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*Xenon Acts by Inhibition of Non–*N*-methyl-*D*-aspartate Receptor–mediated Glutamatergic Neurotransmission in* Caenorhabditis elegans

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Background: **Electrophysiologic experiments in rodents have found that nitrous oxide and xenon inhibit *N*-methyl-D-aspartate (NMDA)–type glutamate receptors. These findings led to the hypothesis that xenon and nitrous oxide along with ketamine form a class of anesthetics with the identical mechanism, NMDA receptor antagonism. Here, the authors ask in *Caenorhabditis elegans* whether xenon, like nitrous oxide, acts by a NMDA receptor–mediated mechanism.**

Methods: **Xenon:oxygen mixtures were delivered into sealed chambers until the desired concentration was achieved. The effects of xenon on various behaviors were measured on wild-type and mutant *C. elegans* strains.**

Results: **With an EC_{50} of 15–20 vol% depending on behavioral endpoint, xenon altered *C. elegans* locomotion in a manner indistinguishable from that of mutants in glutamatergic transmission. Xenon reduced the frequency and duration of backward locomotion without altering its speed or other behaviors tested. Mutation of *glr-1*, encoding a non-NMDA glutamate receptor subunit, abolished the behavioral effects of xenon; however, mutation of *nmr-1*, which encodes the pore-forming subunit of an NMDA glutamate receptor previously shown to be required for nitrous oxide action, did not significantly alter xenon response. Transformation of the *glr-1* mutant with the wild-type *glr-1* gene partially restored xenon sensitivity, confirming that *glr-1* was necessary for the full action of xenon.**

Conclusions: **Xenon acts in *C. elegans* to alter locomotion through a mechanism requiring the non-NMDA glutamate receptor encoded by *glr-1*. Unlike for the action of nitrous oxide in *C. elegans*, the NMDA receptor encoded by *nmr-1* is not essential for sensitivity to xenon.**

THE noble gas xenon has generated increasing interest as a general anesthetic because of its favorable pharmacokinetic parameters and its minimal side effects. The pharmacologic profile of xenon is very similar to that of nitrous oxide, whose predominant mechanism of action seems to be inhibition of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors, thereby reducing excitatory glutamatergic neurotransmission.[1–4] In addition, xenon possesses unique neuroprotective properties that are not shared by other NMDA receptor antagonists such as ketamine or nitrous oxide, both of which may have intrinsic coexisting neurotoxicity.[5,6] Best characterized among xenon's effects are its strong inhibition of NMDA receptor evoked currents[1,7,8] and its potent activation of two-pore domain K^+ channels (TREK-1).[9] Furthermore, electrophysiology experiments with recombinant ion channels expressed in *Xenopus* oocytes showed that xenon inhibits serotonin (5-hydroxytryptamine type 3A) receptors[10] and neuronal acetylcholine receptors.[11] On the other hand, ligand-gated ion channels such as γ-aminobutyric acid type A receptors and glycine receptors, which are very sensitive to volatile anesthetics, have been found to be relatively insensitive to xenon. The data have been mixed for xenon's action on non-NMDA ionotropic glutamate receptors (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid [AMPA] receptors and kainate receptors), with early studies indicating relative insensitivity but others suggesting that xenon can significantly inhibit certain non-NMDA subtypes.[7,8,12,13] The contribution that each of the electrophysiologic effects of xenon makes to its behavioral effects *in vivo* is as yet unclear. In a previous study addressing the question of how nitrous oxide produces its effects *in vivo*, we performed extensive behavioral and pharmacologic testing of wild-type and mutant strains of the nematode and genetic model organism *Caenorhabditis elegans* and found that nitrous oxide requires NMR-1, a subunit of the worm NMDA receptor, to produce behavioral defects identical to those of an *nmr-1* loss-of-function mutant.[4] Given that xenon shares with nitrous oxide the ability to inhibit NMDA receptors, we hypothesized that xenon, like nitrous oxide, acts predominantly through inhibition of NMDA receptors in *C. elegans*.

Materials and Methods

Culture of C. elegans *and Strains*

All strains were grown at 20° on NGM (Nematode Growth Medium) agar plates seeded with OP50 bacteria as described by Brenner.[14] The wild-type strain used was N2 (var. Bristol). VM280—*nmr-1(ak4);lin-15(n765)*; *akEx58*[pNMR-1(+) pLIN-15(+)],[15] VM1494—*glr-1(ky176) dpy-19(n1374);lin-15(n765);akEx244*[pGLR-1(+) pLIN-15(+)],[16] and *nmr-1(ak4);glr-1(ky176)* were

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gifts from Penelope Brockie, Ph.D., Research Associate, and Andres Villu Maricq, Ph.D., Associate Professor (both from the Department of Biology, University of Utah, Salt Lake City, Utah). Only non-muv animals, which represent the animals transformed with the extrachromosomal arrays, were scored in assays with VM280 and VM1494. All other strains were obtained from the Caenorhabditis Genetics Center, which is funded by the National Institutes of Health–National Center of Research Resources.

Behavioral and Pharmacologic Assays

One-day post L4 young adult animals were used for all assays. All anesthetic assays were performed at 22°–23° on NGM agar plates placed into sealed glass chambers. The radial dispersal assays and body bend assays were performed as described.[17] Chemotaxis, pharyngeal pumping, and defecation assays were performed as described previously, except pumping rates were recorded in the absence of bacteria.[18] Reversal frequency and forward and backward locomotion duration were measured as described by Zheng.[19] In short, animals were transferred by platinum wire to agar pads with no bacteria, placed into sealed glass chambers that were then flushed with xenon:oxygen mixture, and allowed to move on the assay plates for 10 min before scoring reversal frequencies. Xenon:oxygen (75%:25%) mixture (kindly provided by Mervyn Maze, M.B., Ch.B., F.R.C.P., F.R.C.A., F.med.Sci., Professor and Chairman, Department of Anesthetics and Intensive Care, Imperial College, Chelsea and Westminster Hospital, London, United Kingdom) was delivered to chambers using a luer-lock fitted tube attached to a luer fitting on special 100-ml glass chambers with inlet and outlet ports. Chambers were flushed with the xenon mixture for 1 min with a flow of approximately 1 l/min to ensure equilibration. The xenon concentration was monitored continuously with a calibrated xenon gas analyzer (Bedfont Scientific, Ltd., Rochester, Kent, United Kingdom)[5] until the xenon concentration exceeded 70%. For the concentration–response experiments, lower xenon concentrations inside the glass chamber were achieved by injecting a predetermined volume of the original 75%:25% xenon: oxygen mixture into the glass chamber with the use of a gastight plastic syringe. The concentration inside the chamber was measured after an 8-min equilibration period by withdrawing 10 ml gas mix from the glass chamber and measuring the xenon concentration in the syringe. Concentrations measured at the end of the assay were within 11% of the nominal beginning concentration, except for one value in the 10% concentration data set where the actual ending value was 12.2%.

Statistical Analysis

Concentration–response data were fitted to the equation $y = \min + (\max - \min)/1 + 10\text{^}[(X - \log EC_{50}) - k]$, where y is the response, X is the concentration, k is the Hill coefficient, and EC_{50} is the agonist concentration for half-maximum response. Curve fitting was performed by nonlinear regression using GraphPad Prism 4.02 software (GraphPad Software Inc., San Diego, CA). Within-group comparisons of normally distributed variables were performed by two-tailed, unpaired *t* tests. $P < 0.05$ was considered statistically significant.

Results

Our first goal was to determine whether xenon produced any behavioral effects in *C. elegans*. From previous experiments, we knew that nitrous oxide altered *C. elegans* locomotion in a manner mimicking loss of glutamatergic transmission, reducing the frequency of reversing direction of locomotion and otherwise not detectably altering behavior.[4] Volatile anesthetics, however, at clinical concentrations profoundly suppress coordinated movement and odorant chemotaxis and at higher concentrations affect feeding and defecation.[18] We presumed that xenon would produce behavioral defects that resembled those of nitrous oxide, volatile anesthetics, or some combination of the two; therefore, we tested the effects of xenon on locomotion, chemotaxis, defecation, and pharyngeal pumping. Like nitrous oxide, 70% xenon did not affect the rate of locomotion, chemotaxis, or defecation but caused a significant reduction in the frequency of reversing the direction of movement and the time spent moving backward (table 1). These effects were dependent on the concentration of xenon (fig. 1), with an EC_{50} of 15.6–20% depending on the behavioral endpoint. The potency for these effects is greater than the minimum alveolar concentration (MAC) of xenon in vertebrates (approximately 70%) and is similar to the EC_{50} reported for the neuroprotective action of xenon against excitatory neurotoxicity in mice (19% for NMDA- and 28% for glutamate-evoked neuronal injury).[6] Besides reducing the frequency of reversing direction of movement, xenon caused a statistically but not behaviorally significant increase in the pharyngeal pumping rate (from 127/min to 137/min). Animals are considered pumping defective if they average a pumping rate slower than 60/min,[18,20] so the effects of xenon on feeding seem to be minor and likely not biologically relevant.

The marked reduction of the frequency of reversing direction of locomotion is a very unusual behavioral phenotype in *C. elegans*, previously only seen in strains with disrupted glutamatergic neurotransmission[15,19,21] or after exposure to nitrous oxide.[4] The fact that xenon caused a significant reduction in reversal frequency and otherwise did not detectably affect worm behavior is consistent with our hypothesis that xenon inhibits glutamatergic neurotransmission in worms. To test this hy-

Table 1. Behavioral Effects of Xenon in *Caenorhabditis elegans*

Behavior	Measurements	Air	Xenon (70 vol%)	*P* Value
Chemotaxis	Chemotaxis index	0.80 (n = 74)	0.77 (n = 93)	NS
Feeding	Pharyngeal pumping rate, pumps/min	127.7 ± 7.1 (n = 10)	137.7 ± 4.1 (n = 10)	0.007
Defecation	pBoc interval, s	37.7 ± 3.9 (n = 10)	38.6 ± 3.8 (n = 10)	NS
	Expulsion interval, sec	39.4 ± 4.9 (n = 10)	38.4 ± 3.9 (n = 10)	NS
Locomotion	Body bends/min	25.4 ± 3.8 (n = 10)	25.5 ± 4.0 (n = 10)	NS
	Reversals/min	3.93 ± 1.61 (n = 27)	2.24 ± 1.32 (n = 17)	0.003
	Time moving backward, %	7.83 ± 3.50 (n = 27)	4.71 ± 2.67 (n = 16)	0.003

Chemotaxis assay measures the ability of worms to reach a spot on the agar plate where a chemoattractant has been placed[18]; chemotaxis index = (No. of animals at chemoattractant spot − No. of animals at control spot)/total number of animals. Feeding and defecation assays were performed as described.[18] pBoc denotes posterior body contraction, like expulsion, a regular step during defecation. pBoc and expulsion intervals measure the time between two contractions or expulsions. One body bend consists of a complete period of the worm's forward sinusoidal motion. Reversal count measures the frequency of reversing of direction of locomotion.[15] All values are presented as mean ± SD. *P* value by two-tailed unpaired *t* test.

n = number of animals tested; NS = not significant.

pothesis, we tested strains carrying null mutations in NMDA and non-NMDA glutamate receptor subunits. In particular, we used worms with a null mutation in *glr-1*, one of eight non–NMDA-type glutamate receptor subunit genes and homolog of the rat GluR1 gene,[16,21] which encodes an essential subunit of the rat AMPA receptor. Furthermore, we tested worms with a null mutation in *nmr-1*,[15] one of two genes encoding for NMDA-type glutamate receptor subunits and the homolog of the rat NR1 gene. We also tested a strain with mutations in both *glr-1* and *nmr-1*. These strains were all previously tested for their nitrous oxide sensitivity, and *nmr-1(ak4)* but not *glr-1(ky176)* was found to be resistant to the behavioral effects of nitrous oxide. Surprisingly, we found that *nmr-1(ak4)* was significantly affected by 70% xenon (figs. 2 and 3). We also measured the effect of 20% xenon on *nmr-1(ak4)* to examine the possibility that the mutation could produce resistance to lower concentrations approximating the wild type EC_{50}, but again, *nmr-1(ak4)* was significantly affected by xenon (%TB Air, 2.52 ± 1.52; 20% xenon, 1.24 ± 1.02; $P < 0.05$, two-tailed *t* test, n = 12). These data are inconsistent with the hypothesis that xenon acts solely by inhibiting the function of NMR-1.

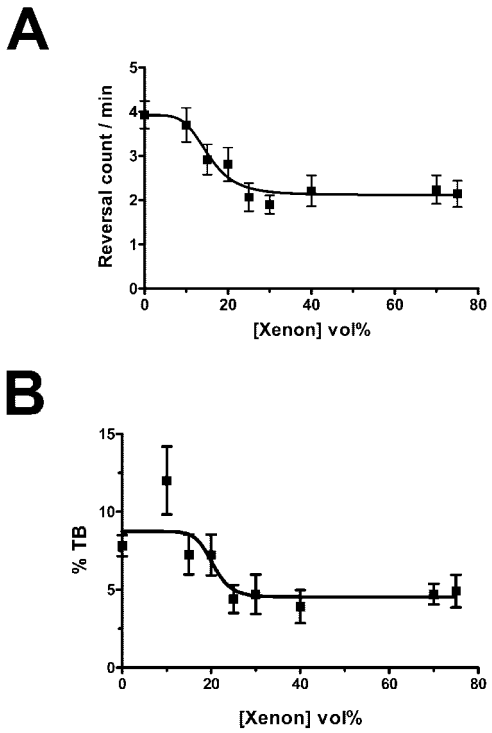

Fig. 1. Dose–response curves of xenon's effects on the control of forward and backward locomotion. Data points for both *A* and *B* are expressed as mean ± SEM of values; n = 27 (0% xenon), 10 (10%), 8 (15%), 12 (20%), 12 (25%), 11 (30%), 11 (40%), 16 (70%), 20 (75%). (A) Concentration–response curve for reversal frequency *versus* xenon concentration. Curve fits to the Hill equation: y = min + (max − min)/1 + 10^[(X − logEC_{50}) − k]. The observed EC_{50} for xenon was 15.3 ± 2.8% (95% confidence interval, 9.8–20.8%), max = 3.92, min = 2.12, k = 4.7. (B) Concentration–response curve for percent time moving backward (%TB) *versus* xenon concentration. Curve fits to the Hill equation: y = min + (max − min)/1 + 10º[(X − logEC_{50}) − k]. The observed EC_{50} for xenon was 20.4 ± 2.4% (95% confidence interval, 15.6–25.1%), max = 8.74, min = 4.53, k = 10.0.

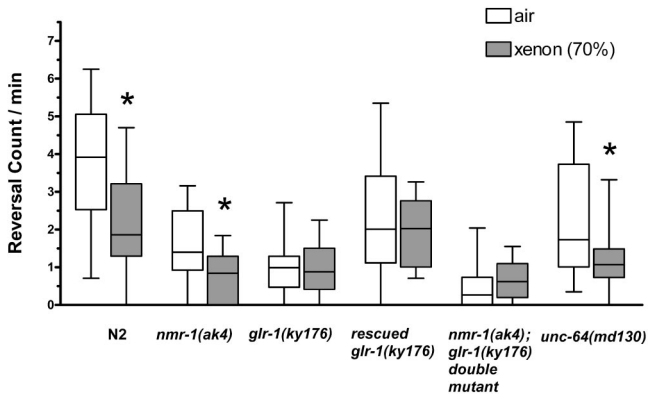

Fig. 2. Effect of loss-of-function mutations in *nmr-1* and *glr-1* on xenon action on reversal frequency. *Box plots* of frequency of reversing direction in air and in 70% xenon for wild-type worms (N2), *N*-methyl-D-aspartate receptor null mutant (*nmr-1(ak4)*), non–*N*-methyl-D-aspartate receptor null mutant (*glr-1(ky176)*), rescued *glr-1(ky176)*, the *nmr-1(ak4);glr-1(ky176)* double mutant, and the volatile anesthetic resistant mutant *unc-64(md130)*. The *box* extends from the 25th to 75th percentile (*lower* and *upper bars*); the *middle line* represents the median, and the *whiskers* represent the range from lowest to highest value. n > 10 animals for all conditions. * Statistically different from air, $P < 0.05$, two-tailed, unpaired *t* test.

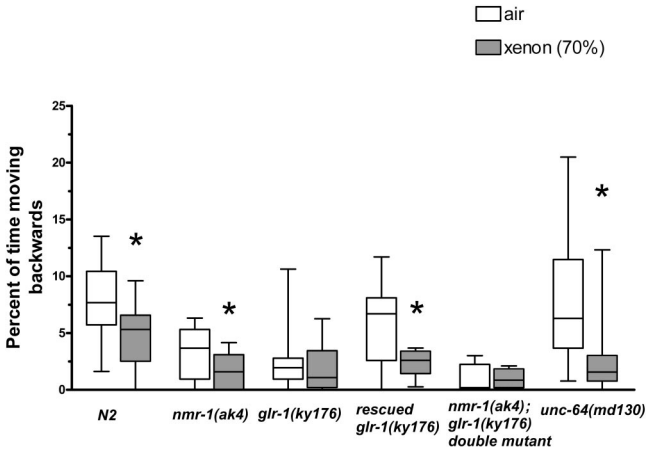

Fig. 3. Effect of loss-of-function mutations in *nmr-1* and *glr-1* on xenon action on time moving backward. *Box plots* of percentage of time moving backward in air and in 70% xenon for wild-type worms (N2), *N*-methyl-D-aspartate receptor null mutant (*nmr-1(ak4)*), non–*N*-methyl-D-aspartate receptor null mutant (*glr-1(ky176)*), rescued *glr-1(ky176)*, the *nmr-1(ak4);glr-1(ky176)* double mutant, and the volatile anesthetic resistant mutant *unc-64(md130)*. The *box* extends from the 25th to 75th percentile (*lower* and *upper bars*); the *middle line* represents the median, and the *whiskers* represent the range from lowest to highest value. n > 10 animals for all conditions. * Statistically different from air, $P < 0.05$, two-tailed, unpaired *t* test.

Even more unexpected were xenon's effects on *glr-1(ky176)*, which was previously shown to be sensitive to nitrous oxide. *Glr-1(ky176)* was not significantly affected by 70% xenon, indicating that its xenon EC_{50} was at least threefold that of wild type. Therefore, GLR-1, a non–NMDA-type glutamate receptor subunit, is required for xenon's action in *C. elegans* (figs. 2 and 3). The *nmr-1(lf);glr-1(lf)* double mutant also seemed resistant to xenon, although the very strong baseline phenotype in the absence of xenon of this mutant makes detection of a xenon effect difficult. To confirm that the xenon resistance was caused by *glr-1(lf)*, we tested a strain containing the *glr-1(ky176)* mutation and an extrachromosomal array carrying the wild-type *glr-1* gene (figs. 2 and 3). In rescued *glr-1(ky176)* worms, xenon caused a significant and profound reduction in the time spent moving backward (fig. 3) but did not cause a significant difference in reversal count (fig. 2). This incomplete rescue by wild-type *glr-1* can be explained by the fact that transformation with extrachromosomal arrays often leads to lower or mosaic expression of the extrachromosomal gene. This transformed strain also did not have a fully wild-type reversal frequency in the absence of xenon (fig. 2), nor did the same array fully rescue the isoflurane resistance[4] or mechanosensory defects of *glr-1(ky176)*.[16] A genetic or pharmacologic separation of the frequency of reversing direction and the fraction of time spent moving backward has not been reported, but these results with xenon imply that the GLR-1 receptor controls these two aspects of locomotion behavior differently, presumably as a function of overall or cell-type–specific expression levels.

To find out whether worms that show a high degree of resistance to volatile anesthetics were also resistant to xenon, we tested *unc-64(md130)*, a strain highly resistant to halothane and isoflurane because of a neomorphic mutation in syntaxin, an essential protein for presynaptic neurotransmitter release.[20,22] This strain was normally sensitive to xenon (figs. 2 and 3), thereby arguing that as with nitrous oxide, the behavioral effects of both drugs are mediated by distinct pathways.

Based on (1) the behavioral effects of xenon, which closely resemble defective glutamatergic signaling; (2) the xenon resistance of *glr-1(null)*; and (3) the partial rescue of the xenon sensitivity of *glr-1(null)* by transformation with wild-type *glr-1*, we conclude that a non-NMDA receptor is required for the behavioral actions of xenon in *C. elegans* and that xenon likely acts to produce its behavioral effects by antagonizing this non-NMDA receptor.

Discussion

The goal of this study was to determine what the behavioral effects, if any, of xenon were in *C. elegans*, and whether xenon altered behavior in *C. elegans*, to test the hypothesis that xenon acted by inhibition of NMDA receptor function. We found that xenon indeed produced behavioral effects consistent with antagonism of glutamatergic neurotransmission in *C. elegans*, but to our surprise, xenon required non-NMDA receptor instead of NMDA receptor function. Our findings provide the first behavioral genetic data to corroborate the notion that xenon inhibits glutamatergic neurotransmission but are in contrast to several electrophysiologic studies in vertebrates that showed the strong inhibitory effect of xenon confined to the NMDA receptor–mediated component of the current.[1,7,8] The effects of xenon on non-NMDA glutamate receptor–evoked currents, in particular AMPA receptor mediated, were markedly smaller in most studies, although results were mixed and dependent on subunit composition and agonist applied and if the receptor was homomeric or heteromeric.[12] A recombinant GluR1–GluR2 receptor showed in fact a substantial inhibition by 80% xenon.[12] A recent study found a strong inhibitory effect of xenon on non-NMDA glutamate receptors in dissociated mouse cortical neurons,[13] so the overall picture of xenon's synaptic effects on glutamate receptors seems more complicated than previously thought.

Ultimately, a critical question to answer with any anesthetic action/mechanism identified in a model system or organism is its role in humans. In this case, how might inhibition of a non-NMDA glutamate receptor contribute to xenon action in humans? There are at least two ways: (1) Action of xenon on a GLR-1 homolog in humans could contribute to the overall efficacy or potency in

producing anesthesia. As pointed out above, some vertebrate GLR-1 homologs have been shown to be antagonized by xenon, and this antagonism should result in nervous system depression and contribute to anesthesia.[12,13] (2) GLR-1 homologs could mediate the neuroprotective effects of xenon found in various vertebrate preparations.[6] Inhibition of glutamatergic signaling has been widely shown in animal and *in vitro* models to protect neurons from excitotoxic injury after hypoxic/ischemic injuries. The potency of xenon at altering locomotion behavior in *C. elegans* is nearer to that for neuroprotection in vertebrates than for the MAC.[6] However, relating potencies across species as divergent as *C. elegans* and humans is complicated not only by the sequence differences in the homologs but also in the relatively different roles of the channels in the two nervous systems. For example, the differences in potency could reflect a higher affinity of xenon for *C. elegans* GLR-1 than its vertebrate counterparts or a greater behavioral effect of the same degree inhibition of GLR-1 compared with that in vertebrates. In terms of the lack of effect of the NMR-1 loss-of-function mutant, one can only conclude that this NMDA receptor is not essential for the action of xenon in *C. elegans*. This result does not necessarily mean that NMDA receptors are not inhibited by xenon in *C. elegans*, only that the effect is not as important for the behavioral effects as GLR-1 inhibition. This result does not necessarily rule out that vertebrate NMDA receptors are essential for xenon action because of the same caveats of molecular divergence and potentially different roles in the two nervous systems. However, this lack of effect of *nmr-1* loss-of-function does allow for one clear and important conclusion: Xenon and nitrous oxide are not mechanistically identical drugs, or at least they have the potential to act on different targets. Given the diversity of glutamate receptors in vertebrates, created not only by different glutamate receptor genes but also by RNA-editing and post-translational modification, the potential for different actions of nitrous oxide and xenon seem to be even greater in vertebrates.

Several questions remain unanswered by this study. Does xenon directly antagonize the GLR-1 receptor, and if so, how efficacious is xenon at antagonizing this receptor if the requirement for GLR-1 in xenon action reflects direct antagonism by xenon? This question cannot be answered fully by behavioral studies. However, the maximum inhibitory effect of xenon on reversal frequency of wild-type animals is approximately twofold, whereas complete loss of GLR-1 genetically produced approximately a fourfold effect. This difference between the reversal frequency of 70% xenon–treated wild-type animals and the *glr-1* loss-of-function mutant is statistically significant ($P < 0.05$, two-tailed t test). This observation indicates that xenon does not antagonize GLR-1 to the point of full loss of function. This is in contrast to nitrous oxide, which produces approximately a threefold reduction in reversal frequency, an effect essentially identical to loss-of-function of NMR-1, which is required for the action of nitrous oxide. Therefore, although the efficacy/potency of a drug against any protein cannot be directly measured in a behavioral assay, our data suggest that xenon is not fully efficacious at antagonizing GLR-1.

In summary, we found that xenon produces behavioral effects consistent with inhibition of glutamatergic neurotransmission in *C. elegans*. The behavioral effects of xenon in *C. elegans* require a non-NMDA receptor, whereas nitrous oxide action in *C. elegans* does not require this receptor; rather, it requires an NMDA receptor. Therefore, these two anesthetics have definitively distinct mechanisms in this organism, suggesting the possibility of nonidentical mechanisms in humans. The structural difference between the *C. elegans* GLR-1 and NMR-1 proteins and between their counterparts in vertebrates known to be antagonized by xenon and nitrous oxide should aid in defining the structural requirements for action of the anesthetics on these important neuronal receptors.

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