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Genetic differences affecting the potency of stereoisomers of halothane

(nematode/genetics/mutations/anesthesia)

M. M. SEDENSKY*†, H. F. CASCORBI*, J. MEINWALD‡, P. RADFORD‡, AND P. G. MORGAN*

*Department of Anesthesiology, University Hospitals of Cleveland and Case Western Reserve University, Cleveland, OH 44106; and ‡Department of Chemistry, Cornell University, Ithaca, NY 14853-1301

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ABSTRACT **The mechanism of action of volatile anesthetics is the subject of some debate. Much of the controversy has centered on whether the site of such actions is purely lipid in nature or may contain a protein target. This report studies the interaction of stereoisomers of halothane on the wild type and on a variety of genetic mutants of *Caenorhabditis elegans*. The mutants studied have previously been shown to have altered sensitivities to volatile anesthetics. In one mutant, *fc34*, (*R*)-halothane [the (+) isomer] was 3 times more potent than its *S* (−) isomer. Other mutants and wild-type animals displayed more modest differences in sensitivity to the enantiomers. The results indicate that a genetic pathway exists in *C. elegans* controlling sensitivity to halothane and that both lipid and protein targets may mediate halothane's effects.**

A striking correlation exists between the potencies of the volatile anesthetics and their lipid solubilities (1–4). This correlation is termed the Meyer–Overton rule; it has been taken as evidence for a purely lipid site of action for volatile anesthetics (5). However, adherence to this relationship does not necessarily rule out a protein target. Franks and Lieb (6) have studied the effects of volatile anesthetics on the protein luciferase and shown that these effects follow the Meyer–Overton relationship. Franks and Lieb (7) have also presented thermodynamic arguments that lipophilic regions of proteins may serve as targets for volatile anesthetics. The nature of the site or sites of action of these anesthetics remains unclear.

A lipid site of action of volatile anesthetics would not be expected to distinguish strongly between stereoisomers of volatile anesthetics (1, 8). Conversely, a protein site of action may exhibit large differences in sensitivities to stereoisomers of particular volatile anesthetics. Previous investigations of the effects of stereoisomers have had varied results (1, 9). Some have demonstrated no difference in the effects of optical isomers, whereas others have noted significant changes. In general, these studies have been hindered by a limited amount of anesthetics and the resulting inability to expose large numbers of animals to the anesthetics (9–13).

The nematode *Caenorhabditis elegans* is a good model for the study of the action of volatile anesthetics. We have previously shown that *C. elegans* is reversibly immobilized by volatile anesthetics and adheres to the Meyer–Overton rule (14, 15). We have isolated several mutants with altered sensitivity to a variety of volatile anesthetics when compared with the wild-type strain, N2 (14–16). Such mutants are useful because they isolate the effects of volatile anesthetics on particular sites of action and can be ordered in a pathway determining sensitivity to volatile anesthetics in *C. elegans* (Fig. 1). Two of these genes, *unc-79* and *unc-80*, confer large increases in sensitivity to halothane, but not to two other volatile anesthetics, enflurane and isoflurane (15). Three other genes, *unc-1*, *unc-7*, and *unc-9*, act downstream from *unc-79* and *unc-80* (16). Mutations in these three genes act as suppressors of *unc-79* and *unc-80* but do not by themselves alter sensitivity to halothane. A third set of genes, represented by the mutations *fc20* and *fc21*, functions downstream from those discussed above (18). These last two mutations, which increase sensitivity to all volatile anesthetics, are epistatic to the aforementioned mutations. They are candidates to represent common sites of action for the volatile anesthetics. We postulated a pathway which includes multiple sites of action for volatile anesthetics in *C. elegans* (16). Other researchers have postulated multiple sites of anesthetic action in different systems (19, 20).

One mutation, *fc34*, gives a unique response to volatile anesthetics (18). This mutation confers increased sensitivity to all volatile anesthetics. Unlike other mutants, animals with this mutation were immobilized with a severe shrinking, often less than half of its initial body length. Other *C. elegans* mutants also can shrink, although none in response to volatile anesthetics. In general, the other "shrinkers" suffer from disruptions of the γ-aminobutyrate (GABA) system (21). For example, *unc-49* is thought to code for the $GABA_A$ receptor, which has been implicated in determining the sensitivity to some volatile anesthetics (1, 21).

If different sites of action vary in their molecular components, some mutants may show a varied response to stereoisomers, while others may not. The existence of multiple sites of anesthetic action suggests that some such sites may be purely lipid in nature, whereas others may have protein components. (*R*)-halothane and (*S*)-halothane have recently become available (generously provided by D. L. Pearson) (22).

We report here the EC_{50} values for N2 and nine mutant strains when exposed to *R* and *S* isomers and the racemate of the volatile anesthetic halothane. Representative strains described above, which confer both high and low sensitivities to racemic halothane, were studied. We find that the mutants differ in their response to the two isomers and that the Meyer–Overton rule is not always conserved. Our results are consistent with the possibility of protein targets at some of the sites of action for volatile anesthetics; however, lipid targets remain a possibility.

METHODS

Nematodes. Nematodes were grown by standard techniques as described by Brenner (17). N2, *unc-1(e580)*, *unc-9(e101)*, *unc-80(e1272)*, and *unc-49(e382)* worms were obtained from the Caenorhabditis Genetics Center. We constructed the double mutant *unc-79;unc-9(ec1;e101)* by standard techniques (17). *unc-79(ec1)*, *fc20*, *fc21*, and *fc34*

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Abbreviation: GABA, γ-aminobutyrate.
†To whom reprint requests should be addressed.

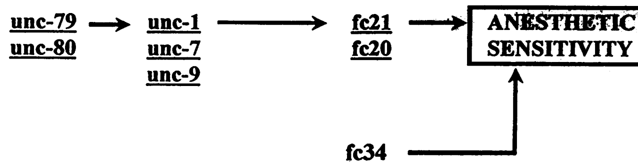

FIG. 1. Genetic pathway showing the interaction of eight genes in determining sensitivity to volatile anesthetics in *C. elegans*. Mutations were identified and scored as described (15, 17). Double mutants were constructed to determine epistasis as described by Brenner (17). Those mutations more distal in the pathway were epistatic to those more proximal. For those mutations altering sensitivities in the same manner, epistasis was determined by scoring interactions with other genes within the pathway and by screening for additive effects.

were isolated in mutational screens for altered sensitivity to volatile anesthetics.

Dose–Response Curves. Nematodes were exposed to the volatile anesthetics in closed glass chambers. Anesthetic was injected in liquid form and allowed to vaporize. Initial injections for N2, *unc-9, unc-1, unc-49,* and *unc-79; unc-9* were estimated to be 20–30% below the EC_{50} of racemic halothane for N2. The initial concentrations of anesthetic for *unc-79, unc-80, fc20, fc21* and *fc34* were established at about 50% of the EC_{50} of racemic halothane for *unc-79(ec1)*. The animals were exposed to one concentration for 2 hr, during which time a steady-state response was reached. Nematodes were then scored as immobile if they did not move for 10 sec when observed through a dissecting microscope. After the cultures were scored for immobility, a sample of gas was taken from the chamber for measurement of the anesthetic concentration by gas chromatography. A second injection of anesthetic was added to the chamber to increase the anesthetic concentration, or some gas was withdrawn from the chamber and replaced with air. The chambers were then allowed to equilibrate for 2 hr. After the culture was scored for immobility and a second gas sample was obtained, the procedure was repeated a third time. After the third concentration point the chambers were opened and the nematodes were allowed to recover. In all cases the animals fully recovered after returning to room air.

The technique of adding anesthetic to chambers already containing anesthetic differs from that used in our previous studies. This approach used a smaller amount of agent. Previously each concentration point was obtained by a single injection of anesthetic into a chamber containing cultures of *C. elegans* (14, 15). No difference in the EC_{50} values or dose–response curves for these cultures in racemic halothane was noted between the two techniques.

Anesthetics. The isomers of halothane were obtained by the methods of Meinwald *et al.* (22). Other than racemate excess, no significant impurities are generated in this synthesis. The *R*-isomer sample contained 3.6% of the *S* isomer; the *S*-isomer sample contained 0.27% of the *R* isomer. The racemic mixture was from a virgin bottle of halothane purchased from Anaquest (Madison, WI). Insufficient amounts of isomers were available to reconstitute the racemate by mixing the pure isomers.

Statistical Methods. EC_{50} values and slope constants were determined by the technique of Waud (23). Waud describes comparing values from different curves by using a normal distribution; thus comparisons between different EC_{50} values were done by ANOVA (23, 24). Statistical methods are described in detail elsewhere (24, 25). Significance was defined as $P < 0.01$. The increased stringency was used to avoid type I errors. Dose–response curves were constructed with at least 30 concentration points, with at least 50 animals scored at each point. Each EC_{50} then represents scoring at least 1500 animals and in several cases >2000 animals. Since the standard errors are dependent on the number of animals scored, they became quite small.

RESULTS

Effects on the Wild-Type Strain, N2. The *R* and *S* forms of halothane differed in their potencies for the wild-type *C. elegans*, N2. The *R* form showed a 12% increase in potency compared with both the *S* form and the racemate (Table 1 and Fig. 2). The simplest interpretation of these data is that in N2 at least one site exists that is particularly sensitive to (*R*)-halothane. It is interesting that the racemic mixture exhibits a potency similar to the less potent form, the *S* isomer. Such a result implies a complicated interaction between the two forms with a site of action or with each other.

Loss-of-function mutations which change different anesthetic sites are candidates for identification of gene products necessary for the difference in anesthetic potency of stereoisomers seen in N2. Therefore, we tested such mutations from each step in our pathway, along with *unc-49* and *fc34*.

Effects of Isomers on *unc-79* and *unc-80* Mutants. Mutations in the genes *unc-79* and *unc-80*, which occupy the most upstream position in the pathway in Fig. 1, confer an increase in sensitivity to halothane. This increase is selective for the very lipid-soluble volatile anesthetics (no increase in sensitivity to enflurane or isoflurane is seen). We reasoned that the *unc-79* and *unc-80* strains may present novel relationships between the stereoisomers, as loss of these gene products unmasks anesthetic sites uniquely sensitive to halothane.

The alleles of *unc-79(ec1)* and *unc-80(e1272)* were similar to each other. In each case the strains showed increased sensitivity to the racemic mixture compared with either isomer alone. In each mutant the potencies of the *R* and *S* isomers were equal to each other (Table 2). Thus, *unc-79* and *unc-80* showed a synergistic interaction between the *R* and *S* forms. This implies an interaction between the two forms of halothane, possibly occupying different sites of action. Since *unc-79(ec1)* and *unc-80(e1272)* represent loss of function of a gene product, halothane must normally produce an excitatory effect via their wild-type products. The synergistic effects seen here occur at some other target than those coded for by the *unc-79* and *unc-80* genes. We also note that in *unc-79* and *unc-80* animals the *R* form is not more potent than the *S* form. This implies that the difference in potencies between the *R* and *S* forms seen in N2 occurs only at the

Table 1. Five strains of *C. elegans* with high EC_{50} values for isomers of halothane

	EC_{50}, % (mean ± SEM)				
Isomer	N2	*unc-1*	*unc-9*	*unc-79;unc-9*	*unc-49*
R	3.13 ± 0.03*	3.02 ± 0.04*	3.07 ± 0.04*	3.10 ± 0.06*	3.25 ± 0.04*†
S	3.55 ± 0.02	3.54 ± 0.09	3.54 ± 0.03	3.54 ± 0.07	3.99 ± 0.13†
Racemate	3.52 ± 0.06	3.59 ± 0.05	3.47 ± 0.06	3.45 ± 0.07	3.89 ± 0.06†

These strains are grouped because they exhibit sensitivities to racemic halothane similar to that of N2, the wild-type strain of *C. elegans* used in this study.
*EC_{50} different from that for racemic halothane, $P < 0.01$.
†EC_{50} different from that of N2 for the same isomer, $P < 0.01$.

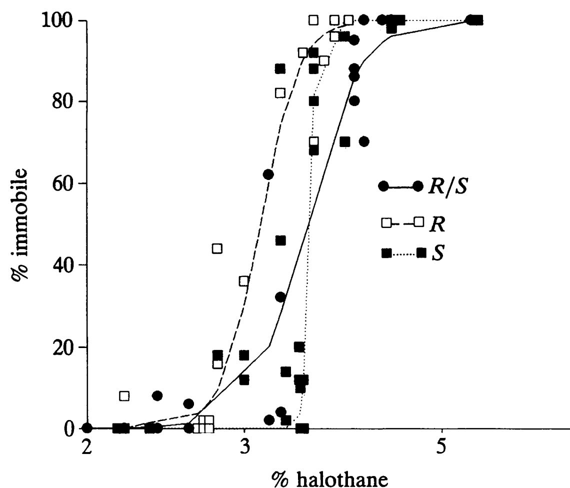

FIG. 2. Dose-response curves for N2 with R and S isomers of halothane and a racemic mixture of the two isomers (R/S) on a semilogarithmic plot. The curves were fitted by a nonlinear regression analysis using the software program SIGMA PLOT (Jandel Scientific, Corte Madera, CA). Note that the form of the curves for all three responses is similar and that N2 is more sensitive to the R isomer than to either the S isomer or the racemate. In addition, no difference in the quality of the response of N2 was noted between the isomers. All curves were fitted to the function $p = D^E/(D^E + K^E)$, where p is the probability of an animal being immobile, D is the dose of anesthetic, K is the ED_{50} and E is the slope constant (23). The curves drawn are cubic spline curves for the theoretical function derived by regression analysis using the same values on the abscissa as were used in the experiments. A continuous curve from the function was not plotted.

higher concentrations and may be the effect of halothane on the wild-type products of *unc-79* or *unc-80*. We tested this hypothesis for *unc-79* (see below).

Effects of Isomers on the Suppressors. The suppressor strains *unc-1* and *unc-9* possess EC_{50} values in racemic halothane identical to that for N2. If these strains maintain the stereospecific potencies of the enatiomers, then they do not eliminate the anesthetic site that can distinguish the enantiomers.

The N2 pattern was repeated in both *unc-1(e580)* and *unc-9(e101)*. In each case the R isomer of halothane was more potent than the S isomer or the racemic mixture (Table 1). We interpret these data to indicate that some other gene product is responsible for the small difference in potencies of the R and S forms of halothane.

We also studied the effects of the isomers on a double mutant, *unc-79;unc-9*. If the wild-type product of the *unc-79* gene were responsible for the difference between the R and S isomers seen in N2 and *unc-9* strains, then the *unc-79* mutation should eliminate the change in potencies in an *unc-9* animal. The wild-type product of the *unc-79* gene is not responsible for this effect, since the difference is maintained in the *unc-79;unc-9* double mutant. In addition, *unc-9* represents a candidate for the wild-type gene product responsible for the synergism seen in the *unc-79* and the *unc-80* mutants. Loss of this gene eliminated the synergism, as seen in the *unc-79;unc-9* double mutant. It is also possible that the synergism between the isomers is present only at low concentrations and is lost at these higher concentrations regardless of the suppressor genes. We favor this interpretation, given the results seen with *fc20* and *fc21*.

Effects of Isomers on *fc20* and *fc21* Mutants. *fc20* and *fc21* represent loss of gene products which unmask responses common to all volatile anesthetics. As candidates for common sites of action they may also present different relationships between the enantiomers, but at apparently different sites than those seen in *unc-79* and *unc-80* strains.

The *fc20* and *fc21* mutants showed equal sensitivities to the R and S isomers, as well as to the racemate, of halothane (Table 2). Both genes are therefore candidates for the synergistic effects seen in the *unc-79* and *unc-80* mutants. The EC_{50} values of all four mutants are in the same general range, which makes a large concentration difference an unlikely cause of loss of the synergistic effects. It may be that the wild-type products eliminated by both *fc20* and *fc21* are necessary for this effect. If the wild-type products of *fc20* and *fc21* are responsible for the synergistic effects seen in *unc-79* and *unc-80* animals, then the double mutants *unc-79;fc20* and *unc-79;fc21* should not show these effects.

Effects of Isomers on "Shrinkers." *fc34* and *unc-49* represent unique opportunities to compare the enantiomers. *fc34* apparently gives rise to a excitatory response to volatile anesthetics. *unc-49* may isolate any response remaining after the $GABA_A$ channel is removed. Both mutations may thus also unmask novel responses to the enantiomers. It should be noted that the immobility caused by *fc34* is qualitatively different from that seen in the other mutants, probably resulting from a different site than those involved in the other strains.

The shrinker *fc34* mutant showed an intriguing response. *fc34* worms were much more sensitive to the R isomer than to the S isomer. The potency of the racemic mixture was between that of the two isomers (Table 2 and Fig. 3). Both isomers caused severe shrinking, although at the widely different doses indicated by their EC_{50}s.

The other shrinker mutant, *unc-49*, was resistant, compared with N2 to all forms of halothane [this differs from the report by Crowder and Thomas (26)]. The *unc-49* mutant also showed increased potency of the (R)-halothane relative to the S form and the racemate (Table 1 and Fig. 4). Not only is the different response to the isomers maintained in *unc-49* animals, but it is actually accentuated. Thus, the wild-type product of this gene is not responsible for the different sensitivities to the isomers. In fact, this gene product seems to actually mask the difference caused by some other gene product. No significant differences were found in the slope constants between the enantiomers or the racemate in any strain. In addition, dose-response curves repeated in successive weeks and by independent observers did not differ (data not shown).

DISCUSSION

A number of investigations have examined the stereospecificity of volatile anesthetic effects. Kendig *et al.* (9) studied the effects of the stereoisomers of halothane on the isolated cervical sympathetic ganglion of the rat and on synthetic phospholipid bilayer membranes. They found no difference between the stereoisomers, consistent with a purely lipid site of anesthetic action. However, the studies were limited by two restrictions. First, the authors had very small quantities of each isomer, which precluded determining the effects in whole animals and also limited the number of trials. A second limitation was that the isomer preparations were not pure: the (−) preparation contained a (+)/(−) (i.e., R/S) ratio of 30/70, whereas the (+) preparation contained a (+)/(−) ratio of 75/25. Small differences in the effects of (+) and (−) forms may have been difficult to identify.

Franks and Lieb (11) exposed an isolated neuron from the molluscan nervous system to stereoisomers of isoflurane. They found that the (+) isomer was twice as effective as the (−) isomer at eliciting a response from a novel anesthetic-activated K^+ channel. Jones and Harrison (12) recently showed that the (+) isomer of isoflurane produced greater increases in inhibitory postsynaptic currents than the (−)

Table 2. Five strains of *C. elegans* with low EC_{50} values for isomers of halothane

Isomer	EC_{50}, % (mean ± SEM)				
	unc-79	*unc-80*	*fc20*	*fc21*	*fc34*
R	1.44 ± 0.06*	1.47 ± 0.06*	1.07 ± 0.05	0.81 ± 0.04	0.62 ± 0.05*†
S	1.32 ± 0.04*	1.39 ± 0.04*	1.25 ± 0.04	0.87 ± 0.04	1.72 ± 0.06*
Racemate	1.06 ± 0.03	1.21 ± 0.05	1.15 ± 0.05	0.93 ± 0.04	1.02 ± 0.03

These strains are grouped because they show an increased sensitivity to racemic halothane compared with N2.

*EC_{50} different from that for racemic halothane, $P < 0.01$.

†EC_{50} different from that for (*S*)-halothane, $P < 0.01$.

isomer in rat hippocampal neurons. It is unknown whether such effects are important in whole animals.

Firestone *et al.* (8) exposed frogs (*Rana pipiens*) to stereoisomers of short-chain alcohols. They could demonstrate no differences in ED_{50} values between any of the isomers. This study had the advantage of assaying the behavioral response of whole animals. The disadvantage was that the alcohols may represent a special class of anesthetics. In a separate study, Firestone *et al.* (10) were also unable to identify a difference between stereoisomers of isoflurane in tadpoles.

Harris *et al.* (13) studied the effects of stereoisomers of volatile anesthetics in whole animals. They injected mice with (+) and (−) isomers of isoflurane and measured dose-dependent changes in anesthetic sleep time. They found a 20–40% increase in sleep time with the (+) isomer compared with the (−) isomer. However, insufficient drug precluded the determination of the EC_{50} values.

Our previous work in *C. elegans* has indicated that this organism is an excellent model for the action of volatile anesthetics (14–16). Nematodes exposed to volatile anesthetics are immobilized and return fully to normal function when removed from the gas. *C. elegans* also follows the Meyer–Overton relationship. However, some differences should be noted between nematodes and most higher organisms. First, *C. elegans* is relatively resistant to volatile anesthetics, requiring 5–10 times the concentrations necessary in mammals for "anesthesia" (27). It may be that immobility is more stringent than the clinical end point used in mammals. It is also possible that *C. elegans* has evolved resistant sites of action as a consequence of living in cool temperatures and exposure to organic substances such as alcohols. A second difference between *C. elegans* and vertebrates is that the relative potencies of isoflurane and enflurane are reversed. In vertebrates, enflurane is less potent than its structural isomer, isoflurane, despite a slightly higher lipid solubility (28). In *C. elegans* enflurane fits the rank order predicted by the Meyer–Overton rule.

The different potencies of the enantiomers observed in this study are unlikely to be due to differences in their metabolism; however, we have no way of measuring this in *C. elegans*. Since we allow the animals to come to a steady-state response, metabolism should play only a small role. We also know that in the two mutants studied by Eckenhoff (29), *unc-79* and *unc-80*, the amount of racemate halothane in the mutants was decreased when they were compared with N2 at identical halothane concentrations. If decreased metabolism, even of only one isomer, caused the difference in sensitivities, one would expect the opposite result.

If multiple sites of action exist for *C. elegans*, then some may be lipid and others protein. In addition, some protein targets may exhibit steric differences and some may not. By isolating the effects of several components controlling sensitivity to volatile anesthetics, it may be possible to identify different types of interaction with stereoisomers. We chose mutations germane to each step in the genetic pathway affecting sensitivity.

This study demonstrates two important points. First, the genetic pathway postulated to control anesthetic response in *C. elegans* is corroborated by the response of each mutant to stereoisomers. Groups of mutations which cause similar patterns of sensitivity to volatile anesthetics are similar to each other in the pattern of their EC_{50} values for the three

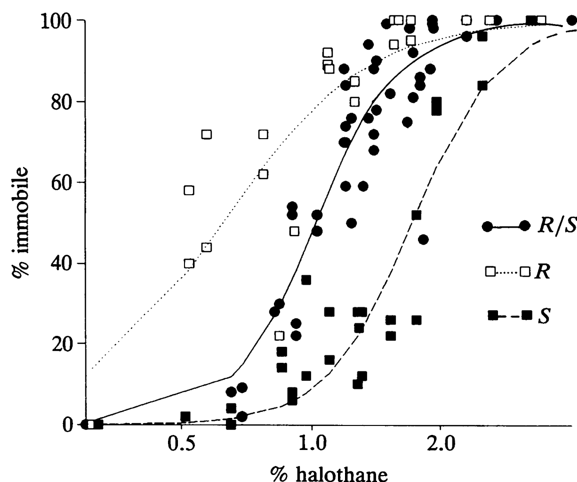

FIG. 3. Dose–response curves for the *fc34* strain with *R* and *S* isomers of halothane and a racemic mixture of the two isomers (*R/S*) on a semilogarithmic plot. As for Fig. 2, the slopes of the curves are similar. Note the large difference between the responses to the different isomers and the racemate. Curves were generated and drawn as in Fig. 2.

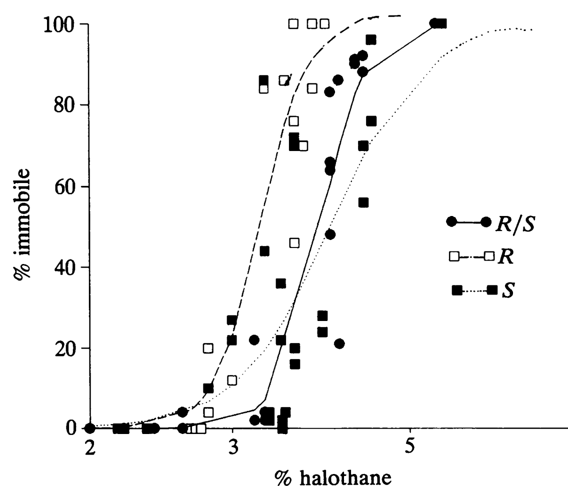

FIG. 4. Dose–response curves for the *unc-49* strain with *R* and *S* isomers of halothane and a racemic mixture of the two isomers (*R/S*) on a semilogarithmic plot. The form of the curves for all three responses is similar. The *unc-49* strain is more sensitive to the *R* form than to the *S* form or the racemate, as noted for N2. However the magnitude of the differences is larger for *unc-49* than seen for N2. In addition, the *unc-49* strain is resistant to all forms of halothane relative to N2. Curves were generated and drawn as in Fig. 2.

forms of halothane. In addition, the epistatic effects of *unc-9* on *unc-79* are seen in these responses also.

Second, it has not escaped our notice that we are confronted with deviations from the Meyer–Overton rule. The most significant differences between isomers was observed in *fc34* worms. While this response is different from the phenomenon of general anesthesia in more complicated animals, it clearly demonstrates that stereoisomers of volatile anesthetics can have very selective effects on the function of the neuromuscular system. The mutation *fc34* has semidominant effects and is probably not a loss-of-function allele. The stereospecific effects of halothane on *fc34* may represent a direct interaction between the anesthetic and this allele's protein product.

Our data indicate that sites of anesthetic action can distinguish steric differences in the halothane molecule. Such results are consistent with protein targets but do not eliminate lipids as a potential site of action of volatile anesthetics. It may be that the multiple sites of action include both lipid and protein targets. Since membrane lipids are a heterogeneous collection of molecules, it is possible that lipid regions in membranes may also be able to differentiate between stereoisomers of volatile anesthetics. It is important to note that the differences between stereoisomers do not eliminate their anesthetic effects. While the configuration of a volatile anesthetic plays a role in its potency, in this case it apparently acts only as a modifier and not as a primary determinant.

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