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Behavioral Effects of Volatile Anesthetics in Caenorhabditis elegans

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Background: **The nematode *Caenorhabditis elegans* offers many advantages as a model organism for studying volatile anesthetic action: It has a simple, well-understood nervous system; it allows the researcher to do forward genetics; and its genome will soon be completely sequenced. *C. elegans* is immobilized by volatile anesthetics only at high concentrations and with an unusually slow time course. Here other behavioral dysfunctions are considered as anesthetic endpoints in *C. elegans*.**

Methods: **The potency of halothane for disrupting eight different behaviors was determined by logistic regression of concentration and response data. Other volatile anesthetics were also tested for some behaviors. Established protocols were used for behavioral endpoints that, except for pharyngeal pumping, were set as complete disruption of the behavior. Time courses were measured for rapid behaviors. Recovery from exposure to 1 or 4 vol% halothane was determined for mating, chemotaxis, and gross movement. All experiments were performed at 20 to 22°C.**

Results: **The median effective concentration values for halothane inhibition of mating (0.30 vol% − 0.21 mM), chemotaxis (0.34 vol% − 0.24 mM), and coordinated movement (0.32 vol% − 0.23 mM) were similar to the human minimum alveolar concentration (MAC; 0.21 mM). In contrast, halothane produced immobility with a median effective concentration of 3.65 vol% (2.6 mM). Other behaviors had intermediate sensitivities. Halothane's effects reached steady-state in 10 min for all behaviors tested except immobility, which required 2 h. Recovery was complete after exposure to 1 vol% halothane but was significantly reduced after exposure to immobilizing concentrations.**

Conclusions: **Volatile anesthetics selectively disrupt *C. elegans* behavior. The potency, time course, and recovery characteristics of halothane's effects on three behaviors are similar to its anesthetic properties in vertebrates. The affected nervous system molecules may express structural motifs similar to those on vertebrate anesthetic targets. (Key words: Anesthetics, volatile: halothane. Behavior. Genetics, behavioral. *Caenorhabditis elegans*. Drug mechanisms. Theories of anesthesia. Volatile anesthetics.)**

VOLATILE anesthetics affect the function of multiple ligand and voltage-gated ion channels essential for proper metazoan nervous system function (reviewed in Franks and Lieb[1] and in Pocock and Richards[2]). Either one or some combination of these molecular perturbations presumably contributes to the mixture of behavioral effects called general anesthesia. However, no direct link has been made between these *in vitro* effects and behavioral dysfunction. Furthermore, without specific well-characterized behavioral antagonists, *in vitro* techniques are fundamentally incapable of making a causal connection between molecular and behavioral effects.

Genetic approaches are well suited to address the functional role of particular molecules in a drug's mechanism of action in an intact organism. Not only can the hypothetical role of a gene or protein be tested directly by reverse genetic techniques (*e.g.*, transgenic knockout mice) but previously unexpected components of the drug mechanism can be uncovered by classical and quantitative forward genetics. The latter approach is particularly useful for describing a complex mechanistic pathway with multiple components. Furthermore, the location and nature of missense mutations can give insight into where and how a drug binds. However, genetics has thus far been underused to define anesthetic action. This may stem in part from the apparent lack of genetic variance in humans and the very small variance in mouse lines that have been examined.[3-5] Thus the feasibility of mapping, cloning, and

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characterizing the genes responsible for these small phenotypic differences is very low. Invertebrate models offer considerable practical and theoretical advantages for genetic studies of anesthetic mechanisms and one obvious disadvantage. Their short life cycles, high fecundity, small and well-mapped genomes, and inexpensive maintenance all facilitate application of forward genetics. Another theoretical advantage is based on the concern that mutation of relevant genes may result in lethality or such severe nervous system dysfunction that the behavior or drug effect cannot be tested. Invertebrate organisms with their simpler growth requirements may circumvent these problems of behavioral pleiotropy. For example, null mutations that knock out a major type A γ-aminobutyric acid receptor in *Caenorhabditis elegans* are not only viable but also have only subtle locomotion defects and thus can be tested for their anesthetic sensitivity.[6,7] The obvious problem with invertebrate models is their relatively large evolutionary distance from humans. Although many genes are highly homologous between invertebrates and humans, invertebrates may lack the homologues of vertebrate anesthetic targets or these homologues may operate in behavioral pathways other than response to a noxious stimulus. Thus invertebrate genetics should be viewed as a route toward identifying functionally relevant genes for subsequent reverse genetic testing in vertebrate models.

The fruit fly, *Drosophila melanogaster*, and the nematode, *Caenorhabditis elegans*, are the two most highly developed invertebrate genetic systems and both have been screened for mutations that alter anesthetic sensitivity. *Drosophila* is rendered unresponsive to an intense beam of light at a median effective concentration (EC_{50}) of halothane of 0.410 vol% or an aqueous concentration of 0.29 mM (human minimum alveolar concentration [MAC] = 0.21 mM).[8] Nash and coworkers have screened for halothane-resistant (*har*) mutations and isolated strains that appear mildly resistant by a loss of negative geotaxis endpoint[9] but appeared hypersensitive to halothane by the response to light endpoint.[8] Thus careful characterization of anesthetic endpoints is essential before screening for anesthetic mutants.

More extensive mutant screens have been performed by Morgan, Sedensky, and coworkers in *C. elegans*.[10–14] They isolated several mutants that are significantly hypersensitive to halothane-induced immobility.[10,14] They have also isolated mutations that genetically interact with many of the former mutations.[11,13] Positional cloning of one of the genes, *unc-79*, revealed a novel sequence indicative of a transmembrane glycoprotein (P. Morgan, personal communication, January 20, 1996).

Although lack of spontaneous movement seems to be a natural anesthetic endpoint, the halothane EC_{50} for immobility is 3.2[10] to 3.5[6,7] vol% or a calculated aqueous concentration of about 2.5 mM at 20°C, which is 12 times the aqueous concentration produced by human MAC at 37°C.[15] In electrophysiologic studies of vertebrate tissue, these aqueous concentrations affect a much larger set of proteins than those altered at human MAC (reviewed in Franks and Lieb[1,16]), Furthermore, the onset of immobility is peculiarly slow, with steady-state not reached until after 2 h of anesthetic exposure.[7] Here we examine the potency, time course, and recovery characteristics of halothane-induced changes in most of the well-described *C. elegans* behaviors. Our hypothesis is that other *C. elegans* behaviors may be controlled by anesthetic targets that are reversibly affected at anesthetic concentrations and with time courses similar to vertebrate anesthetic targets.

Materials and Methods

Nematodes

General methods to maintain *C. elegans* were as described by Brenner.[17] All strains were grown at 20°C on NGM[17] agar plates with the *Escherichia coli* strain OP50[17] as a food source. Some of the strains used were obtained from the Caenorhabditis Genetics Center, which is funded by the National Institutes of Health's National Center for Research Resources. CB4855 was a gift from Jonathan Hodgkin (MRC, Cambridge, UK). Standard nomenclature, as set forth by Horvitz and colleagues,[18] was used throughout the study.

Concentration–Response Curves and Statistics

Anesthetic behavioral endpoints, as described below, were plotted against anesthetic concentration, and data were fitted iteratively to the equation $y = \min + (\max - \min)/(1 + [x/x^{50}]^{-k})$ where y = the anesthetic behavioral endpoint, x = [Hal], k = slope to estimate EC_{50}s, slopes, and standard errors of the estimate. All curves were estimated from a minimum of six data points; replicate data were pooled for final curve estimates. Comparisons between curves for statistical difference of EC_{50}s were performed by simultaneous curve fitting as described by De Lean and associates.[19] All other significance testing was performed by one-way analysis

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of variance followed by, if indicated, Student's paired *T* test. The significance level was set at $P < 0.01$.

Behavioral Assays

All behaviors except mechanosensation were scored in sealed glass chambers at 20 to 22°C as described previously by Morgan and Cascorbi.[10] All animals except those in the chemotaxis assay were scored on NGM agar plates seeded with OP50 *E. coli*; except where noted, well-fed young adult *C. elegans* var. Bristol strain N2 (wild type) hermaphrodites were used. Except for mechanosensation assays, anesthetics were delivered through a sealable port in liquid form onto the lid of the sealed chamber and allowed to evaporate. Vapor-phase concentrations were measured by gas chromatography and calculated by interpolating between known standards. Mechanosensation assays were performed in a glove box and scored as described below. Halothane was delivered at 5-l diluent flow from a copper kettle vaporizer, and the concentration was determined by sampling from multiple sites within the glove box to ensure uniformity. For kinetic measurements, t = 0 was set as the time when anesthetic was added. Recovery from anesthetics was determined by transferring animals from the incubation plates to fresh plates and assaying after recovery in air for at least 16 h. For mating recovery, males were transferred to fresh plates with new L4 *dpy-11(e224)* hermaphrodites and allowed to mate over the next 3 days before scoring.

Locomotion. Immobility was scored as described by Morgan and Cascorbi[10] except that animals remaining immobile for 5 rather than 10 s were scored as immobilized. After a 2-h anesthetic exposure, at least 30 animals were scored per data point. For time course measurements, 20 animals per set were scored with three independent sets per time point. Coordinate movement was scored by two different methods. Ten animals per anesthetic concentration were scored by an observer blinded to anesthetic concentration for either normal or uncoordinated movement. Coordinate movement was also scored by the radial dispersion assay.[20] After washing three times with M9 buffer[17] and once with distilled water, 15 to 60 animals in 10 μl distilled water were placed in the center of a 9.5-cm plate of NGM agar seeded at the edge with a 0.5-cm ring of OP50 *E. coli*. The plates were then placed in the glass chambers, and anesthetic was added immediately on drying and initial dispersal of the animals. After 40 min, the number of animals in the bacterial ring was divided by the total number of animals on the plate to determine the radial dispersion index.

Mating. Mating efficiency was scored as described by Hodgkin[21] with the following modifications. Two N2 males were placed on a 3.5-cm NGM seeded plate with two L4 hermaphrodites or females of a different, easily distinguishable strain. Five such mating trial plates were placed in each glass chamber and halothane was added immediately. After a 20- to 24-h incubation period, males were removed and the fraction of plates with cross progeny scored 3 or 4 days later. Anesthetic concentration was measured at the beginning and end of the incubation period to ensure against chamber leakage. For mating efficiency curves, the fraction of plates with cross progeny was plotted against the anesthetic concentration at the end of the assay. For mating against *dpy-11(e224)*, 35 pooled data points (four independent experiments) were used for halothane mating efficiency and 12 points (two independent experiments) each were used for isoflurane, enflurane, and desflurane. For mating against *unc-51(e369)*, 40 pooled data points (four independent experiments) were used. Ten points each were used for the *fog-2(q71)* and CB5855 curves. Male mating behavioral steps were scored as described by Liu and Sternberg[22] except with N2 young adult males mating *unc-51(e369)* hermaphrodites. After a 1-h incubation period, the fraction of sloppy or missed turns for the turning endpoint and the fraction of passed vulva for the vulval location endpoint were scored. A total of 662 turns and 307 vulval locations by 143 males were scored, divided among ten halothane concentrations with a minimum of 21 turns, six vulval locations by seven males per point. For time course measurements, turns and vulva location behavior were scored over 5-min periods with three to eight animals scored independently during each period. Sperm counts of potentially mated *fog-2(q71)* females were performed by Normarski microscopy, with animals mounted on a 5% agar pad with 5 mM sodium azide for immobilization.

Chemotaxis. Chemotaxis assays were performed as described by Bargmann and associates[23] with the following modifications. Young adults were washed three times with 1 ml S-basal,[24] once with 1 ml distilled water, and then resuspended in the same fluid. Animals were pelleted in each case by allowing them to settle at 1*g*. Thirty to 100 washed animals in 10 μl H_2O were placed in the center of a 9.5-cm chemotaxis plate[23] that had been spotted at the control pole (1.5-cm-diameter circle at the edge of the plate) with 1 μl ethanol along

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with 1 μl 1M sodium azide and at the attractant pole (1.5-cm-diameter circle at the opposite edge of the plate) with 1 μl of a 1:100 dilution of the chemoattractant in 100% ethanol along with 1 μl 1M sodium azide. Anesthetic was added immediately after placement of the animals. After 2 hours, the chemotaxis index (CI) was scored. CI = (number of animals at the attractant pole − the number of animals at the control pole)/ Total number of animals on the plate. All chemoattractants were obtained from Sigma Chemical Company (St. Louis, MO).

Defecation/Pharyngeal Pumping. Defecation and pharyngeal pumping rates were scored using an event-recording computer program as described by Liu and Thomas.[25] For defecation, a step in the cycle was scored as defective if it was not observed during a 60-s period. Each animal was scored for four cycles each with ten animals per anesthetic concentration. For pharyngeal pumping, the number of terminal bulb oscillations was counted in each animal for 30 s. Ten animals per anesthetic concentration were scored, and an animal was scored as defective if the mean pumping rate was slower than 1 Hz, which is the intrinsic rate of a denervated pharynx.[26] Animals were preincubated in anesthetic for 1 h before scoring either defecation or pumping, except for time-course measurements that are relative to the time when anesthetic was added to the chambers.

Egg Laying. Five animals per anesthetic concentration divided among five plates were incubated in anesthetic for 4 h. Eggs were counted at 2 and 4 h. Animals on a plate were scored as egg-laying defective if no eggs were laid during the second 2-h period. For egg-laying rates, the eggs laid during the first 2 h were plotted against the halothane concentration.

Mechanosensation. Ten animals per anesthetic concentration were scored for light-touch sensitivity as described by Chalfie and Sulston.[27] After a 2-h equilibration in halothane in a glove box as described previously, each animal was stroked transversely five times just posterior to the pharynx with an eyelash glued to a toothpick. The mean fraction of strokes not provoking response ± standard error of the mean (SEM) for the ten animals was plotted.

Results

Halothane-induced Locomotion Defects

Mutations in many loci of *C. elegans* result in movement defects of various kinds ranging from relatively subtle qualitative defects, such as shrinking, fainting, kinking, looping, hyperactivity, coiling, and twitching, which are usually the result of defects in nervous system components, to immobility, which is often due to defects in muscle components (reviewed in Bargmann[28] and Waterston[29]). Halothane induces a sluggish, loopy, occasionally kinking uncoordinated movement at lower concentrations and immobility at higher concentrations. Figure 1 compares the potency of halothane for inducing uncoordinated movement and immobility. Halothane is approximately ten times more potent at producing uncoordinated movement than immobility. The calculated aqueous EC_{50} for uncoordinated movement is 0.26 mM, compared with 2.6 mM for immobility; human MAC is 0.21 mM. To confirm our subjective assessment of uncoordinated movement, we examined the ability of anesthetized animals to move from the center of an agar plate to a bacterial ring 4 cm away.[20] Table 2 summarizes these data for halothane and three other volatile anesthetics. The EC_{50} in the radial dispersion assay is similar to that obtained by subjective assessment.

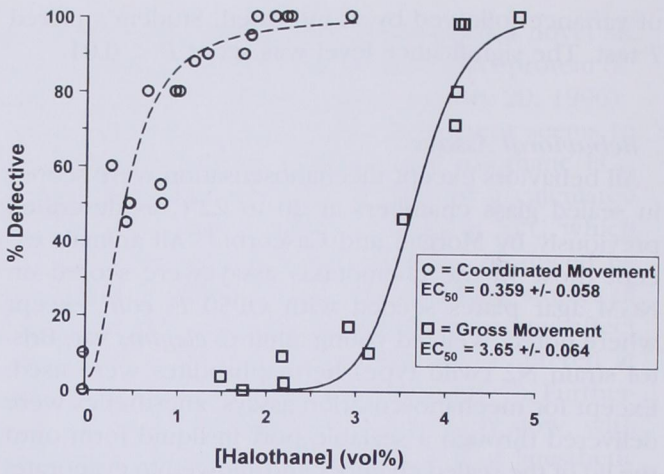

Fig. 1. Halothane-induced locomotion defects. For the loopy/sluggish uncoordinated movement defect induced by halothane, 10 young adult animals per data point were scored by an observer blinded to drug concentration. For immobilization, 30 to 50 animals per data point were scored for lack of spontaneous movement of any type during a 5-s period. Median effective concentrations and standard errors are estimated by logistic regression.

Halothane-induced Mating Defects

Male mating is a complex multistep process that has been dissected at a cellular level[22] and is beginning to be examined by molecular genetic approaches.[30–32] On

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Table 1. Halothane Potency for Male Mating Defects

Mating Pair	Female Mobility	Mating Efficiency		Turning		Vulval Location	
		EC_{50} (vol%)	Slope	EC_{50} (vol%)	Slope	EC_{50} (vol%)	Slope
N2 × *unc*-51(e369)	Paralyzed	0.92 ± 0.02	7.8 ± 1.3	0.28 ± 0.03	2.5 ± 0.64	0.33 ± 0.07	1.2 ± 0.33
N2 × *dpy*-11(e224)	Slow	0.30 ± 0.02	3.5 ± 0.4	ND	—	ND	—
CB4855 × *dpy*-11(e224)	Slow	0.24 ± 0.03	2.7 ± 0.8	ND	—	ND	—
N2 × *fog*-2(q71)	Fast	0.11 ± 0.04	1.3 ± 0.5	ND	—	ND	—

Mating efficiency is the fraction of mating trials (2 males × 2 females) with cross-progeny; a turn was scored as defective if it was sloppy or missed; if the vulva was passed, location was scored as defective.

ND = not done.

contact with a hermaphrodite, a wild type male apposes his tail (the specialized mating organ) to the hermaphrodite, backs until reaching the nose or tail, turns around the nose or tail while never losing contact with his mate, and on contacting the vulva with his tail, stops, finely adjusts his position, inserts his spicules, and transfers sperm.[22] We measured the global ability to accomplish all of these steps by measuring mating efficiency as defined by the fraction of crosses resulting in cross progeny.[21] Different strains of hermaphrodites with various mobilities were used as mates to assess halothane's effects on male movement compared with other mating steps. The results are shown in table 1. Halothane's potency in stopping mating is hermaphrodite dependent, which suggests that male mobility defects are part of the behavioral dysfunction. To preclude a toxic effect on sperm as an explanation for the lack of cross progeny, *fog-2(q71)* females (who carry a mutation that prevents spermatogenesis) were scored by Normarski microscopy for male sperm after exposure to halothane. In no instance were females with sperm found on plates that grew no progeny (n = 24). We also examined the effect on the strain CB4855 (aka Mr. Vigorous) whose males have been shown to be naturally better maters than N2 (J. Hodgkin, personal communication, January 11, 1996). The similar sensitivity of CB4855 shows that merely being a better mater does not render this strain resistant to halothane. We directly examined halothane's effects on turning and vulval location behaviors in N2 males mated against *unc-51(e369)* hermaphrodites (table 1). These paralyzed hermaphrodites were used as mates to avoid any confounding effect of hermaphroditic movement on scoring of male mating behavior. Halothane is approximately equipotent at disrupting both turning and vulval location mating behaviors, with EC_{50}s similar to mating efficiency against *dpy-11(e224)*, a mobile but slow mate, and to loss of coordination.

Halothane-induced Chemotaxis Defects

C. elegans senses and taxes to multiple chemoattractants.[23,33] Specialized multicellular sensory structures

Table 2. Summary of Behavioral Effects of Volatile Anesthetics in *Caenorhabditis elegans*

Behavior	EC_{50} (mean ± SEM) (vol%)				Effect Time (min)	% Recovery	
	Halothane	Isoflurane	Enflurane	Desflurane		p̄ 1%	p̄ 4%
Mating	0.30 ± 0.02	0.63 ± 0.02	0.71 ± 0.08	4.1 ± 0.3	10	97	47
Chemotaxis	0.34 ± 0.01	0.89 ± 0.05	0.97 ± 0.08	5.1 ± 0.6	<30	100	53
Coordinated movement	0.32 ± 0.05	0.86 ± 0.09	1.1 ± 0.07	—	<20	—	—
Defecation	1.2 ± 0.05	2.5 ± 0.14	1.9 ± 0.02	—	10	—	—
Pumping	1.8 ± 0.02	3.1 ± 0.07	3.6 ± 0.001	—	10	—	—
Egg laying	2.1 ± 0.10	—	—	—	—	—	—
Mechanosensation	2.2 ± 0.07	—	—	—	—	—	—
Gross movement	3.6 ± 0.06	6.5 ± 0.05	4.7 ± 0.04	—	120	100	88

Halothane potency/recovery for mating is efficiency of N2 males × *dpy*-11(e224); time course is N2 males × *unc*-51(e369); chemotaxis is against isoamyl alcohol; coordinated movement data are from radial diffusion assays; defecation data are for expulsion; p = % of recovery to air control after exposure to 1 or 4 vol% halothane.

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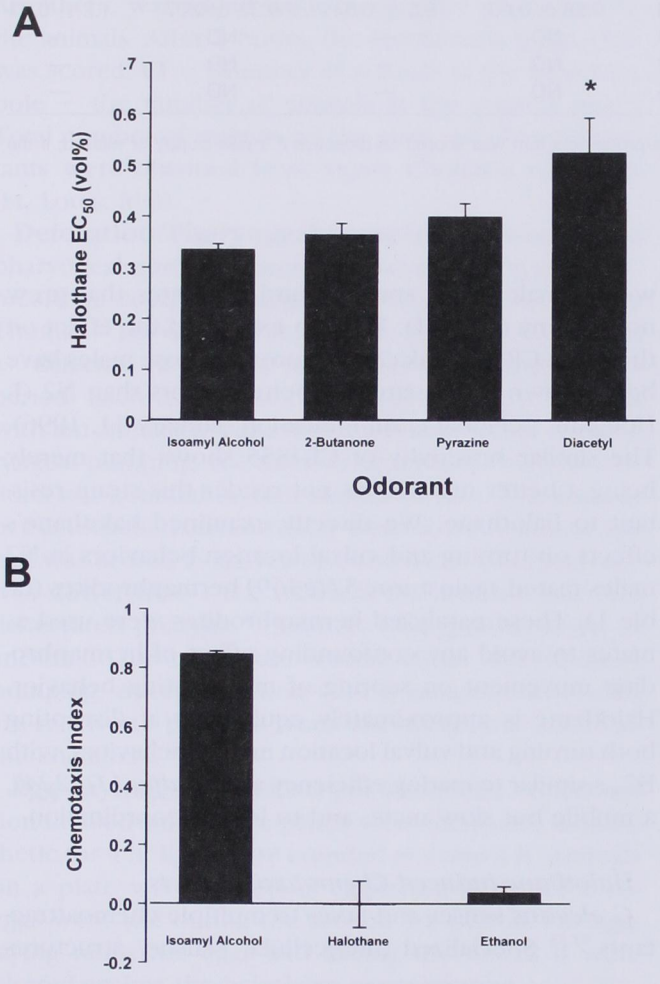

Fig. 2. Halothane-induced chemotaxis defects. Thirty to 100 animals per halothane concentration were scored for chemotaxis during a 2-h period to the odorants noted. The chemotaxis index[23]: (number of animals at the attractant − number of animals at the control)/total number of animals was plotted against halothane concentration to obtain median effective concentrations (EC_{50}s). (*A*) Halothane EC_{50}s ± SEM against chemotaxis to four distinct classes of odorants. The EC_{50}s ± SEM are from pooled experiments: Isoamyl alcohol = 0.34 ± 0.01 slope = −2.5 ± 0.2 n = 88 *d*ata *p*oints, 8 *i*ndependent *e*xperiments; 2-Butanone = 0.36 ± 0.02 slope = −3.6 ± 1.4 n = 18 dp, 3 ie; Pyrazine = 0.4 ± 0.03 slope = −2.4 ± 0.42 n = 24 dp, 2 ie; Diacetyl = 0.52 ± 0.07 slope = −2.6 ± 0.6 n = 29 dp, 3 ie. (* − Diacetyl EC_{50} ≠ Isoamyl alcohol EC_{50} @ $P < 0.01$). (*B*) Chemotaxis indices to isoamyl alcohol, halothane, or ethanol. A 1:100 dilution of isoamyl alcohol, halothane in ethanol, or ethanol itself was spotted at the attractant circle and chemotaxis tested immediately (n = 16 for isoamyl alcohol; n = 4 for halothane; n = 8 for ethanol). Halothane is not significantly different from ethanol, both of which are different from isoamyl alcohol ($P < 0.01$).

are required for chemotaxis, and each neuron and odorant receptor has been shown to mediate taxis only to specific classes of odorants.[23] Figure 2A shows the potency of halothane to completely abolish chemotaxis to representative odorants from four distinct classes. Halothane is essentially equipotent at abolishing chemotaxis to three odorants but is significantly less potent against chemotaxis to diacetyl ($P < 0.01$); however, the effects on chemotaxis to all four odorants are within the anesthetic concentration range for vertebrate anesthesia. To rule out that halothane was acting as an odorant itself, chemotaxis assays were performed with halothane dissolved in ethanol as the attractant. *C. elegans* immediately orients and begins moving to authentic odorants.[23] Thus an affinity for one pole of the plate would be expected if halothane were attractive or repulsive, even if a halothane gradient is maintained only for a few minutes. Figure 2B shows that halothane is neither an attractant nor a repellent. Furthermore, direct observation of animals did not demonstrate orientation toward halothane.

Halothane-induced Defecation and Pharyngeal Pumping Defects

Both defecation and pharyngeal pumping are highly repetitive and stereotypical behaviors. Young adults invariably defecate every 40 to 50 s, with each cycle composed of a posterior body contraction(pBoc), an anterior body contraction (aBoc), and then expulsion of gut contents (exp).[34] Figure 3A shows the potency of halothane against pBoc and expulsion. Expulsion (EC_{50}: 1.2 ± 0.05 vol%) is more sensitive than pBoc (EC_{50}: 1.5 ± 0.05 vol%) to halothane. The sensitivity of aBoc was not formally scored but appeared to be similar to that of pBoc. Pharyngeal pumping is very rapid in the presence of bacteria, with 0.2 to 0.3 s between pumps.[26] By ablating with a laser all the neurons innervating the pharynx, Avery and Horvitz[26] showed that the denervated pharynx continues to pump, but at an average rate of less than 1 Hz. Thus an average pumping rate of less than 1 Hz for 30 s was set as defective. Figure 3B shows that pumping is made defective by halothane, with an EC_{50} of 1.8 ± 0.02 vol%. However, the rate of pumping itself does not clearly fit a sigmoidal curve and continues to slow until movement and pumping completely cease at halothane concentrations greater than 3 vol%.

Halothane-induced Egg-Laying Defects

Animals were scored as egg-laying defective if, after a 2-h preincubation, no eggs were laid during the subse-

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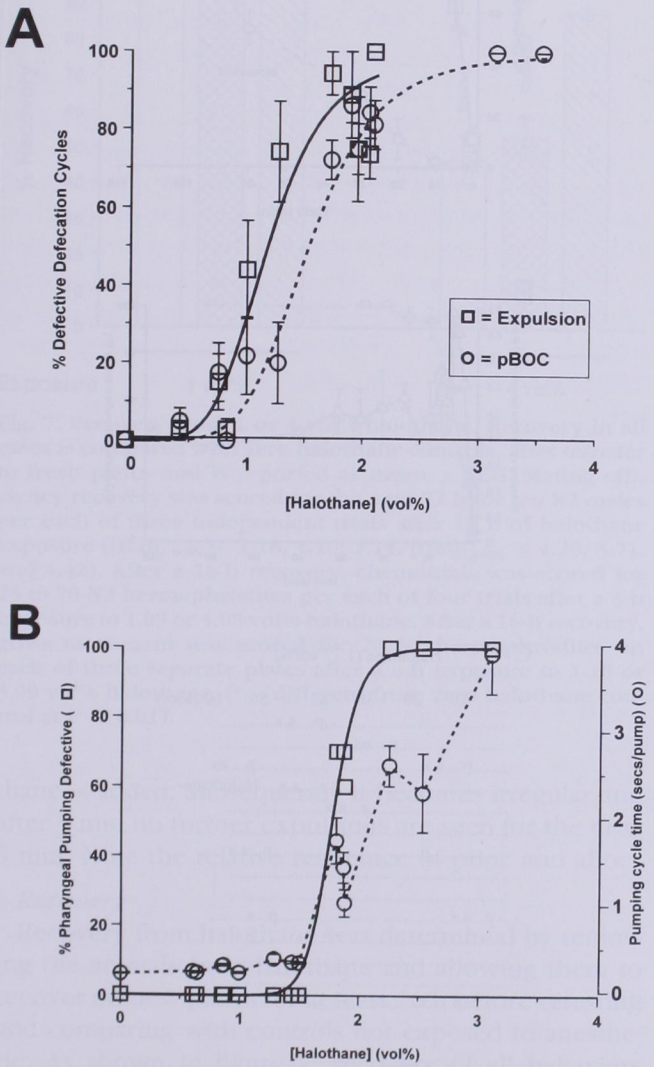

Fig. 3. Halothane-induced defecation and pharyngeal pumping defects. (*A*) Defecation occurs every 42 ± 4.7 s[34] and is composed of three steps: *p*osterior *bo*dy *c*ontraction (pBoc), *a*nterior *bo*dy *c*ontraction (aBoc), followed by *exp*ulsion (exp) of gut contents. A step in the cycle was scored as defective if it was not observed during a 60-s period (4 sd greater than mean cycle time in air). Each animal was scored for four cycles. The mean ± SEM of the fraction of defective cycles for ten animals per [Hal] is plotted. (*B*) Pharyngeal pumping (eating behavior) consists of a rapid anterior/posterior oscillatory movement of the pharynx at a rate of 3 to 5 pumps/s in the presence of food. Animals were scored as pumping defective if the mean pumping rate during the 30-s observation period was slower than 1 hz, which is the rate of a denervated pharynx.[26] The percentage of pumping-defective animals (n = 10 at each [Hal]) along with the cycle time in seconds/pump (mean ± SEM) for those ten animals is plotted.

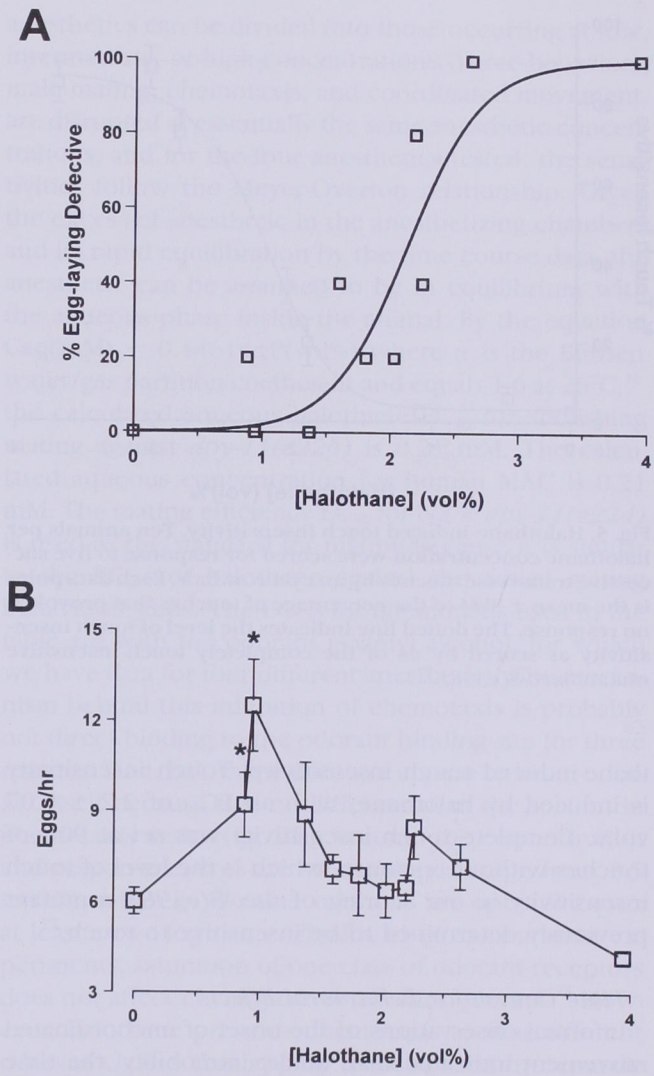

Fig. 4. Halothane-induced egg-laying defects. (*A*) After a 2-h halothane preincubation, five animals/[Hal] were scored for egg laying. Animals that layed no eggs in the next 2 h were scored as defective. (*B*) During the initial 2-h preincubation, the number of eggs laid per animal was counted. Each data point is the mean ± SEM for five animals (* – different from 0 halothane control at $P < 0.01$).

quent 2-h period. Figure 4A shows halothane's potency for stopping egg laying. However, the effect is complex because in the first 2 h halothane actually stimulates egg laying at lower concentrations (fig. 4B).

Halothane-induced Touch Insensitivity

C. elegans respond to light touch near their noses by reversing forward movement and backing up.[27] Figure 5 shows the concentration and response curve for halo-

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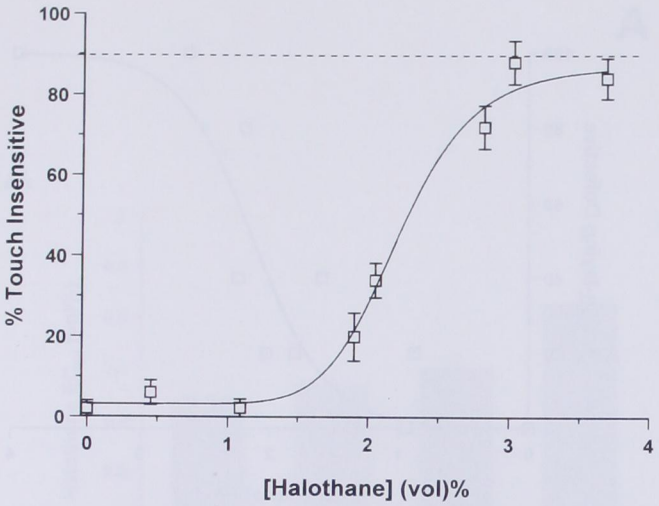

Fig. 5. Halothane-induced touch insensitivity. Ten animals per halothane concentration were scored for response to five successive transverse touches by an eyebrow hair. Each data point is the mean ± SEM of the percentage of touches that provoked no response. The dotted line indicates the level of touch insensitivity as scored by us of the completely touch-insensitive mutant *mec-8(e398)*.[27]

thane-induced touch insensitivity. Touch insensitivity is induced by halothane, with an EC_{50} of 2.2 ± 0.07 vol%. Complete touch insensitivity was set at 90% of touches without response, which is the level of touch insensitivity in our scoring of *mec-8(e398)*, a mutant previously determined to be insensitive to touch.[27]

Time Course for Behavioral Effects

Informal observations of the onset of uncoordinated movement indicated that, unlike immobility, the time course was rapid. The time course was measured for halothane's effect on five behavioral endpoints: male turning, male vulval location, gross movement, pumping rate, and expulsion (fig. 6). The time courses were measured at EC_{90-98} for each behavior except for pumping rate, which does not fit a sigmoidal curve as shown in figure 3B. Halothane produced a steady-state effect for all behaviors within 10 min except for gross movement, which, as reported previously,[7] required at least 2 h to reach steady state (figs. 6A and B). Because pumping rate continues to slow until stopping at immobilizing concentrations of halothane, the initial overshoot effect on pumping rate may be due to locally high concentrations as the halothane evaporates over the animals. Figure 6C shows a record for defecation before and after administration of 1.77 vol% halothane. Defecation is regular until and one cycle after halo-

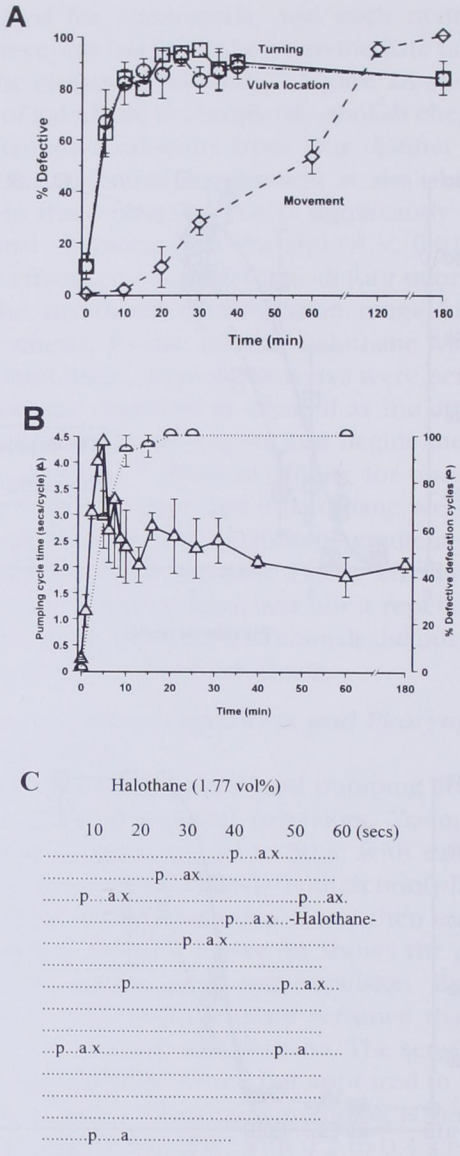

Fig. 6. Time course of halothane-induced behavioral defects. (*A*) For 5-min intervals ending at the time point, three to eight N2 males in each of four independent trials were scored for turning and vulval location behavior as above ([Hal] = 1.08, 1.06, 1.02, and 0.96 vol% ≈ $EC_{96} - 3 \times EC_{50}$). Gross movement was scored as above for three sets of 20 N2 hermaphrodites in a single chamber ([Hal] = 4.82 vol% ≈ $EC_{98} - 1.3 \times EC_{50}$). (*B*) For 1-min intervals ending at the time point, pumping cycle time (seconds/cycle) was scored for one to three animals in each of three trials ([Hal]> = 2.0, 2.2, and 2.1 vol%). For 5-min intervals ending at each time point, the percentage of defective cycles for the expulsion step of defecation was scored for one to five animals in each of three trials ([Hal] = 1.9, 1.8, and 1.9 vol% ≈ $EC_{90} - 1.5 \times EC_{50}$). (*C*) A representative defecation record, 4 min before and 9.5 min after addition of halothane. Each line represents 1 min. *P* = pBoc, a = aBoc, x = expulsion. All data points are means ± SEM.

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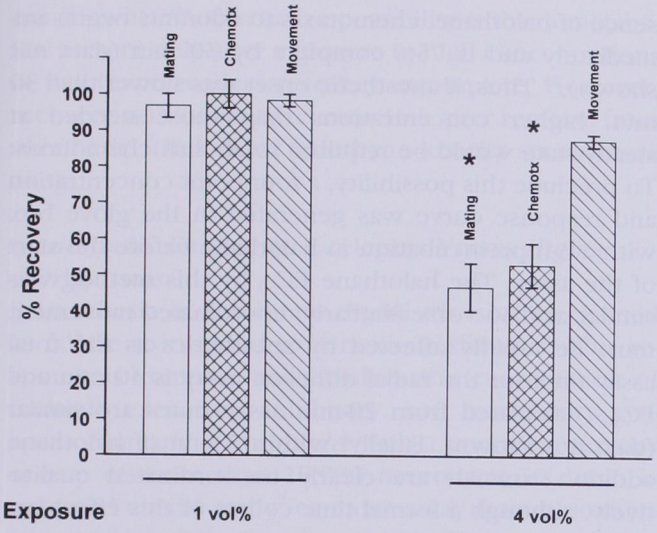

Fig. 7. Recovery from 1 or 4 vol% halothane. Recovery in all cases is compared with zero halothane controls, after transfer to fresh plates and is reported as mean ± SEM. Mating efficiency recovery was scored for the next 72 h for ten N2 males per each of three independent trials after 16 h of halothane exposure ($[Hal]_{1\ vol\%}$ = 1.18, 1.10, 1.14; $[Hal]_{4\ vol\%}$ = 4.20, 3.71, and 4.42). After a 16-h recovery, chemotaxis was scored for 25 to 70 N2 hermaphrodites per each of four trials after a 3-h exposure to 1.09 or 4.08 vol% halothane. After a 16-h recovery, gross movement was scored for 20 N2 hermaphrodites on each of three separate plates after a 6-h exposure to 1.10 or 3.99 vol% halothane. (* – different from zero halothane control at $P < 0.01$).

thane is added. Subsequently it becomes irregular and after 5 min no further expulsions are seen for the next 5 min. Note the relative resistance of pBoc and aBoc.

Recovery

Recovery from halothane was determined by removing the animals from halothane and allowing them to recover on new plates for at least 16 h before retesting and comparing with controls not exposed to anesthetic. As shown in figure 7, recovery of all behaviors tested was essentially complete after exposure to 1 vol% halothane but was significantly reduced ($P < 0.01$) after immobilizing concentrations for mating and chemotaxis. Gross movement was the most robust behavior at 88% recovery and was not significantly different from that seen in control animals or those exposed to 1 vol% halothane (fig. 7).

Discussion

Volatile Anesthetics Are Selectively Potent in Disrupting C. elegans Behaviors

Table 2 summarizes the anesthetic potency, time course, and recovery data. The behavioral effects of anesthetics can be divided into those occurring at low, intermediate, or high concentrations. Three behaviors, male mating, chemotaxis, and coordinated movement, are disrupted at essentially the same anesthetic concentrations; and for the four anesthetics tested, the sensitivities follow the Meyer-Overton relationship. Given the excess of anesthetic in the anesthetizing chambers and its rapid equilibration by the time course data, the anesthetic can be assumed to be in equilibrium with the aqueous phase inside the animal. By the equation $Caq(mM) = 0.44614\alpha P(vol\%)$ where α is the Bunsen water/gas partition coefficient and equals 1.6 at 20°C,[16] the calculated aqueous halothane EC_{50} for abolishing mating against *dpy-11(e224)* is 0.21 mM. The calculated aqueous concentration for human MAC is 0.21 mM. The mating efficiency EC_{50} for N2 × *dpy-11(e224)* is used as the standard for mating because it is similar to the EC_{50}s of halothane against the actual mating behavioral steps. The EC_{50}s for chemotaxis used in the table are with the odorant isoamyl alcohol, for which we have data for four different anesthetics. The mechanism behind this inhibition of chemotaxis is probably not direct binding to the odorant binding site for three reasons. First, halothane does not appear to be an odorant itself because it is no different than the solvent in chemotaxis assays (fig. 2B). Second, the concentration and response curves are steep (slopes range from 2.5 to 6.7), whereas all other odorants are operative over at least a two-log range. Finally, in cross-saturation experiments, saturation of one class of odorant receptors does not affect chemotaxis to another odorant.[23] However, halothane prevents chemotaxis to four different odorant classes that appear to be sensed by separate odorant receptors with distinct neuronal distributions.[23] Thus halothane most likely acts at some sensory or motor integrative step common to all chemotaxis, although binding to a structural motif distinct from the odorant binding site and common to all four receptor classes is possible. The similar sensitivities of the three behavioral endpoints could be due to three mechanisms: (1) identical anesthetic targets operating for all three behaviors, (2) different targets with similar anesthetic sensitivities for each behavior, (3) identical behavioral effect measured in three different way; that is, the uncoordinating effects of anesthetics might be entirely responsible for loss of mating and chemotactic abilities. We cannot preclude any of these mechanisms, although explanation 1 or 2 is more consistent with the data.

Defecation, pumping, egg laying, and mechanosensa-

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tion are intermediately sensitive to anesthetics. Three points are noteworthy. First, as for *C. elegans* behavior in general, specific aspects of defecation behavior are more sensitive to anesthetics than are others. Similarly, mutations have been isolated that specifically affect expulsion.[34] Second, pumping only ceases at immobilizing concentrations of anesthetic. Given that pumping continues after denervation, albeit in an uncoordinated and slow fashion,[26] the ceasing of pumping by immobilizing halothane concentrations is probably mediated by direct effects on pharyngeal muscle either by decreasing excitability of pacemaker muscle cells or by disrupting gap junction–mediated signaling between muscle cells. Finally, the stimulation of egg laying by halothane is reminiscent of serotonin-induced egg laying. This serotonin effect is complex but is mimicked by loss-of-function mutations in the *C. elegans* Goα gene, *goa-1*.[31,32] Intriguingly, the halothane-induced loopy uncoordinated movement, male turning defects, and pharyngeal pumping defects are also qualitatively similar to *goa-1* loss-of-function mutants.

High concentrations of halothane produce immobility in *C. elegans*, as has been shown previously by Morgan, Sedensky, and coworkers.[10] The calculated aqueous halothane EC_{50} is 2.6 mM, or about 12 times MAC. These concentrations alter the function of a much larger set of proteins than those affected at MAC.[1] However, anesthetic targets in *C. elegans* could be so structurally different from those in vertebrates that higher concentrations might be required. The appropriate sensitivities of other behaviors, their time courses, and recovery profiles argue against this explanation.

Time Course of Behavioral Effects

The rapid time course of halothane's effects on several behaviors indicates that the cuticle of *C. elegans* does not pose a significant kinetic barrier to entry of anesthetic or that anesthetic enters through the multiple interruptions in the cuticle (pharynx, anus, sensilla, excretory ducts, and vulva [in hermaphrodites]). The rapid time course also suggests that anesthetics disrupt neuronal signaling rather than transcription, protein synthesis, or cellular integrity. However, the latter mechanisms might underlie the relatively slow onset of anesthetic-induced immobilization. While the time courses for mating behavior, defecation, pumping, and gross movement were formally measured, those for chemotaxis and coordinated movement can only be estimated from the rapidity of their assays. In the absence of halothane, chemotaxis to odorants begins immediately and is 75% complete by 30 min (data not shown).[23] Thus, if anesthetic onset was slower than 30 min, higher concentrations than those needed at steady-state would be required to abolish chemotaxis. To preclude this possibility, a four-point concentration and response curve was generated in the glove box with a 3-h preincubation in halothane before the start of the assay. The halothane EC_{50} by this method was similar at 0.36 vol%. Similarly, coordinated movement must be rapidly affected by anesthetics as the total assay time for the radial diffusion assay is 40 min and EC_{50}s calculated from 20-min assay times are similar (data not shown). Finally, within 5 min of halothane addition, animals are clearly uncoordinated qualitatively, although a formal time course of this effect has not been performed.

A caveat necessary for these experiments is that the time courses were measured at the EC_{90-98} for each behavior; however, because the slopes of the concentration and response curves ranged from 1.2 (vulval location) to 13 (immobility), the relationship of the EC_{90} to the EC_{50} differed for each behavior, as noted in the figure legend. For immobility, time courses were measured at six different concentrations ranging from the EC_5 to the EC_{98}, for which the data are shown in figure 6. At all concentrations, the time to steady-state for immobility was not related to the halothane concentration and was not reached until at least 2 h (data not shown). For other behaviors, only time courses around the EC_{90} were measured; nevertheless, this one concentration is sufficient to conclude that halothane gains rapid access to the nervous system and disrupts the neuronal circuits mediating mating, defecation, and pumping, with a time course similar to vertebrate anesthesia.

Recovery from Halothane

Full recovery after exposure to 1 vol% concentrations of halothane rules out irreversible toxicity as a mechanism for the nervous system dysfunction and is consistent with anesthetic action in vertebrates. However, after exposure to immobilizing concentrations, recovery is incomplete for mating and chemotaxis and tends to be lower for gross movement as well. Although starvation during exposure to high halothane concentrations is a possible explanation for this poor recovery, short-term starvation (4 h) of the same length as the halothane exposure for the chemotaxis recovery experiments does not alter the chemotaxis index (data

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not shown). An alternative possibility is that a metabolite present only after exposure to high concentrations of halothane renders recovery incomplete. However, recovery was incomplete even 36 h after removal from anesthetic. Thus direct halothane toxicity seems more likely.

Anesthetic Endpoints: C. elegans Versus *Higher Organisms*

Anesthesia in vertebrate organisms is often defined as failure to respond to a noxious stimulus. This response is complex and involves the coordination of multiple excitatory, inhibitory, and modulatory synapses. Similarly, *Drosophila* with a complex nervous system of more than 10,000 neurons in its brain is rendered unable to fly[9] or move away from a noxious stimulus[8] by clinically relevant volatile anesthetic concentrations, presumably due to its inability to control or coordinate the multiple neurons involved in flight and response to a stimulus. On the other hand, *C. elegans* is a much simpler organism at a cellular level, with only 302 neurons, four of which are required for gross, albeit uncoordinated movement.[35] Gross backward movement requires only the function of the interneurons AVA and AVD, whereas forward movement requires AVB and PVC. Even with ablation of both AVB and PVC, animals are capable of slow, uncoordinated movement driven by head foraging, which is controlled by different neurons. These interneurons innervate motorneurons through and are innervated by both chemical synapses and gap junctions; however, the chemical synapses are probably modulatory while the gap junctions directly drive the interneurons and motorneurons.[35] Thus for halothane to induce immobility, dysfunction of at least one of four processes appear to be required: the intrinsic excitability of the command interneurons, motorneurons, or muscle; gap junction–mediated signaling to the interneurons, motor neurons, or muscle; neuromuscular synaptic transmission; or excitation contraction coupling of the muscle itself. Intrinsic excitability, gap junction electrical coupling, cholinergic neuromuscular junctions, and muscle contraction have been shown in other systems to be relatively resistant to volatile anesthetics.[2,36] Thus the high concentration of halothane required for immobilization of *C. elegans* is not at all surprising. On the other hand, the behavioral specificity of low *versus* intermediate anesthetic concentrations more likely represents distinct neuronal distributions of synaptic molecules. The hypothesis, based on their pharmacologic dissimilarities, that the mating defects and immobility produced by halothane are through distinct mechanisms is supported by preliminary genetic evidence that the loci controlling the two endpoints do not overlap.[37]

Conclusions

Genetics provides the tools to identify the molecules whose functional disruption is actually responsible for anesthetic-induced nervous system depression. Other methods can determine which neuronal molecules are affected at relevant anesthetic concentrations and the nature of anesthetic binding sites; however, without genetics, they can never establish which, if any, of these *in vitro* anesthetic effects produces behavioral dysfunction in an intact nervous system. *C. elegans* is one of only two model organisms with the necessary genetic power to bridge this molecular-to-behavioral gap. We applied the pharmacologic standards of potency, time course, and recovery, which are rightly used to judge the relevancy of any molecular effect of anesthetics, to the behavioral effects of anesthetics in *C. elegans*. The observation that specific behaviors are inhibited by clinically relevant concentrations of halothane in *C. elegans* suggests that these behaviors are controlled by molecules that may share structural motifs with vertebrate anesthetic targets. The detailed understanding of the neuronal circuits controlling these anesthetic-sensitive behaviors coupled with the model's genetic power should allow identification of the functionally operant anesthetic targets in *C. elegans* and thereby guide subsequent directed genetic testing in vertebrate models.

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