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Mutations Conferring New Patterns of Sensitivity to Volatile Anesthetics in Caenorhabditis elegans

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Background: **We previously described the use of the nematode *Caenorhabditis elegans* as a genetic model for studying the mechanism of action of volatile anesthetics. All previous strains of *C. elegans* with altered responses to anesthetics have been identified by screening the response to halothane. The current study was designed to identify classes of mutations by screening for alterations in sensitivity to enflurane, isoflurane, and diethylether.**

Methods: **Nematodes were mutated and the resulting mutant strains were screened for immobility in low doses of enflurane, isoflurane, or diethylether. Concentrations of halothane, enflurane, isoflurane, and diethylether that anesthetized 50% of the animals were determined in all mutations. Interactions of some new mutations with previously identified mutations were determined by construction of double mutants.**

Results: **Mutations in six genes were identified and were divided into two classes. One class primarily affected sensitivity to enflurane and isoflurane; a second class affected sensitivity to all of the volatile anesthetics studied. The effects of the latter group dominated the effects of previously identified mutations.**

Conclusions: **The interaction of these mutations indicates that multiple sites of anesthetic action exist and that there are at least three such sites. A pathway for control of sensitivity to volatile anesthetics is proposed. (Key words: Anesthetics, volatile: diethylether; enflurane; halothane; isoflurane. Animals, nematodes: *Caenorhabditis elegans*. Genetics.)**

THE sites and mechanisms of action of volatile anesthetics remain unknown. Meyer[1] and Overton[2] described the relation between the potencies and lipid solubilities of these anesthetics, generally termed the Meyer-Overton relation. It states that the log of the concentrations anesthetizing 50% of the population (EC_{50}s) of gaseous anesthetics is directly related to the log of their oil–gas partition coefficients in all species and for all volatile anesthetics. The slope of this line always closely approximates −1.[3] Such a relation had suggested that all volatile anesthetics exert their effect at a single type of site, for all volatile anesthetics in all species,[3] a model termed the "unitary hypothesis" of general anesthesia.

The unitary hypothesis has been called into doubt by several studies.[4–9] These reports indicate that volatile anesthetics act at more than one site of action. They also demonstrate that the volatile anesthetics have differing characteristic patterns of effects at these sites.

We have previously reported mutations in six genes which specifically alter sensitivity to volatile anesthetics in the nematode *Caenorhabditis elegans*.[4] Two such mutations, *unc-79* and *unc-80*, greatly increase sensitivities to one group of anesthetics (halothane, chloroform, methoxyflurane, and thiomethoxyflurane) but not to others (enflurane, isoflurane, fluroxene, and flurothyl). A qualitatively different alteration in sensitivity to diethylether is also seen with *unc-79* and *unc-80*.

The mutations *unc-1, unc-7, unc-9,* and *unc-24* exhibit a different profile of altered sensitivities. Each of these four mutations acts as a suppressor of the increased sensitivities of *unc-79* and *unc-80* to halothane, chloroform, methoxyflurane, and thiomethoxyflurane; that is, they return the increased sensitivities to normal. By themselves, *unc-1, unc-7, unc-9,* and *unc-24* each cause an increased sensitivity only to diethylether. These mutations do not suppress the altered sensitivities of *unc-79* and *unc-80* to diethylether. We have postulated that multiple sites of action of volatile anesthetics must exist in *C. elegans* to explain these mutations.[4]

All of the mutations mentioned above were isolated by screening the response of mutants to the anesthetic, halothane.[4,10,11] Other sites not affected by any identified mutations may control sensitivities to the group of volatile anesthetics represented by enflurane, isoflurane, fluroxene and flurothyl, or cause larger changes

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in sensitivity to diethylether. To characterize other sites of action of volatile anesthetics in *C. elegans,* we attempted to identify mutations affecting sensitivities to the anesthetics enflurane, isoflurane, and diethylether.

We report here the identification of new genetic mutations which alter sensitivities to enflurane, isoflurane, or diethylether. However, and somewhat surprisingly, some of the new mutations make the animal very sensitive to *all* the volatile anesthetics we have tested. The results of these studies strengthen the theory that multiple sites of anesthetic action do exist, but also indicate that some gene products may act on sites or in pathways common to all anesthetics. The molecular characterization of anesthetic sites remains a prerequisite for understanding the mode of action of volatile anesthetics. These mutations should serve as tools for identifying, at the molecular level, the different sites of action of volatile anesthetics.

Materials and Methods

Nematodes

Certain nematodes were obtained from the *Caenorhabditis* Genetics Center (Columbia, MO). These included *unc-79* (*e1068*), *unc-80* (*e1272*), and the wild-type (*i.e.,* nonmutated) *C. elegans* strain (N2). These strains are the canonical strains for these mutations and the wild type; they therefore were used to test all other isolates and also were used as genetic markers. *m48* was supplied as the double mutation *m48;unc-24* (Don Riddle, Columbia, MO). Methods for raising, handling, and performing genetic crosses with *C. elegans* have been described elsewhere in detail.[12]

Mutagenesis

Nematodes were exposed to the potent mutagen ethylmethanesulfonate as described by Brenner.[12] The exposure to ethylmethanesulfonate was sufficient to generate, on average, one mutation per genome. The responses of offspring were screened in the first generation and second generation to identify dominant or recessive mutations respectively. All newly isolated mutants were mated to the N2 strain, and mutant animals were collected from the second-generation offspring. This procedure was repeated twice, such that three genetic outcrosses were performed on each new mutation. This was done to isolate single mutations in an animal effecting the changes in responses to anesthetics.

Screening

More than 10,000 mutated hermaphrodite animals treated as above were screened for immobility in enflurane, isoflurane or diethylether at concentrations corresponding to 50–60% of the respective EC_{50}s of N2.[4] At these doses N2 animals show an increase in activity, though uncoordinated, and are easily distinguishable from immobile animals. Immobile hermaphrodites were removed from the anesthetic; those regaining mobility were individually plated to produce isogenic offspring, which were retested. If the offspring also showed an increase in sensitivity, the strains were outcrossed, as described above, and studied further.

Ethylmethanesulfonate-treated animals also were screened for immobility in doses of enflurane, isoflurane, and diethylether corresponding to 150% of the EC_{50}s for N2. Animals moving in these concentrations were isolated and outcrossed as described above.

Dose–Response Curves

Procedures for exposing nematodes to anesthetics, scoring their responses, and measuring anesthetic concentrations, have been described in detail previously.[4,10,11] All measurements were done at 1 atmosphere at 22–24°C. Fifty nematodes of each strain were scored for mobility for ten seconds at each concentration of anesthetic. Immobility was used as the end point for anesthesia and in all cases immobility was reversible within 10–15 minutes of removal from the anesthetics. Each dose response curve consisted of a minimum of 12 concentrations which were expressed in percent volume at 1 atm and 22°C in air. EC_{50}s were the percents volume at which 50% of the animals were immobile for greater than ten seconds (for example, as listed in table 1). When animals that carried two mutations (*i.e., unc-79 fc21*) were scored, simultaneous cultures of animals carrying either mutation alone (*i.e., unc-79* or *fc21*) were scored in the same anesthetic chamber. Thus, the EC_{50}s of new mutations like *fc20, fc21,* and *fc34* differ slightly between tables. The EC_{50}s in tables 1 and 2 are the most accurate (800–1,000 animals scored) for each mutation, whereas those in tables 3–8 are used as controls for the double mutants (400 animals of each genotype).

Genetics

We crossed *fc20, fc21,* and *fc34* with other genetic markers on known chromosomes to identify their position on the genetic map. These methods are described elsewhere in detail.[12] Mutations are mapped by first

Table 1. EC_{50}s and Slope Constants for Four Strains of *C. elegans* in Four Anesthetics

Strain		H	E	ISO	DE
N2	EC_{50}s	3.2 ± 0.1	6.1 ± 0.1	7.1 ± 0.1	6.5 ± 0.2
N2	Slope constants	12.5 ± 4.1	18.1 ± 6.1	21.3 ± 5.6	16.8 ± 4.0
fc23	EC_{50}s	2.9 ± 0.1	4.5 ± 0.2*	4.2 ± 0.2*	6.0 ± 0.3
fc23	Slope constants	11.6 ± 3.0	9.6 ± 5.0	10.1 ± 6.6	10.7 ± 3.8
fc30	EC_{50}s	2.7 ± 0.1	4.2 ± 0.2*	4.0 ± 0.2*	5.8 ± 0.3
fc30	Slope constants	13.4 ± 4.3	8.8 ± 4.4	12.5 ± 5.7	11.4 ± 4.4
m48	EC_{50}s	2.8 ± 0.1	4.4 ± 0.2*	4.4 ± 0.2*	6.0 ± 0.3
m48	Slope constants	14.1 ± 5.0	7.8 ± 4.0	13.1 ± 5.5	11.9 ± 5.1

EC_{50}s ± SE and slope constants ± SE for four strains of *C. elegans* (*fc23, fc30, m48,* N2) in four anesthetics. H = halothane; E = enflurane; ISO = isoflurane; DE = diethylether.

* EC_{50} different from that of N2, $P < 0.01$.

performing crosses between N2 males and the mutant hermaphrodites. First-generation hermaphrodites are scored to determine if the mutations are dominant. If the mutation is recessive, the presence of the mutant phenotype in first-generation males (genotype X0) indicates that the mutation lies on the X chromosome.

By the above criteria, *fc21* was shown to be on the X chromosome (see Results). To determine its approximate position on the X chromosome, *fc21* was mated to the double mutant *lon-2unc-7* (see reference 12 for *C. elegans* genetic nomenclature). We scored Lon-2 non Unc-7 animals and Unc-7 non Lon-2 animals for the presence of *fc21*. If all Lon-2 non Unc-7 animals carry *fc21,* and Unc-7 non Lon-2 animals do not carry *fc21* then *fc21* ties to the right of *unc-7*. If all Unc-7 non Lon-2 animals and no Lon-2 non Unc-7 animals carry *fc21,* then *fc21* lies to the left of *lon-2*. If a mix occurs then *fc21* lies between the two markers. (Note that in *C. elegans* only one recombination event occurs per X chromosome.)

fc21 was also mapped over deletions on the X chromosome. In particular we used *mnDf41* which deletes part of the *let-2* cluster on the right end of the X chromosome. We crossed the double-mutant males *lon-2fc21* to the viable strain *mnDp1/+;unc-3mnDf41*. We scored the offspring for increased sensitivity to halothane, interpreted as *fc21/mnDf41*. The offspring of these self-fertilizing hermaphrodites were scored for the reappearance of the *lon-2fc21* phenotype to prove that the parent was in fact the heterozygote. By self fertilization, the strain *mnDp1/+;unc-3mnDf41* gave no offspring having an increased sensitivity to halothane.

Mutant strains containing non–X-linked mutations are crossed to an array of mutations, one from each linkage group. In these studies we used the dumpy (*dpy*) mutations *dpy-5* (chromosome I), *dpy-10* (II), *dpy-17* (III), *dpy-13* (IV), and *dpy-11* (V). We then scored the second-generation offspring for presence of the *dpy* and anesthetic-sensitivity gene in the same animals. Presence of anesthetic sensitivity in approximately 25% of the Dpy animals indicates that the new mutation is *not* on that linkage group. Presence of the increased sensitivity in less than 25% of the animals indicates linkage to that chromosome and gives an approximate map distance relative to the *dpy* gene. More detailed mapping must follow to closely map the genes.

We also performed crosses to construct the double mutants *unc-79;fc20, unc-79;fc21, fc34;unc-79, fc20;unc-80, unc-80;fc21,* and *fc34;unc-80*. We also constructed the double mutants *fc20;unc-1, fc34;unc-1, unc-1 fc21, fc20;unc-9, fc34;unc-9,* and *unc-9 fc21*. We exposed each of these strains to four anesthetics, halothane, enflurane, isoflurane, and diethylether, to determine their dose response curves.

Anesthetics

Halothane was purchased from Halocarbon Laboratories (North Augusta, SC). Enflurane, and isoflurane were purchased from Anaquest (Madison, WI) and diethylether from Fisher Scientific (Fair Lawn, NJ).

Statistical Methods

Regression analysis, EC_{50}s, slope constants, and standard errors (SEs) were calculated using the methods described by Waud.[13] The dose response curves fit the classic logistic distribution described by Waud. For each anesthetic, the EC_{50}s of all strains were compared using an analysis of variance to see if they satisfied the null hypothesis ($P < 0.05$). If this analysis indicated

Table 2. EC_{50}s and Slope Constants for Five Strains of *C. elegans* in Four Anesthetics

Strain		H	E	ISO	DE
N2	EC_{50}s	3.1 ± 0.05	6.2 ± 0.1	7.0 ± 0.1	6.6 ± 0.3
N2	Slope constants	10.8 ± 3.1	16.8 ± 7.1	14.1 ± 4.1	17.1 ± 7.4
fc20	EC_{50}s	1.2 ± 0.1*	2.9 ± 0.2*	3.6 ± 0.1*	4.8 ± 0.3*
fc20	Slope constants	3.0 ± 1.7†	3.8 ± 2.1†	3.4 ± 1.8†	10.8 ± 4.1
fc21	EC_{50}s	1.1 ± 0.1*	2.6 ± 0.2*	1.3 ± 0.2*‡	4.8 ± 0.3*
fc21	Slope constants	2.8 ± 2.1†	3.3 ± 1.1†	2.6 ± 1.9†	13.4 ± 4.1
fc28	EC_{50}s	1.3 ± 0.1*	2.8 ± 0.2*	3.6 ± 0.2*	5.0 ± 0.3*
fc28	Slope constants	3.1 ± 1.5*	3.6 ± 1.8†	2.9 ± 1.6†	12.1 ± 4.6
fc34	EC_{50}s	1.4 ± 0.1*	2.8 ± 0.2*	3.5 ± 0.2*	5.0 ± 0.3*
fc34	Slope constants	1.5 ± 1.0†	1.7 ± 1.1†	1.9 ± 0.9†	12.0 ± 3.3

EC_{50}s ± SE and slope constants ± SE for five strains of *C. elegans* (*fc20, fc21, fc28, fc34,* N2) in four anesthetics. Abbreviations are as in table 1.

* EC_{50} different from that of N2, $P < 0.01$.

† Slope constant different from that of N2, $P < 0.01$.

‡ EC_{50} different from that of *fc20, fc28, fc34,* $P < 0.01$.

that a difference existed within the group, we compared the individual mean values of each strain. Comparison of EC_{50}s for the different strains was performed by using the Studentized range (Q) for multiple comparisons.[14] Significance was defined as $P < 0.01$. The increased stringency was used to avoid Type I errors (detecting a difference when none actually existed). Variances for the differences between EC_{50}s were calculated by adding the variances of each EC_{50} involved. Because the EC_{50}s for *fc20, fc21,* and *fc34* in tables 3–8 were used as controls only, they were not compared with N2 or the other previously described single mutants (*unc-1, unc-9, unc-79,* and *unc-80*).

Results

Screens

Enflurane. Four mutations were isolated that immobilize worms in 3.5% enflurane (EC_{50} for N2, which is the normal worm, is 6.0%). These mutations were *fc20, fc21, fc23,* and *fc30.* In addition we screened a suppressor of *unc-24, m48,*[15] in enflurane, and found it to have an increase in sensitivity. No mutation resistant to 9% enflurane was isolated.

Diethylether. Three new mutations caused immobility to 3.5% diethylether (EC_{50} for N2, 6.8%). These mutations were *fc31, fc32,* and *fc34. fc31* and *fc32* were found to be new alleles of *unc-1,* which we already had shown to be sensitive to diethylether. Their dose response profile was not studied further at this time. No mutation resistant to 9% diethylether was isolated.

Isoflurane. Two mutations were identified in 3.5% Isoflurane (EC_{50} for N2, 6.8%). One, *fc26,* was a new allele of *unc-80* with a different pattern of sensitivity than seen in previous alleles, and will be discussed in a separate report. Another, *fc24,* was quite sluggish in air, sensitive to all anesthetics, and was not studied at this time. No mutation resistant to 9.5% isoflurane was isolated.

Dose–Response Curves

Mutants Sensitive Primarily to Enflurane and Isoflurane. Table 1 lists the EC_{50} values and slopes constants (mean ± SE) for four strains, N2, *fc23, fc30,* and *m48,* in four volatile anesthetics. We grouped *fc23, fc30,* and *m48,* because each shows increased sensitivities to enflurane and isoflurane, but no significant change in sensitivities to halothane and to diethylether.

Mutants Sensitive to All Volatile Anesthetics. Table 2 lists the EC_{50} values and slope constants (mean ± SE) for the five strains N2, *fc20, fc21, fc28,* and *fc34.* We grouped these mutants because each shows an increased sensitivity to anesthetics from all four groups despite being vigorous movers in air. It was also noted that the slope constants of this group were significantly lower than that of N2.

Genetics

Mapping. *fc20* was not observed to be X-linked. Crosses with *dpy* markers gave the following ratios for cosegregation in the second generation: *dpy-5* (I), (43/150) *fc20; dpy-10* (II), (29/84) *fc20; dpy-17* (III), (58/213) *fc20; dpy-13* (IV), (23/98) *fc20;* and *dpy-*

Table 3. The Effect of *fc20* on EC_{50}s and Slope Constants of *unc-79* and *unc-80* in Four Anesthetics

Strain		H	E	ISO	DE
N2	EC_{50}s	3.2 ± 0.1	6.3 ± 0.2	7.1 ± 0.3	6.5 ± 0.2
N2	Slope constants	10.9 ± 3.0	13.9 ± 6.1	16.1 ± 6.0	15.8 ± 4.4
unc-79	EC_{50}s	1.1 ± 0.05	6.7 ± 0.1	6.7 ± 0.3	4.6 ± 0.2
unc-79	Slope constants	3.4 ± 1.4	18.9 ± 7.0	19.3 ± 6.8	12.0 ± 3.0
unc-80	EC_{50}s	1.3 ± 0.1	6.4 ± 0.2	6.6 ± 0.3	4.7 ± 0.2
unc-80	Slope constants	4.1 ± 1.3	19.4 ± 6.2	21.3 ± 5.9	10.5 ± 4.1
fc20	EC_{50}s	1.3 ± 0.3	3.0 ± 0.3	3.0 ± 0.2	5.3 ± 0.2
fc20	Slope constants	3.1 ± 1.9	3.4 ± 2.1	3.8 ± 1.9	10.4 ± 3.8
unc-79;fc20	EC_{50}s	1.1 ± 0.2*	2.9 ± 0.2*	2.8 ± 0.2*†	5.1 ± 0.1*
unc-79;fc20	Slope constants	3.3 ± 2.3§	3.1 ± 1.8§¶	3.3 ± 2.1§¶	9.8 ± 4.1
fc20;unc-80	EC_{50}s	1.4 ± 0.2*	3.0 ± 0.2*‡	3.1 ± 0.2*‡	5.3 ± 0.2*
fc20;unc-80	Slope constants	3.9 ± 2.1§	2.9 ± 1.1§**	3.4 ± 2.1§**	10.0 ± 3.1

EC_{50}s ± SE and slope constants ± SE for 12 strains of *C. elegans* in four anesthetics. Abbreviations are as in table 1. In this table the EC_{50}s for N2, *fc20*, *fc21*, and *fc34* differ slightly from other tables in this article. This is because these strains were scored strictly as controls while testing the double mutants. Thus, they varied slightly from the cultures done on other days and the data in different experiments were not grouped.

* EC_{50} different from that of N2, $P < 0.01$.

† EC_{50} different from that of *unc-79*, $P < 0.01$.

‡ EC_{50} different from that of *unc-80*, $P < 0.01$.

§ Slope constant different from that of N2, $P < 0.01$.

¶ Slope constant different from that of *unc-79*, $P < 0.01$.

** Slope constant different from that of *unc-80*, $P < 0.01$.

11 (V), (20/137) *fc20*. Only crosses with *dpy-11* were notably different from 25% (14.6%). These data indicate that *fc20* is on chromosome V approximately 38 map units from *dpy-11*. Distances of this magnitude are unreliable and more detailed mapping must be done to localize this gene. Thus, *fc20* is located

Table 4. The Effect of *fc21* on EC_{50}s and Slope Constants of *unc-79* and *unc-80* in Four Anesthetics

Strain		H	E	ISO	DE
N2	EC_{50}s	3.2 ± 0.1	6.3 ± 0.2	7.1 ± 0.3	6.5 ± 0.2
N2	Slope constants	10.9 ± 3.0	13.9 ± 6.1	16.1 ± 6.0	15.8 ± 4.4
unc-79	EC_{50}s	1.1 ± 0.05	6.7 ± 0.1	6.7 ± 0.3	4.6 ± 0.2
unc-79	Slope constants	3.4 ± 1.4	18.9 ± 7.0	19.3 ± 6.8	12.0 ± 3.0
unc-80	EC_{50}s	1.3 ± 0.1	6.4 ± 0.2	6.6 ± 0.3	4.7 ± 0.2
unc-80	Slope constants	4.1 ± 1.3	19.4 ± 6.2	21.3 ± 5.9	10.5 ± 4.1
fc21	EC_{50}s	1.5 ± 0.4	2.7 ± 0.3	1.3 ± 0.3	4.8 ± 0.15
fc21	Slope constants	3.4 ± 2.0	3.1 ± 1.9	4.1 ± 2.5	1.4 ± 4.8
unc-79;fc21	EC_{50}s	1.4 ± 0.1*	2.8 ± 0.2*†	1.4 ± 0.1*†	4.7 ± 0.2*
unc-79;fc21	Slope constants	3.3 ± 1.9§	3.8 ± 2.3§¶	3.9 ± 2.1§¶	10.8 ± 4.3
unc-80;fc21	EC_{50}s	1.2 ± 0.2*	2.7 ± 0.2*‡	1.3 ± 0.3*‡	5.5 ± 0.2*
unc-80;fc21	Slope constants	3.6 ± 2.2§	3.3 ± 2.1§**	3.8 ± 1.8§**	9.8 ± 5.5

EC_{50}s ± SE and slope constants ± SE for 12 strains of *C. elegans* in four anesthetics. Abbreviations are as in table 1. In this table the EC_{50}s for N2, *fc20*, *fc21*, and *fc34* differ slightly from other tables in this article. This is because these strains were scored strictly as controls while testing the double mutants. Thus, they varied slightly from the cultures done on other days and the data in different experiments were not grouped.

* EC_{50} different from that of N2, $P < 0.01$.

† EC_{50} different from that of *unc-79*, $P < 0.01$.

‡ EC_{50} different from that of *unc-80*, $P < 0.01$.

§ Slope constant different from that of N2, $P < 0.01$.

¶ Slope constant different from that of *unc-79*, $P < 0.01$.

** Slope constant different from that of *unc-80*, $P < 0.01$.

Table 5. The Effect of *fc34* on EC_{50}s and Slope Constants of *unc-79* and *unc-80* in Four Anesthetics

Strain		H	E	ISO	DE
N2	EC_{50}s	3.2 ± 0.1	6.3 ± 0.2	7.1 ± 0.3	6.5 ± 0.2
N2	Slope constants	10.9 ± 3.0	13.9 ± 6.1	16.1 ± 6.0	15.8 ± 4.4
unc-79	EC_{50}s	1.1 ± 0.05	6.7 ± 0.1	6.7 ± 0.3	4.6 ± 0.2
unc-79	Slope constants	3.4 ± 1.4	18.9 ± 7.0	19.3 ± 6.8	12.0 ± 3.0
unc-80	EC_{50}s	1.3 ± 0.1	6.4 ± 0.2	6.6 ± 0.3	4.7 ± 0.2
unc-80	Slope constants	4.1 ± 1.3	19.4 ± 6.2	21.3 ± 5.9	10.5 ± 4.1
fc34	EC_{50}s	1.5 ± 0.3	3.0 ± 0.4	2.9 ± 0.4	4.9 ± 0.3
fc34	Slope constants	2.1 ± 1.1	3.0 ± 1.9	1.8 ± 1.1	12.2 ± 4.3
fc34;unc-79	EC_{50}s	0.7 ± 0.2*†§	3.1 ± 0.2*†	2.2 ± 0.15*†	4.8 ± 0.2*
fc34;unc-79	Slope constants	1.4 ± 0.9¶	1.9 ± 1.0¶**	1.7 ± 1.5¶**	12.4 ± 4.5
fc34;unc-80	EC_{50}s	0.9 ± 0.2*‡§	2.8 ± 0.2*‡	2.1 ± 0.2**	4.7 ± 0.2*
fc34;unc-80	Slope constants	1.1 ± 0.9¶	1.3 ± 1.1¶††	1.4 ± 1.2¶††	14.9 ± 4.4

EC_{50}s ± SE and slope constants ± SE for 12 strains of *C. elegans* in four anesthetics. Abbreviations are as in table 1. In this table the EC_{50}s for N2, *fc20, fc21,* and *fc34* differ slightly from other tables in this article. This is because these strains were scored strictly as controls while testing the double mutants. Thus, they varied slightly from the cultures done on other days and the data in different experiments were not grouped.

* EC_{50} different from that of N2, $P < 0.01$.

† EC_{50} different from that of *unc-79*, $P < 0.01$.

‡ EC_{50} different from that of *unc-80*, $P < 0.01$.

§ EC_{50} different from that of *fc34*, $P < 0.01$.

¶ Slope constant different from that of N2, $P < 0.01$.

** Slope constant different from that of *unc-79*, $P < 0.01$.

†† Slope constant different from that of *unc-80*, $P < 0.01$.

on chromosome V. It fails to complement another newly isolated mutation, *fc28,* and thus is allelic to that mutation. *fc28* was isolated in a separate screen for increased sensitivity to halothane. Each allele is recessive and confers a very mild abnormal motion described as kinked.[12]

fc21 confers a decreased brood size (temperature-sensitive trait), and a slight decrease in locomotion. Because 60 of 60 first-generation males in a cross with N2 were anesthetic-sensitive, *fc21* was noted to be X-linked. *fc21* was mapped to the right portion of the X chromosome by recombination with *unc-7* and *lon-2* using the double mutant *lon-2 unc-7* (see Materials and Methods). Ten of 10 Lon-2 non Unc-7 contained *fc21,* whereas 0 of 11 Unc-7 non Lon-2 contained *fc21.* Thus, *fc21* lies to the right of *unc-7,* on the right end of the X chromosome, near a region known as the *let-2* cluster. Crosses between *lon-2fc21* males and the strain *mnDp1/+;unc-3 mnDf41* gave 10 anesthetic sensitive offspring. These animals exhibited a decreased brood size (1–7) and slow locomotion. 30% of their offspring were long animals indicating they were indeed the heterozygote *lon-2fc21/unc-3mnDf41.*

fc23 and *fc30* are recessive mutations with different phenotypes in air. *fc23* worms are short and exhibit vigorous motion. *fc30* worms are normal in length and their motion is mildly slow in air. We have not mapped *fc23* and *fc30* but by complementation studies they are not alleles of each other or any other gene isolated in these studies. *m48* is a dominant mutation that suppresses *unc-1* and *unc-9*[15] and maps to chromosome I, near *dpy-5.*

We mapped *fc34* by crossing these mutant animals with *dpy* markers as with *fc20.* We obtained the following results: *dpy-5* (I), 74 of 329 *fc34;dpy-10* (II), 19 of 200 *fc34;dpy-17* (III), 52 of 209 *fc34;dpy-13* (IV), 69 of 211 *fc34;dpy-11* (V), and 42 of 150 *fc34.* We interpret these data to indicate that *fc34* lies on chromosome II about 30 map units from *dpy-10.* The high percent of Dpy animals that are anesthetic sensitive seen with *dpy-13* is curious and may represent difficulty scoring the "shrinker" phenotype of *fc34* in a Dpy background. We are presently finely mapping *fc34* using PCRable markers to finalize its position. The motion of *fc34* worms is absolutely normal in air. In addition to the pronounced increase in sensitivity to all volatile anesthetics, *fc34* confers an additional abnormal phenotype. In the presence of anesthetics these worms shorten dramatically along their longitudinal axis. This shrinking is semidominant; it occurs in a milder form in the heterozygote

Table 6. The Effect of *fc20* on EC_{50}s and Slope Constants of *unc-1* and *unc-9* in Four Anesthetics

Strain		H	E	ISO	DE
N2	EC_{50}s	3.2 ± 0.2	6.5 ± 0.3	7.2 ± 0.4	6.6 ± 0.2
N2	Slope constants	14.1 ± 5.3	18.9 ± 6.1	20.1 ± 6.0	17.2 ± 6.4
unc-1	EC_{50}s	3.4 ± 0.2	6.4 ± 0.2	7.0 ± 0.3	4.7 ± 0.2
unc-1	Slope constants	15.1 ± 4.9	16.9 ± 5.3	19.3 ± 5.5	16.4 ± 5.1
unc-9	EC_{50}s	3.5 ± 0.2	6.4 ± 0.2	6.8 ± 0.3	4.6 ± 0.2
unc-9	Slope constants	14.8 ± 4.1	19.3 ± 6.3	21.5 ± 7.1	15.4 ± 5.6
fc20	EC_{50}s	1.3 ± 0.3	3.0 ± 0.3	3.0 ± 0.2	4.8 ± 0.2
fc20	Slope constants	3.1 ± 1.6	3.9 ± 2.4	4.2 ± 2.1	10.9 ± 4.0
fc20;unc-1	EC_{50}s	1.2 ± 0.2*†	3.2 ± 0.2*†	2.8 ± 0.2*†	5.0 ± 0.2*
fc20;unc-1	Slope constants	2.9 ± 1.7§¶	3.6 ± 2.3§¶	3.9 ± 2.3§¶	11.4 ± 4.1
fc20;unc-9	EC_{50}s	1.5 ± 0.2*‡	2.8 ± 0.1*‡	3.2 ± 0.2*‡	5.0 ± 0.2*
fc20;unc-9	Slope constants	2.8 ± 1.4§**	3.8 ± 1.9§**	4.4 ± 1.9§**	9.8 ± 4.8

EC_{50}s ± SE and slope constants ± SE for 12 strains of *C. elegans* in four anesthetics. Abbreviations are as in table 1. In this table the EC_{50}s for N2, *fc20*, *fc21*, and *fc34* differ slightly from other tables in this article. This is because these strains were scored strictly as controls done on different days than those in tables 2 and 3 and the data in different experiments were not grouped.

* EC_{50} different from that of N2, $P < 0.01$.

† EC_{50} different from that of *unc-1*, $P < 0.01$.

‡ EC_{50} different from that of *unc-9*, $P < 0.01$.

§ Slope constant different from that of N2, $P < 0.01$.

¶ Slope constant different from that of *unc-1*, $P < 0.01$.

** Slope constant different from that of *unc-9*, $P < 0.01$.

and at higher doses of anesthetic.† This shrinking is reminiscent of two previously identified mutants in *C. elegans, unc-25* and *unc-49*. These animals shrink in response to touch and have been shown to involve the γ-aminobutyric acid system.[16] However, *unc-25* and *unc-49* show no unusual responses to anesthetics[17,‡] and *fc34* does not shorten to touch. We have also noted a lesser tendency of *fc20* and *fc28* to shorten in response to volatile anesthetics.

Gene Interactions. We wished to establish whether the three mutations *fc20, fc21,* and *fc34* were epistatic to (*i.e.,* dominated or controlled) the sensitivities of *unc-79, unc-80, unc-1,* and *unc-9*. As noted in the methods, we constructed the double mutants containing these genes with those of *unc-79, unc-80, unc-1,* and *unc-9*. The sensitivities of these 12 strains to volatile anesthetics are represented in tables 3–8. We found that in most cases *fc20, fc21,* and *fc34,* controlled the response of the double mutants to anesthetics: *unc-9fc21,* behaved like *fc21* in enflurane, not like *unc-9* and not like a combination of the two mutations. In addition, we found no additive effects between *fc20* or *fc21* and *unc-79* and *unc-80* even though both groups increase sensitivity to halothane and diethylether. The increased sensitivities of *fc20* and *fc21* to enflurane were epistatic to the resistance of *unc-79* to enflurane. Thus, we found that the double mutants behaved as *fc20* or *fc21*. In two cases, however, the responses were additive. *fc34* had additive effects with *unc-79* and with *unc-80*. The double mutants had larger increases in sensitivity to isoflurane than either single mutant.

The triple mutant animals *unc-79;unc-1fc21, unc-79;unc-7fc21,* and *unc-79;unc-9fc21* were constructed to further demonstrate epistasis. In all cases the triple mutant had EC_{50}s similar to those of *fc21* alone.

Discussion

We have isolated seven new mutations in six genes that change sensitivity to volatile anesthetics in *C. elegans*. These mutations are strikingly different from any others identified in this model system. One group of mutations shows an increase in sensitivity to enflurane, isoflurane, and diethylether. They strengthen our previous hypothesis concerning multiple sites of action of volatile anesthetics in *C. elegans*. A second group of mutations, which are quite vigorous in air, confers a

† Morgan PG, Sedensky MM: Unpublished data. 1993.

‡ Morgan PG, Sedensky MM: Unpublished data. 1991.

Table 7. The Effect of *fc21* on EC_{50}s and Slope Constants of *unc-1* and *unc-9* in Four Anesthetics

Strain		H	E	ISO	DE
N2	EC_{50}s	3.2 ± 0.2	6.5 ± 0.3	7.2 ± 0.4	6.6 ± 0.2
N2	Slope constants	14.1 ± 5.3	18.9 ± 6.1	20.1 ± 6.0	17.2 ± 6.4
unc-1	EC_{50}s	3.4 ± 0.2	6.4 ± 0.2	7.0 ± 0.3	4.7 ± 0.2
unc-1	Slope constants	15.1 ± 4.9	16.9 ± 5.3	19.3 ± 5.5	16.4 ± 5.1
unc-9	EC_{50}s	3.5 ± 0.2	6.4 ± 0.2	6.8 ± 0.3	4.6 ± 0.2
unc-9	Slope constants	14.8 ± 4.1	19.3 ± 6.3	21.5 ± 7.1	15.4 ± 5.6
fc21	EC_{50}s	1.5 ± 0.4	2.7 ± 0.3	1.3 ± 0.3	5.2 ± 0.15
fc21	Slope constants	3.9 ± 2.1	4.0 ± 1.9	1.8 ± 0.6	10.4 ± 4.3
fc21;unc-1	EC_{50}s	1.4 ± 0.2*†	3.1 ± 0.2*‡	1.5 ± 0.2*†	5.3 ± 0.2*
fc21;unc-1	Slope constants	3.4 ± 1.8§¶	3.6 ± 2.1§¶	2.1 ± 1.1§¶	10.1 ± 3.9
fc21;unc-9	EC_{50}s	1.4 ± 0.2*‡	3.1 ± 0.2*‡	1.4 ± 0.2*‡	5.3 ± 0.1*
fc21;unc-9	Slope constants	3.6 ± 2.0§**	3.1 ± 2.3§**	2.3 ± 1.4§**	11.4 ± 5.5

EC_{50}s ± SE and slope constants ± SE for 12 strains of *C. elegans* in four anesthetics. Abbreviations are as in table 1. In this table the EC_{50}s for N2, *fc20, fc21,* and *fc34* differ slightly from other tables in this article. This is because these strains were scored strictly as controls done on different days than those in tables 2 and 3 and the data in different experiments were not grouped.

* EC_{50} different from that of N2, $P < 0.01$.

† EC_{50} different from that of *unc-1*, $P < 0.01$.

‡ EC_{50} different from that of *unc-9*, $P < 0.01$.

§ Slope constant different from that of N2, $P < 0.01$.

¶ Slope constant different from that of *unc-1*, $P < 0.01$.

** Slope constant different from that of *unc-9*, $P < 0.01$.

large increase in sensitivity to all volatile anesthetics tested. These latter mutations may affect a final common pathway shared by all volatile anesthetics.

We previously postulated that in *C. elegans* multiple sites of action exist for volatile anesthetics.[10] All of the mutations upon which that conclusion was drawn were identified by screening for altered responses in halothane; none was markedly changed in response to enflurane, isoflurane, or diethylether. The pattern of anesthetic response of *fc23, fc30,* and *m48* fills in this gap, and extends the possible range of sites of volatile anesthetics. Mutations with other patterns of anesthetic response (perhaps sensitive to isoflurane but not enflurane), may still be found in *C. elegans.* In *Drosophila melanogaster,* Krishnan and Nash have identified mutations which alter sensitivities to some volatile anesthetics to different degrees than to others.[8] They conclude that in *D. melanogaster,* multiple sites of action of volatile anesthetics best explain their results. Two groups studying mice strains selected for altered sensitivity to ethanol showed altered sensitivity to some anesthetics and not others.[5–7] Thus genetic dissection of multiple sites of action of volatile anesthetics is not limited to the invertebrates, and is most encouraging for future comparative studies. A complete genetic pathway controlling anesthetic response in a simple model is of fundamental importance for future studies in more complicated organisms.

We did not expect to find mutations like *fc20, fc21,* and *fc34,* which are exquisitely sensitive to all volatile anesthetics tested. Because they are vigorous in air, it seems unlikely that they possess globally disrupted nervous or muscular systems. At present, we have not identified the tissue(s) in which these genes function. However, by mosaic analysis it is known that two other genes important to anesthetic response in *C. elegans, unc-79* and *unc-7,* do exert their effects through the nervous system.[18] These new mutations, regardless of where they function, should serve to identify common features of pathways profoundly affected by volatile anesthetic and have corollaries within the CNS, peripheral nervous system, or muscular systems of other organisms.

We do not know if any of the newly isolated mutations represent total loss of function of the normal gene product (a null allele), or a newly acquired function (for a complete discussion of null *vs.* nonnull mutations see reference 19). In the case of *fc21,* we have placed this allele over a deficiency in the region and found that this animal is more sensitive to halothane and decreased in viability compared with *fc21* alone. In addition *fc21* is sensitive to temperature; its phenotypes are more severe

Table 8. The Effect of *fc34* on EC_{50}s and Slope Constants of *unc-1* and *unc-9* in Four Anesthetics

Strain		H	E	ISO	DE
N2	EC_{50}s	3.2 ± 0.2	6.5 ± 0.3	7.2 ± 0.4	6.6 ± 0.2
N2	Slope constants	14.1 ± 5.3	18.9 ± 6.1	20.1 ± 6.0	17.2 ± 6.4
unc-1	EC_{50}s	3.4 ± 0.2	6.4 ± 0.2	7.0 ± 0.3	4.7 ± 0.2
unc-1	Slope constants	15.1 ± 4.9	16.9 ± 5.3	19.3 ± 5.5	16.4 ± 5.1
unc-9	EC_{50}s	3.5 ± 0.2	6.4 ± 0.2	6.8 ± 0.3	4.6 ± 0.2
unc-9	Slope constants	14.8 ± 4.1	19.3 ± 6.3	21.5 ± 7.1	15.4 ± 5.6
fc34	EC_{50}s	1.5 ± 0.3	3.0 ± 0.4	2.9 ± 0.4	5.2 ± 0.3
fc34	Slope constants	4.0 ± 2.1	4.3 ± 2.3	4.4 ± 1.9	13.1 ± 4.0
fc34;unc-1	EC_{50}s	1.5 ± 0.2*†	2.8 ± 0.2*†	3.0 ± 0.2*†	5.3 ± 0.2*
fc34;unc-1	Slope constants	4.2 ± 2.3§¶	4.4 ± 2.8§¶	4.9 ± 2.1§¶	13.4 ± 4.8
fc34;unc-9	EC_{50}s	1.3 ± 0.2*‡	3.2 ± 0.1*‡	3.3 ± 0.2*‡	5.4 ± 0.1*
fc34;unc-9	Slope constants	3.1 ± 2.4§**	4.1 ± 2.5§**	4.1 ± 2.4§**	10.1 ± 5.0

EC_{50}s ± SE and slope constants ± SE for 12 strains of *C. elegans* in four anesthetics. Abbreviations are as in table 1. In this table the EC_{50}s for N2, *fc20*, *fc21*, and *fc34* differ slightly from other tables in this article. This is because these strains were scored strictly as controls done on different days than those in tables 2 and 3 and the data in different experiments were not grouped.

* EC_{50} different from that of N2, $P < 0.01$.

† EC_{50} different from that of *unc-1*, $P < 0.01$.

‡ EC_{50} different from that of *unc-9*, $P < 0.01$.

§ Slope constant different from that of N2, $P < 0.01$.

¶ Slope constant different from that of *unc-1*, $P < 0.01$.

** Slope constant different from that of *unc-9*, $P < 0.01$.

at 25°C than 20°C or 15°C (Data not shown). Thus *fc21* is not a null allele; however, its phenotype is most consistent with a partial loss of function. *fc34* has semidominant effects which also makes it unlikely to be a null. However, there are many instances where a heterozygote null has an altered phenotype.[19] A similar argument applies to *m48,* which is a true dominant mutation.[15] In the case of *fc20, fc23,* and *fc30,* we do not yet know if they are true null mutations, though in the case of *fc20* we have identified one other identical allele at a frequency consistent with a loss of function.

We think that *fc20, fc21,* and *fc34* represent a most interesting class of mutants because they raise the possibility that all volatile anesthetics, even if they interact with different sites, share a final common pathway. We believe that *fc20* and *fc21* are most likely to be at least partial loss of function mutations. Assuming they represent loss of function alleles, we have placed these two mutations genetically downstream of any other previously identified mutations. We did this by constructing animals that carry two mutations, each of which changes anesthetic response in a unique manner. If the two mutations work along separate genetic pathways, then such an animal will display features of both mutations; in other words, the behaviors will be additive. If the mutations represent early and late steps in a common pathway, the doubly mutant animal will exhibit the behavior of the mutation that is the late step, because the earlier-acting mutation requires the activity of the second step to exert its effect. This phenomenon, the control or dominance of one mutation over another, is termed epistasis. In our model, *fc20* and *fc21* are epistatic to *unc-79, unc-80, unc-1,* and *unc-9.* It must be noted that, in particular, *fc20* may not occupy this position if it is shown to not represent a loss of function allele.

On the other hand, *fc34* has some additive effects with *unc-79* and *unc-80;* the EC_{50} of the double mutant in isoflurane is much less than that of either mutation in isolation. Thus *fc34* may affect neuromuscular components other than those affected by *unc-79* or *unc-80.* As noted above, *fc34* is unlikely to be a loss of function allele and its position relative to the pathway is unclear. Thus we have been conservative and placed it in a parallel position.

Based on the interactions reported here, we have modeled a genetic pathway controlling anesthetic response in *C. elegans,* incorporating all currently identified mutations (fig. 1) This model is primarily a guide for future studies; we are undertaking molecular and genetic analyses to further understand the nature of these mutations.

Genetic variation is a powerful tool for identifying how anesthetics function; one observes or generates a behavioral change and undertakes a molecular analysis

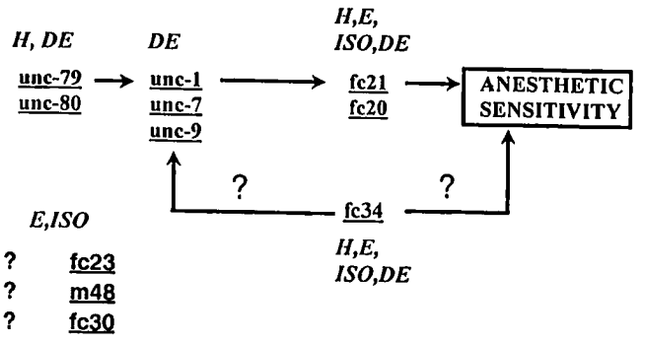

Fig. 1. A genetic pathway controlling sensitivity to volatile anesthetics in *Caenorhabditis elegans*. The genes involved are grouped by their patterns of sensitivity and by their interactions with each other. The anesthetic sensitivities affected by mutations in these genes are listed above the genes. Question marks = precise position of these genes in this pathway is unknown.

of the gene causing that change. No preconceptions are necessary about where or what these anesthetics sites are. However, the weakness of the approach is that despite the unequivocal causal relation between the response and the mutation, one cannot predict how far upstream that mutation is from the elusive site of anesthetic action. Hence it is important to isolate an array of mutations, to obtain as extensive and clear a picture as possible of anesthetic sites and their interactions, in a simple model. As a model, *C. elegans* has many outstanding features; however, it is not without its limitations. The normal animal is relatively resistant to volatile anesthetics in comparison with other species, with an EC_{50} 5–10 times that of the minimum alveolar concentration in humans. This may represent an evolutionary adaptation of this ancient organism to living at cool temperatures and being exposed to organic compounds like alcohols and alkanes within its environment, or the choice of endpoint defining anesthesia in worms. In addition, the relative rank of potencies of isoflurane and enflurane is slightly different in worms than in humans. This difference is a result of the failure of mammals to adhere to the Meyer-Overton relation in regard to the chemical isomers, enflurane, and isoflurane. Enflurane is slightly more lipid soluble than isoflurane (O/G 96 *vs.* 91 for isoflurane)[20] yet is less potent than isoflurane in humans and in dog and mouse models. Unlike these more complicated species, *C. elegans* generates the rank order of volatile anesthetic potencies predicted by the Meyer-Overton relation. We think that there are multiple sites of action for volatile anesthetics. It seems likely that enflurane has an excitatory affect in mammals that does not exist in *C. elegans;* this in turn causes its more perfect adherence to the Meyer-Overton relation. Simple models are simpler than complex ones. Far from eliminating *C. elegans* as a model, we believe this strengthens it as a manageable system, without the complicating response to enflurane.

Overall, we think that drawbacks of *C. elegans* are outweighed by the animal's impeccable adherence to the Meyer-Overton relation[20] the reversibility of our anesthetic endpoint, the qualitative behavior of the animals in various anesthetic agents, and the maintenance of a cut-off effect.[21] The mutations reported here both extend our global picture of anesthetics sites and their interactions in *C. elegans,* and pinpoint a pathway common to all volatile anesthetics. Molecular mechanisms stemming from these genetics studies and those in other model systems may eventually be used to probe more complicated organisms like mammals. At present no data exist to indicate large variations in sensitivity to volatile anesthetics in humans.[22,23] However, such variation may be very rare; we are unaware of any systematic search for such individuals.

In conclusion, we have found two new patterns of sensitivity to volatile anesthetics in *C. elegans.* These data further support a model involving multiple sites of action for these agents. The possibility of one common interacting pathway is suggested. Such a pathway may have implications for similar types of interactions in vertebrates.

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