone, N2 pheromone, and pheromone made from *daf-22(m130lf)* on halothane sensitivity. *daf-22(m130lf)* disrupts production of dauer pheromone (GOLDEN and RIDDLE 1985). Halothane sensitivity was measured by halothane concentration/response curves against the dispersal behavior.

TABLE 1

Effect of mutations in dauer pathway genes on pheromone-induced halothane resistance

Genotype	Halothane EC_{50} Without pheromone	With pheromone	Fold change[a]
N2 (wild type)	0.42 ± 0.03	0.95 ± 0.02	2.3[b]
daf-22(m130)	0.48 ± 0.03	0.70 ± 0.05	1.5[b]
daf-11(m47)	0.29 ± 0.06	0.30 ± 0.07	1.0
daf-21(p673)	0.64 ± 0.12	0.62 ± 0.07	1.0
osm-1(p808)	0.56 ± 0.07[c]	0.78 ± 0.03	1.4
osm-3(p802)	0.50 ± 0.03	0.52 ± 0.03	1.0
osm-5(p813)	0.50 ± 0.01	0.50 ± 0.01	1.0
osm-6(p811)	0.33 ± 0.01	0.33 ± 0.01	1.0
che-2(e1033)	0.42 ± 0.03	0.31 ± 0.01	0.74
che-3(e1124)	0.52 ± 0.04	0.42 ± 0.03	0.81
che-11(e1810)	0.35 ± 0.01	0.37 ± 0.01	1.1
che-13(e1805)	0.42 ± 0.01	0.31 ± 0.01	0.74
daf-10(e1387)	0.39 ± 0.07	0.38 ± 0.04	1.0
daf-1(m40)	0.21 ± 0.02[d]	0.37 ± 0.06	1.8[b]
daf-4(e1364)	0.38 ± 0.06	0.95 ± 0.17	2.5[b]
daf-7(e1372)	0.20 ± 0.08[d]	0.36 ± 0.07	1.8[b]
daf-8(e1393)	0.13 ± 0.07[d]	0.38 ± 0.01	2.9[b]
daf-3(e1376)	0.63 ± 0.06[c]	0.92 ± 0.16	1.5[b]
daf-5(e1386)	0.55 ± 0.06[c]	0.80 ± 0.11	1.5[b]
daf-12(m20)	0.59 ± 0.04[c]	0.91 ± 0.06	1.5[b]

[a] Ratio of the halothane EC_{50} with N2 pheromone to the halothane EC_{50} without pheromone.

[b] A significant increase in halothane EC_{50} with pheromone *vs.* without ($P < 0.05$); statistical significance determined by simultaneous curve fitting (WAUD 1972; DELEAN *et al.* 1978).

[c] Significantly resistant compared to N2 without pheromone condition ($P < 0.05$).

[d] Significantly hypersensitive compared to N2 without pheromone condition ($P < 0.05$).

quired for the function of the amphid neurons and found that, as for dauer formation, these mutants are Pir defective (Table 1). Thus, the Pir activity requires the normal function of the amphid neurons, which likely detect the pheromone. *daf-11* and *daf-21* code for homologs of transmembrane guanylyl cyclase and HSP-90, respectively, both of which function upstream of the cilium structure genes to regulate dauer formation (BIRNBY *et al.* 2000). Both *daf-11* and *daf-21* mutants are Pir defective (Table 1), a result consistent with the proposed role of these genes in the function of amphid neurons. Reduction-of-function mutants in the TGFβ-SMAD arm (*daf-1*, *daf-4*, *daf-3*, and so on; PATTERSON and PADGETT 2000) of the dauer pathway were normally responsive to pheromone-induced resistance to halothane (Table 1). However, mutants reducing TGβ signaling (*daf-1*, *daf-7*, and *daf-8*) did increase native halothane sensitivity (*i.e.*, in the absence of pheromone), and *daf-3*, *daf-5*, and *daf-12* mutants, which suppress the Daf-c phenotypes of *daf-1*, *daf-7*, and *daf-8*, were significantly halothane resistant. Thus, while pheromone-induced VA resistance is not particularly sensitive to alterations in TGFβ-SMAD signaling, native VA sensitivity appears to be.

***goa-1* signaling pathway:** Various clues suggested the *C. elegans* Go pathway as a candidate for a mediator of pheromone's antagonism of anesthetics. G-protein α-subunits transduce pheromone action in yeast (SONG and DOHLMAN 1996; DAVIS and DAVEY 1997; LEBERER *et al.* 1997). We have previously found that a dominant-negative mutation in the *goa-1* gene, which codes for the α-subunit of Go (MENDEL *et al.* 1995; SEGALAT *et al.* 1995), and gain-of-function mutations in *egl-10*, which codes for an RGS protein negatively regulating GOA-1 (KOELLE and HORVITZ 1996), confer a twofold resistance to halothane, similar in magnitude to that produced by pheromone (VAN SWINDEREN *et al.* 2001). We tested mutants in the *goa-1* pathway to determine their role, if any, in pheromone signaling. We found that all

TABLE 2

Effect of Goα pathway mutations on Pir

Genotype	Halothane EC_{50}[a]		Fold change	Mutation
	Without pheromone	With pheromone		
N2	0.42 ± 0.03	0.95 ± 0.02	2.3*	None
goa-1(sy192)	1.07 ± 0.04[b]	0.80 ± 0.08	0.7	Dominant negative in Goα
goa-1(n363)	0.43 ± 0.03	0.52 ± 0.05	1.2	Null
goa-1(n1134)	0.47 ± 0.04	0.43 ± 0.04	0.9	Null
goa-1(pk62)	0.45 ± 0.05	0.48 ± 0.01	1.1	Rf
egl-10(nIs51)	1.06 ± 0.04[b]	0.88 ± 0.04	0.8	Gf in RGS for Goα
egl-10(n480)	0.52 ± 0.01	0.47 ± 0.04	0.9	Rf
egl-10(md176)	0.45 ± 0.12	0.29 ± 0.05	0.6	Rf
sag-1(sy428)	0.28 ± 0.02	0.24 ± 0.02	0.9	Lf in DGK-1
eat-16(sy438)	0.77 ± 0.04[b]	0.85 ± 0.08	1.1	Lf in RGS for Gqα

Rf, reduction of function; Gf, gain of function; Lf, loss of function. *Significant Pir, $P < 0.05$.

[a] All EC_{50}'s were calculated from at least three independent experiments with at least six halothane concentrations/experiment.

[b] Significantly resistant compared to N2 without pheromone condition.

mutations that reduce GOA-1 signaling are Pir defective, including the normally halothane-sensitive *goa-1*(null) alleles, the already halothane-resistant dominant-negative *goa-1(sy192)* allele, and *egl-10(gf)* alleles (Table 2). Interestingly, *egl-10(lf)* mutants are also Pir defective. Thus, both diminished and enhanced Goα signaling disrupt pheromone's halothane antagonism. The regulation of *C. elegans* locomotion by GOA-1 is dependent on the normal function of diacyl glycerol kinase, coded for by *sag-1* (*s*uppressor of *a*ctivate *goa-1*), also known as *dgk-1* (Hajdu-Cronin *et al.* 1999). We tested a *sag-1(rf)* mutant to determine if pheromone signaling also utilized this pathway. Indeed, *sag-1(sy428)* was found to be Pir defective (Table 2). Like *goa-1*(null) mutants, *sag-1(sy428)* is not halothane resistant in the absence of pheromone, indicating that pheromone does not produce halothane resistance solely by inhibiting the GOA-1/DGK-1 pathway. Goα acts antagonistically with Gqα to regulate transmitter release, Goα negatively regulating release and Gqα positively regulating it (Hajdu-Cronin *et al.* 1999; Lackner *et al.* 1999; Miller *et al.* 1999; Nurrish *et al.* 1999). *eat-16(sy438)* is a reduction-of-function mutant isolated as a suppressor of *goa-1(gf)* (Hajdu-Cronin *et al.* 1999). *eat-16* codes for an RGS protein that functions to negatively regulate Gqα and positively regulate Goα. Thus, we predicted that *eat-16(rf)* mutants should phenocopy the Pir-defective phenotype of *goa-1(lf)* and *egl-10(gf)*. Indeed, *eat-16(sy438)* is Pir defective. Moreover, like the dominant-negative *goa-1(sy192)* and *egl-10(gf)*, but unlike *goa-1(lf)*, *sy438* is halothane resistant in the absence of pheromone. These data all support a role for Goα and Gqα in pheromone's antagonism of halothane.

Neurotransmitter/receptor pathways: What might be the neurotransmitter/receptor systems through which pheromone antagonizes halothane? We tested neurotransmitter/receptor mutants that might reasonably affect Pir (Table 3). Multiple lines of evidence suggest that VAs may act in part in the vertebrate nervous system by enhancing GABAergic signaling (Jones *et al.* 1992; Franks and Lieb 1994, 1998; Mihic *et al.* 1997; Koltchine *et al.* 1999; Jenkins *et al.* 2001). We hypothesized that pheromone might produce resistance by modulating the effect of VAs on GABAergic signaling. To examine this hypothesis, we measured the Pir phenotypes of *unc-25(lf)* and *unc-49(lf)*, which each abolish GABA neurotransmission in *C. elegans* neurons by defects in GABA synthesis and in the $GABA_A$ receptor, respectively (McIntire *et al.* 1993; Bamber *et al.* 1999; Richmond and Jorgensen 1999). We found that neither *unc-25 (e156lf)* nor *unc-49(e382lf)* altered Pir nor were these mutants halothane resistant in the absence of pheromone (Table 3). These results eliminate the possibility of the $GABA_A$ receptor mediating halothane action against locomotion in *C. elegans*. Another reasonable candidate for a neurotransmitter pathway either mediating or modulating Pir is the serotonergic pathway. In *C. elegans*, serotonin signals through GOA-1 and could be the neurotransmitter that pheromone is modulating (Mendel *et al.* 1995; Segalat *et al.* 1995; Nurrish *et al.* 1999). However, *bas-1(ad446)*, which lacks serotonin immunoreactivity, *cat-2(e1112)*, which is defective in dopamine synthesis, and *cat-4(e1141)*, which disrupts both dopamine and serotonin-mediated signaling (Loer and Kenyon 1993), were all wild type for Pir (Table 3). Thus, the neurotransmitter system through which pheromone modulates halothane's action on locomotion is unclear.

Presynaptic machinery: Several lines of evidence indicate that VAs inhibit the release of neurotransmitters in both vertebrates and *C. elegans* (Zorychta and Capek 1978; Takenoshita and Takahashi 1987; Kullmann *et al.* 1989; Miao *et al.* 1995; Perouansky *et al.* 1995;

TABLE 3

Effect of neurotransmitter/receptor mutations on Pir

Strain	Halothane EC_{50}[a] Without	With	Fold change	Mutation
N2	0.42 ± 0.03	0.95 ± 0.02	2.3*	None
unc-49(e382)	0.37 ± 0.05	0.75 ± 0.13	2.0*	Rf in GABA receptor
unc-25(e156)	0.30 ± 0.02	0.52 ± 0.07	1.7*	Null in GAD
cat-2(e1112)	0.33 ± 0.06	1.01 ± 0.05	3.1*	Rf in dopamine biosynthesis
bas-1(ad446)	0.56 ± 0.13	1.15 ± 0.20	2.1*	Rf in serotonin biosynthesis
cat-4(e1141)	0.38 ± 0.11	0.75 ± 0.14	2.0*	Rf in dopamine/serotonin biosynthesis
glr-1(n2461)	0.64 ± 0.04	1.08 ± 0.13	1.7*	Rf in glutamate receptor

Rf, reduction of function; Gf, gain of function; GAD, glutamic acid decarboxylase. *Significant Pir, $P < 0.05$.

[a] All EC_{50}'s were calculated from at least three independent experiments with at least six halothane concentrations/experiment.

Schlame and Hemmings 1995; MacIver *et al.* 1996; van Swinderen *et al.* 1999, 2001; Nishikawa and MacIver 2000). In *C. elegans*, mutations that reduce transmitter release are VA hypersensitive and those that increase release are resistant (van Swinderen *et al.* 1999, 2001). The extreme exception is *unc-64(md130)*, which has reduced transmitter release yet is markedly resistant to halothane and other VAs, more so than that produced by pheromone treatment or by *goa-1(rf)* mutants (van Swinderen *et al.* 1999). These large allelic differences that cannot be explained by an indirect effect on transmitter release implicate syntaxin or syntaxin-binding proteins as an essential, perhaps binding, component of the VA mechanism. We investigated the effect of mutations in genes involved in presynaptic release of neurotransmitter on the Pir phenotype (Table 4). Both the VA-resistant *unc-64* syntaxin allele *md130* and the VA hypersensitive allele *js21* responded to pheromone by increasing their halothane EC_{50}'s (Table 4). A double-mutant strain carrying both *goa-1*(null) and *unc-64(md130)* combined the properties of the two mutants. The strain was halothane resistant in the absence of pheromone like *unc-64(md130)* but was unresponsive to pheromone like *goa-1(n363)*. These results indicate that unlike *goa-1(lf)* mutations, the mechanism whereby syntaxin mutations alter VA sensitivity does not disrupt pheromone's mechanism, and the resistance of *unc-64 (md130)* does not depend on pheromone signaling. Likewise, reduction-of-function mutations in *ric-4* and *snb-1*, which code for the syntaxin-binding SNARE proteins SNAP-25 and VAMP (Rand and Nonet 1997; Nonet *et al.* 1998), respectively, do not block Pir.

To test whether pheromone itself alters transmitter release, we measured pheromone's effects on aldicarb sensitivity. Aldicarb is an acetylcholinesterase inhibitor that is routinely used to assess the effect of mutations on transmitter release (Rand and Nonet 1997). Mutations or drugs that confer resistance to aldicarb generally reduce neurotransmitter release at the neuromuscular junction while aldicarb hypersensitivity is found in mutants with increased cholinergic neurotransmission. We have previously shown that VAs induce aldicarb resis-

TABLE 4

Effect of presynaptic machinery mutants on Pir

Strain	Halothane EC_{50}[a] Without	With	Fold change	Mutation
N2	0.42 ± 0.03	0.95 ± 0.02	2.3	
snb-1(md247)	0.11 ± 0.04[b]	0.19 ± 0.06	1.7*	Rf in VAMP
ric-4(md1088)	0.22 ± 0.04[b]	0.66 ± 0.08	3.0*	Rf in SNAP-25
unc-64(js21)	0.17 ± 0.03[b]	0.45 ± 0.05	2.6*	Rf in syntaxin
unc-64(md130)	1.06 ± 0.11[c]	2.08 ± 0.23	2.0*	Rf in syntaxin
goa-1(n363);unc-64(md130)	1.33 ± 0.06[c]	1.40 ± 0.06	1.1	*goa-1(null);unc-64(rf)*

Rf, reduction of function. *Significant Pir, $P < 0.05$.

[a] All EC_{50}'s were calculated from at least three independent experiments with at least six halothane concentrations/experiment.

[b] Significantly hypersensitive compared to N2 under "without pheromone" condition at $P < 0.05$.

[c] Significantly resistant compared to N2 under "without pheromone" condition at $P < 0.05$.

tance, indicating that VAs inhibit acetylcholine release; *unc-64(md130)* but not *goa-1(lf)* blocks VA-induced aldicarb resistance, suggesting that VAs act downstream of GOA-1 but upstream of UNC-64 to inhibit transmitter release (van Swinderen *et al.* 1999, 2001). Pheromone might produce VA resistance by increasing transmitter release and thereby indirectly antagonizing VAs. However, pheromone had no effect on native aldicarb sensitivity (Figure 4A) nor did it alter the potency of VAs to induce aldicarb resistance (Figure 4B). Thus, as for locomotion, pheromone does not appear to alter indirectly the effects of halothane on cholinergic neurotransmitter release in the absence of VAs.

DISCUSSION

The mechanism of volatile anesthetics has long been postulated to be nonspecific. Nonspecific theories of anesthesia propose that volatile anesthetics imbed into membranes and disrupt membrane structure, thereby altering the function of numerous membrane-associated proteins. These theories predict that high-level resistance to anesthetics cannot be achieved by altering a single mechanism. The lack of specific antagonism of volatile anesthetics by a drug has heretofore been a pharmacologic argument for nonspecific theories of anesthesia. Pheromone's antagonism of halothane now demonstrates that pharmacologic antagonism of VAs is biologically possible.

A pathway for pheromone's effects on halothane action consistent with the genetic data is shown in Figure 5. The pathway is drawn to summarize and discuss the data, but given the lack of testable null mutants in syntaxin and syntaxin-interacting proteins as well as other issues discussed below, the pathway should be considered a working model at this point. Pheromone-induced halothane resistance is dependent on the normal function of the amphid sensory neurons. If pheromone constitutively antagonized VAs through the amphid neurons, then the cilium structure mutants that disrupt amphid function would be expected to have abnormal VA sensitivities. However, the cilium structure mutants have normal halothane sensitivities in the absence of applied pheromone, indicating that amphid neurons regulate VA sensitivity only in the presence of high pheromone concentrations. At least from an anatomical standpoint, the amphid neurons might reasonably modulate VA effects on locomotion, given that they synapse onto interneurons that coordinate locomotion (Chalfie *et al.* 1985; White *et al.* 1986).

We have previously shown that *goa-1*(null) mutants are isoflurane but not halothane resistant, whereas *sy192*, a dominant-negative *goa-1* allele, and mutants in the RGS-protein-coding genes, *egl-10* and *eat-16*, are both halothane and isoflurane resistant (van Swinderen *et al.* 2001). Pheromone also produces both halothane (Figure 2) and isoflurane resistance (data not shown). How do pheromone, *sy192*, and *egl-10(gf)* produce halothane resistance when *goa-1*(null) mutations do not? Given that loss-of-function mutants in *eat-16*, which normally negatively regulates *egl-30*, are both halothane and isoflurane resistant and pheromone unresponsive, we speculate that pheromone, *sy192*, and *egl-*

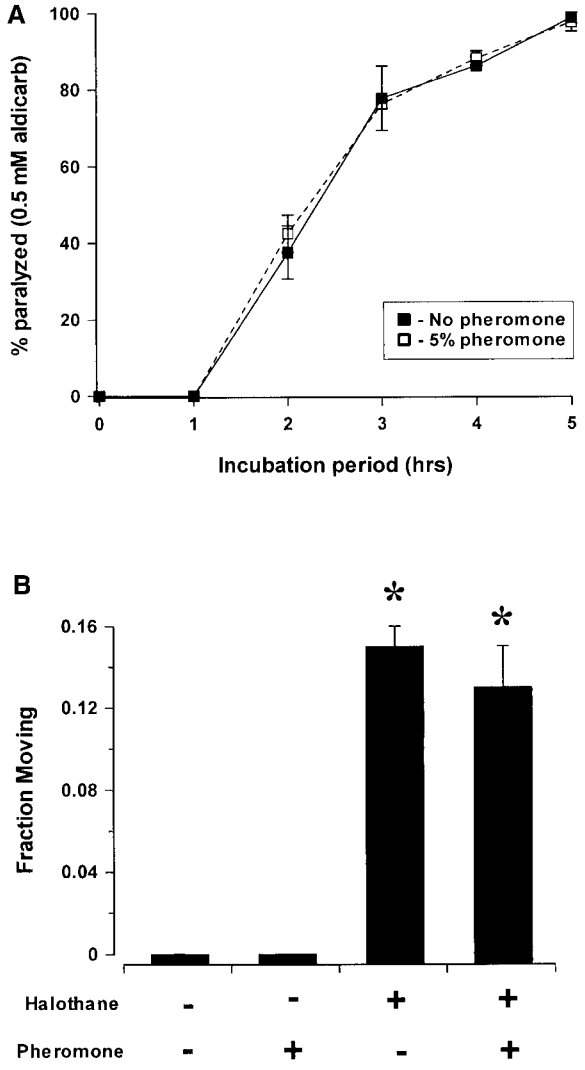

Figure 4.—Effect of pheromone on aldicarb action. Young adult N2 were placed on plates containing 0.5 mM aldicarb that either did or did not contain, in addition, 5% pheromone. (A) 30–50 animals per condition were scored for paralysis (lack of movement in response to touch) immediately and at 1, 2, 3, 4, and 5 hr after placement on the plates. The percentage paralyzed represents mean ± SEM for two independent experiments for each condition. The percentage paralyzed on pheromone-containing plates was not significantly different from the percentage without pheromone at any timepoint. (B) N2 were preincubated for 4 hr on aldicarb plates with or without pheromone and then moved to a 0.5-cm-diameter circle marked on the plates. The plates were then placed into chambers containing either no halothane or 0.2–0.5 vol% halothane, and the fraction of animals (n = 20–40/plate) moving out of the circle after 1 hr was scored as the movement index. Halothane produced a significant increase in the movement index (*$P < 0.05$ by ANOVA). The movement indices for the pheromone-containing plates were not significantly different from the no-pheromone plates.

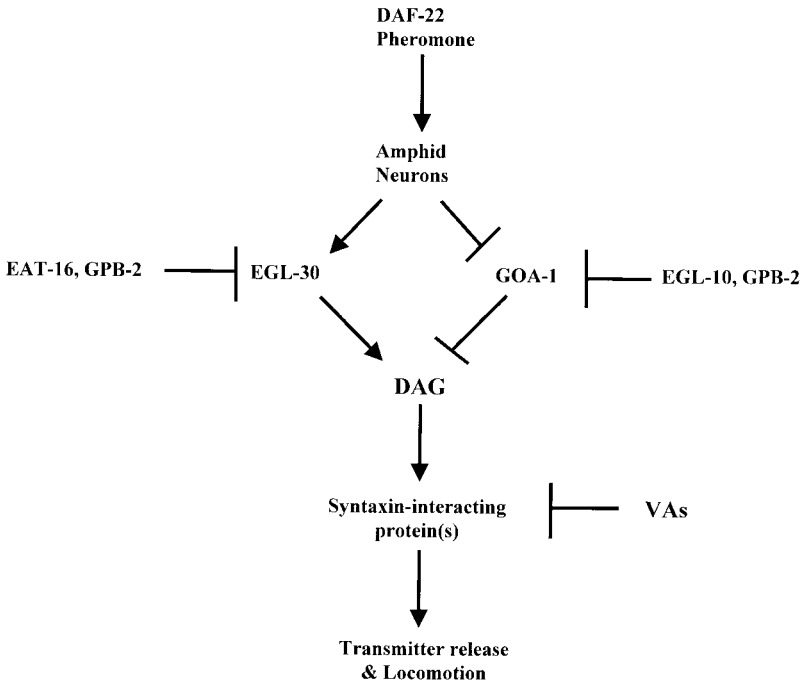

FIGURE 5.—Proposed pathway for pheromone-induced VA resistance. Transduction of the pheromone signal requires and is presumably sensed by the amphid sensory neurons. Pheromone signaling through the amphid neurons or in the neuronal pathway downstream of amphid neurons requires the normal activity of both Goα- and Gqα-coupled pathways. The proposal of a positive modulation by amphid neurons on Gqα activity, coded for by *egl-30*, and an inhibitory modulation on Goα signaling is based on the VA resistance of *goa-1(lf)* and *egl-30(gf)* (VAN SWINDEREN *et al.* 2001). GOA-1, EGL-30, and their RGS proteins, EGL-10 and EAT-16, respectively, have previously been shown to modulate VA action against locomotion and transmitter release (VAN SWINDEREN *et al.* 2001). On the basis of the high-level resistance of a dominant-negative form of syntaxin (VAN SWINDEREN *et al.* 1999), VAs are shown to inhibit directly the function of a syntaxin-interacting protein that positively regulates transmitter release; however, the data are also consistent with VAs enhancing the function of a negative regulator of release.

10(gf) alter both *goa-1* and *egl-30* signaling. A mechanism whereby this might occur has recently been delineated through the characterization of mutants of *gpb-2*, which codes for $G\beta_5$-like protein. GPB-2 was shown to interact with both EGL-10 and EAT-16 (CHASE *et al.* 2001; ROBATZEK *et al.* 2001; VAN DER LINDEN *et al.* 2001) and thereby enhance the GTPase activity of both RGS proteins. EGL-10 overexpression not only inhibits the function of GOA-1 but also enhances the function of EGL-30 by sequestering GPB-2 away from EAT-16 (CHASE *et al.* 2001; ROBATZEK *et al.* 2001; VAN DER LINDEN *et al.* 2001). Likewise, *eat-16(lf)* enhances *egl-30* function while inhibiting that of *goa-1*. In light of these new findings, the stronger anesthetic and locomotion phenotypes of *goa-1 (sy192)* compared to *goa-1*(null) can be explained by a dominant-negative action against GPB-2 and thereby enhancement of EGL-30 function. Thus, we propose that the halothane resistance of pheromone, *sy192*, and the RGS mutants is produced primarily by increasing the activity of Gqα and that the *goa-1*(null) mutants block the effect of pheromone by increasing Gqα activity to a level that cannot be further increased by pheromone.

An instructive aspect of pheromone's action is its lack of effect on the behavior and synapses that are disrupted by VAs. Importantly, this specificity indicates that pheromone does not antagonize VAs by indirectly enhancing locomotion and transmitter release. However, it is puzzling how pheromone does this. Two explanations are reasonable. One possibility is that pheromone actually binds to VA targets and directly inhibits the binding of VAs without altering the normal function of the VA target. While certainly appealing, this explanation seems unlikely, given that the Pir is abolished by *goa-1* null mutations, which themselves are not halothane resistant; therefore, GOA-1 cannot be the primary target for halothane. For pheromone to be a direct antagonist, one would have to postulate that the binding of pheromone is dependent on the activity of *goa-1*. The fact that mutants that disrupt sensory neurons are Pir defective would suggest that the pheromone acts primarily there, whereas motor neurons are the most likely cellular site for VA effects against locomotion and cholinergic neurotransmission (VAN SWINDEREN *et al.* 1999). We have previously shown that the high-level halothane and isoflurane resistance produced by *unc-64(md130)* is partially diminished by a *goa-1*(null) mutant (VAN SWINDEREN *et al.* 2001). However, pheromone-induced resistance is additive to *md130*'s. Like the *goa-1*(null); *unc-64(md130)* data, this result shows that the resistance activity of the *md130* product can be modulated. These data also show that pheromone does not simply inhibit *goa-1* function [*i.e.*, pheromone does not phenocopy *goa-1*(null)]. Our current working hypothesis is that pheromone, Goα, and Gqα signaling alter the structure (perhaps by changing the phosphorylation state) or abundance of a protein or proteins to which VAs and the dominant-negative truncated syntaxin bind. Investigations are underway to identify this VA target.

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