Differential Modulation of Human *N*-Methyl-D-Aspartate Receptors by Structurally Diverse General Anesthetics

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N-Methyl-D-aspartate (NMDA) receptors have a presumed role in excitatory synaptic transmission and nociceptive pathways. Although previous studies have found that inhaled anesthetics inhibit NMDA receptor-mediated currents at clinically relevant concentrations, the use of different experimental protocols, receptor subtypes, and/or tissue sources confounds quantitative comparisons of the NMDA receptor inhibitory potencies of inhaled anesthetics. In the present study, we sought to fill this void by defining, using the two-electrode voltage-clamp technique, the extent to which diverse clinical and aromatic inhaled anesthetics inhibit the NR1/NR2B subtype of the human NMDA receptor expressed in *Xenopus laevis* oocytes. At 1 minimum alveolar anesthetic concentration (MAC), anesthetic compounds reversibly inhibited NMDA receptor currents by 12 ± 6% to 74 ± 6%. These results demonstrate that equianesthetic concentrations of inhaled anesthetics can differ considerably in the extent to which they inhibit NMDA receptors. Such differences may be useful for defining the role that this receptor plays in producing the *in vivo* actions of general anesthetics.

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N-Methyl-D-aspartate (NMDA) receptors are ligand-gated cation channels that comprise one of the major subclasses of glutamate receptors. These receptors have presumed roles in excitatory synaptic transmission, learning, memory, nociceptive pathways, and muscular movement modulated at the spinal cord level (1,2). They have also been implicated as mediators of ischemic neuronal injury (3).

The NMDA receptor is a heteromeric protein assembly comprising at least two of seven known subunit types: the NR1, NR2 (A, B, C, and D), and NR3 (A and B) subunits. Proper receptor assembly requires the NR1 subunit (which binds the requisite coagonist glycine) and at least one NR2 subunit (which binds the agonist glutamate). The NR1/NR2B subtype is important to pain perception, and the NR2B subunit may be a useful target for novel anesthetics (4).

Diverse inhaled anesthetics inhibit NMDA receptor function, suggesting a role for these receptors as important *in vivo* targets for such anesthetics. For example, although 80% xenon had little effect on currents in cultured hippocampal neurons mediated by γ-aminobutyric acid type A receptors, 80% xenon decreases NMDA-activated currents by approximately 60% in such neurons (2). Using a radioligand binding assay, Martin et al. (5) reported that halothane, chloroform, diethyl ether, methoxyflurane, and enflurane decrease binding of the NMDA receptor antagonist MK-801 (which requires opening of the ion channel) to rat brain homogenates, suggesting that these inhaled anesthetics inhibit glutamate-mediated channel opening. Using receptors expressed in oocytes, Hollman et al. (6) found that isoflurane, sevoflurane, and desflurane inhibited 50% of NMDA receptor-mediated currents at concentrations close to 1 minimum alveolar anesthetic concentration (MAC).

Although these and other studies indicate that a variety of inhaled anesthetics inhibit NMDA receptor-mediated currents, such studies typically used different experimental protocols, anesthetic concentrations, receptor subtypes, and/or tissue sources, thereby confounding quantitative comparisons of the NMDA receptor inhibitory potencies of inhaled anesthetics. The present study sought to fill this void, by defining the

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extent to which a range of structurally diverse inhaled anesthetics inhibits the NR1/NR2B subtype of the human NMDA receptor, under identical experimental conditions and at equal anesthetizing concentrations.

Methods

Adult female *Xenopus laevis* frogs (Xenopus One, Ann Arbor, MI) underwent surgery for removal of oocytes as described previously (7). The MA General Hospital Animal Care Committee approved all procedures.

Linearized cDNA templates encoding the NR1 and NR2B subunits of the NMDA receptor (kindly provided by Dr. Stuart Forman, Harvard Medical School, Boston, MA) were transcribed into capped messenger RNA using SP6 or T7 RNA polymerase kits (Ambion, Inc., Austin, TX). After treatment with collagenase IA for 1 h, stage V and VI oocytes were manually defolliculated and injected with mRNA (5–10 ng for each subunit at a ratio of 1:1). Injected oocytes were kept in ND-96 incubation solution (96 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 10 mM HEPES, pH 7.5) containing 5 U/mL of penicillin and 5 μg/mL of streptomycin at 17°C for at least 72 h before electrophysiological experiments.

All electrophysiological recordings were performed at room temperature (22–24°C) using the whole-cell, two-electrode voltage-clamp technique. Oocytes were placed in a 0.04-mL recording chamber and impaled with capillary glass electrodes filled with 3 M KCl and possessing open tip resistances <5 MΩ. Oocytes were then voltage-clamped at –50 mV using a GeneClamp 500B amplifier (Molecular Devices, Sunnyvale, CA), and were constantly perfused with ND96 recording buffer (96 mM NaCl, 2 mM KCl, 2 mM $BaCl_2$, and 10 mM HEPES, pH 7.5) at a rate of 4 mL/min using a closed-syringe superfusion system. Buffer perfusion was controlled using a six-channel valve controller (Warner Instruments, Hamden, CT) interfaced with a Digidata 1322A data acquisition system (Molecular Devices), and driven by a personal computer (Dell, Round Rock, TX). Current responses were recorded using Clampex 9.0 software (Molecular Devices), and processed using a Bessel (eight-pole) low-pass filter with a –3 dB cutoff at 1.56 Hz using Clampfit 9.0 software (Molecular Devices). The perfusion apparatus was made from gas-tight glass syringes and Teflon tubing to minimize absorptive and evaporative loss of anesthetic drugs. In parallel experiments, gas chromatographic analysis of solutions entering the oocyte chamber indicated that such loss was $<15\%$.

Volatile anesthetic solutions were prepared by adding an excess of drug to a sealed bottle containing buffer solution and stirring overnight. These saturated solutions of known concentration were then diluted using gas-tight syringes to yield the final desired anesthetic concentration. For the gaseous anesthetics xenon and cyclopropane, the anesthetic gas was bubbled at a rate of 100–120 mL/min for at least 3 min through 150 mL of buffer solution in a 250-mL glass bottle sealed with a Teflon septum. Lines for gas inlet and outlet were introduced through the septum. The resulting solution was sealed and continuously stirred to allow equilibration for at least 30 min. The anesthetic gas was then bubbled through the buffer solution again for at least 2 min further to ensure saturation. The saturated solution was subsequently diluted to the final desired concentration using gas-tight syringes. For the aromatic anesthetics, the drug was added to a glass bottle and weighed, and the volume of buffer necessary to make a 2 MAC solution was calculated and added to the bottle. After the addition of buffer, the bottle was quickly sealed using a Teflon-coated cap, and the resulting solution was stirred overnight. Care was taken to minimize the amount of air in the bottle (typically $<10\%$ of the total volume). The resulting 2 MAC solution was diluted using gas-tight syringes to yield a final anesthetic concentration of 1 MAC.

For each experiment, the oocyte was perfused for 30 s with buffer solution containing the agonist mixture (100 μM NMDA and 10 μM glycine) to generate a control current. After at least 2 min of recovery, the oocyte was first perfused with buffer solution containing the test anesthetic for 60 s, and then perfused with buffer solution containing both the agonist mixture and the test anesthetic for 30 s. After another recovery period, the agonist mixture was again applied to the cell for 30 s to ensure reversibility of any anesthetic-induced change in current response. Peak current responses were recorded, and the magnitude of current modulation was determined using the average of the two control experiments (before and after application of anesthetic).

The MAC of anesthetics in rats typically exceeds that in humans (8). Therefore separate studies were performed for each anesthetic at concentrations equivalent to 1 MAC in humans (8,9) and in rats (8,10). Because 1 MAC for xenon is 1.6 atm in rats (a concentration of xenon that is unattainable using our experimental protocol), we fitted data using 0.7 atm and 1.0 atm xenon to a Hill equation (assuming a Hill coefficient of 1 and complete inhibition at large xenon concentrations), and extrapolated the results to estimate the inhibitory potency of xenon at 1.6 atm.

The aromatic drugs were studied only at concentrations equal to 1 MAC in rats (11), because human data are unavailable for these drugs. MAC values were converted to aqueous concentrations using the equation $C_{aq} = 0.44614\alpha P$, where C_{aq} is the aqueous millimolar concentration of anesthetic, α is the aqueous/gas partition coefficient at 37°C, and P is the gaseous

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Table 1. Aqueous Concentrations and Partial Pressures of Anesthetics Corresponding to 1 MAC

Anesthetic	Human MAC	Rat MAC
Xenon	2.3 mM, 70 % atm	5.4 mM, 161 % atm*
Cyclopropane	0.84 mM, 9.2 % atm	1.46 mM, 16 % atm
Enflurane	0.52 mM, 1.68 % atm	0.68 mM, 2.2 % atm
Isoflurane	0.28 mM, 1.15 % atm	0.35 mM, 1.45 % atm
Desflurane	0.60 mM, 6.0 % atm	0.76 mM, 7.71 % atm
Halothane	0.22 mM, 0.76 % atm	0.35 mM, 1.24 % atm
Sevoflurane	0.34 mM, 2.05 % atm	0.48 mM, 2.80 % atm
Benzene	NA	1.3 mM, 1.01 % atm
Fluorobenzene	NA	1.1 mM, 1.12 % atm
1,2-Difluorobenzene	NA	0.83 mM, 0.61 % atm
1,4-Difluorobenzene	NA	0.69 mM, 0.64 % atm
1,2,4-Trifluorobenzene	NA	0.78 mM, 0.97 % atm
1,3,5-Trifluorobenzene	NA	0.71 mM, 2.22 % atm
Pentafluorobenzene (PFB)	NA	0.41 mM, 1.25 % atm
Hexafluorobenzene (HFB)	NA	0.29 mM, 1.61 % atm

Statistical analysis was performed using Graphpad Prism v4.02 (Graphpad Software Inc., San Diego, CA). *P* values were generated using a paired, two-tailed *t*-test. All anesthetics were tested on 4 oocytes except for 1,2,4-trifluorobenzene, which was tested on 8 oocytes.

NA = human MAC data are unavailable for the volatile aromatic anesthetics.

* Inhibition at 5.4 mM (1.6 atm) xenon (i.e., 1 MAC for rats) was extrapolated from data using smaller xenon concentrations (see Methods).

partial pressure of anesthetic in percentage of atmosphere (12). The final aqueous concentrations of the anesthetics studied are listed in Table 1.

Cyclopropane and all of the aromatic anesthetics were purchased from Aldrich Chemical (Milwaukee, WI). The other inhaled anesthetics were purchased from the following companies: halothane from Halocarbon Laboratories (River Edge, NJ), sevoflurane from Abbott Laboratories (North Chicago, IL), isoflurane and desflurane from Baxter Healthcare Corp. (Deerfield, IL), enflurane from Anaquest Inc. (Liberty Corner, NJ), and xenon from BOC Gases (Murray Hill, NJ). NMDA and glycine were purchased from Sigma-Aldrich (St. Louis, MO).

Results

Typical current responses evoked by NMDA and glycine in the absence and presence of sevoflurane, isoflurane, and xenon (at concentrations equivalent to 1 MAC in humans) are shown in Figure 1. The second control current evoked by NMDA and glycine after drug washout is shown for each experiment to illustrate the reversibility of anesthetic effects. For the experiments shown in the figure, sevoflurane, isoflurane, and xenon inhibited NMDA currents by 12%, 31%, and 39%, respectively.

Figure 2 shows analogous traces obtained in the absence and presence of pentafluorobenzene, fluorobenzene, and benzene at concentrations of 1 MAC in rats. The second control experiment shows the reversibility of anesthetic-induced effects. For the experiments shown, pentafluorobenzene, fluorobenzene, and benzene inhibited currents evoked by NMDA and glycine by 9%, 60%, and 76%, respectively.

All 15 anesthetics reversibly inhibited NMDA receptor currents. The magnitude of current inhibition observed for each anesthetic is summarized in Table 2 and Table 3 (data presented as mean ± SD). Among the clinical anesthetics, xenon produced the greatest inhibition at 1 MAC, whereas sevoflurane produced the least, and the difference was highly significant for humans and for rats ($P < 0.0001$). NMDA receptor inhibition by aromatic anesthetics also varied greatly, with benzene producing approximately sixfold greater inhibition at 1 MAC than hexafluorobenzene ($P < 0.0001$).

Discussion

Hollman et al. (6) reported that the halogenated ethers isoflurane, sevoflurane, and desflurane all inhibit NMDA receptors to similar degrees at clinically relevant concentrations, leading the authors to conclude that volatile anesthetics are "mainly equipotent" in their effects on NMDA receptors, and to suggest that these receptors play an important role in producing anesthesia. We now show that a range of inhaled (volatile and gaseous) anesthetics possessing greater structural diversity may inhibit NMDA receptors to different extents at equianesthetic concentrations. These results imply that if the NMDA receptor plays a role in producing the state of general anesthesia, the contribution of NMDA receptor inhibition to the overall anesthetic state varies from drug to drug.

We previously reported that volatile aromatic anesthetics inhibit NMDA receptors with potencies that correlate strongly with their abilities to engage in cation-π interactions, but not with their hydrophobicities (13), suggesting that electrostatic interactions between these anesthetics and their receptor binding

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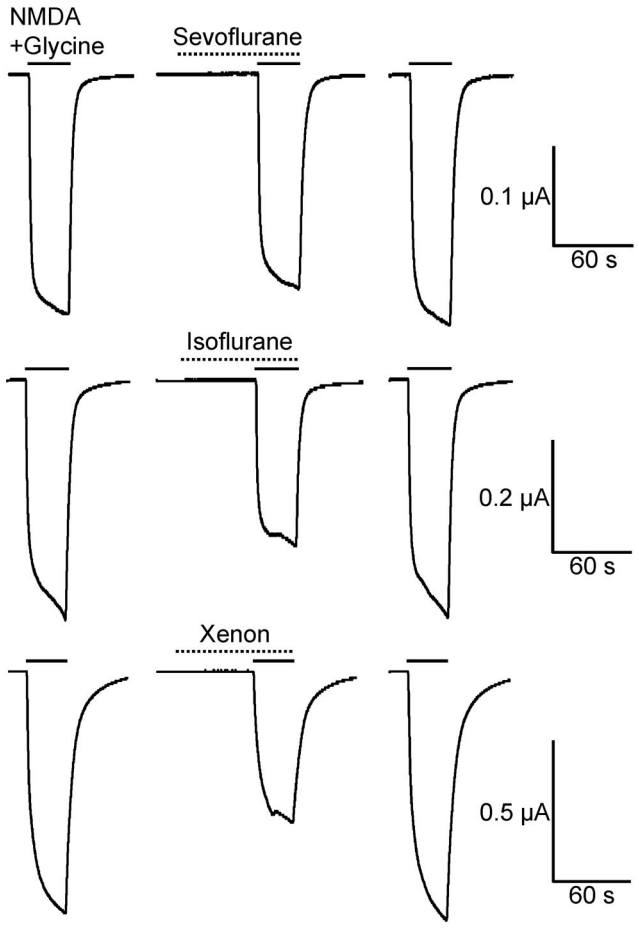

Figure 1. In these representative current traces, the solid line overlies traces elicited by application of 100 μM *N*-methyl-D-aspartate (NMDA) and 10 μM glycine to NR1/NR2B NMDA receptors, and the dotted line overlies traces resulting with applications of sevoflurane, isoflurane, and xenon, at concentrations equivalent to 1 MAC in humans. The control currents evoked by NMDA and glycine before (left) and after (right) anesthetic exposure illustrate the reversibility of anesthetic effects.

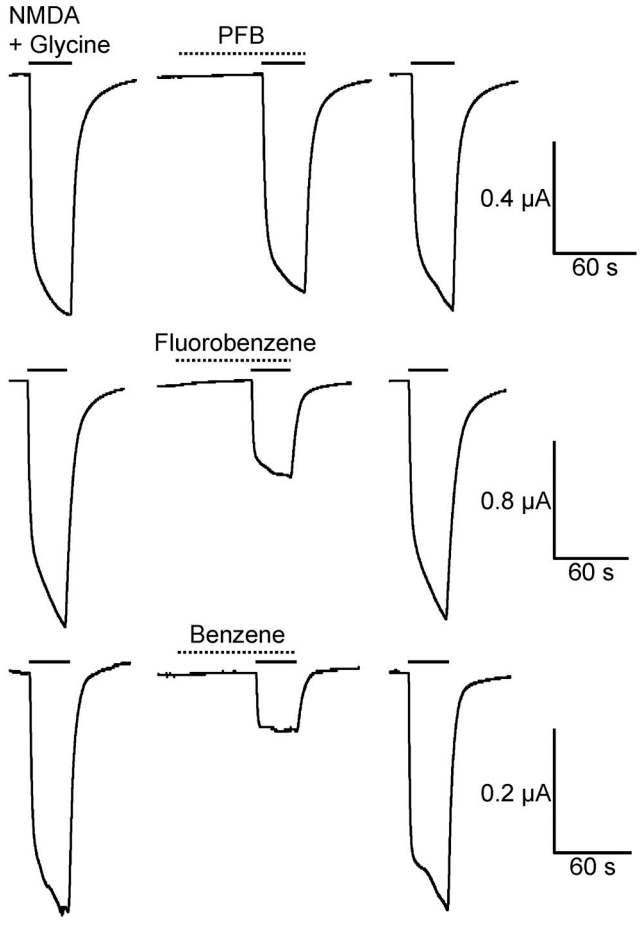

Figure 2. In these representative current traces, the solid line overlies traces elicited by application of 100 μM *N*-methyl-D-aspartate (NMDA) and 10 μM glycine to NR1/NR2B NMDA receptors, and the dotted line overlies traces resulting with applications of pentafluorobenzene (PFB), fluorobenzene, and benzene at concentrations equivalent to 1 MAC in rats.

sites modulate binding affinity and, hence, inhibitory potency. Conversely, Peoples and Weight (14) reported that the inhibitory potencies of n-alcohols for the NMDA receptor increase from methanol to pentanol, consistent with a critical role for hydrophobic interactions. It is noteworthy that the electrostatic properties of n-alcohols (e.g., dipole moment, hydrogen-bonding capacity) are invariant; therefore, these studies could not evaluate the importance of electrostatic interactions in modulating the NMDA receptor inhibitory potencies of alcohols.

The present study shows that equianesthetic concentrations of aromatic anesthetics that have the greatest abilities to engage in cation-π interactions (e.g., benzene, fluorobenzene, and 1,2-difluorobenzene) produce the greatest degree of NMDA receptor inhibition. There is no obvious analogous correlation between the ability of clinical anesthetics to inhibit NMDA receptors and their capacity to engage in electrostatic interactions. In fact, xenon and cyclopropane produce the greatest degree of inhibition at 1 MAC, but have neither a dipole moment nor the ability to

Table 2. Percentage Inhibition of *N*-Methyl-D-aspartate (NMDA)-Evoked Currents by Clinical Anesthetics at 1 MAC

	Inhibition	
Anesthetic	Human MAC	Rat MAC
Xenon	39 ± 1%	59%*
Cyclopropane	36 ± 4%	51 ± 5%
Enflurane	32 ± 4%	34 ± 2%
Isoflurane	28 ± 3%	27 ± 3%
Desflurane	25 ± 6%	30 ± 5%
Halothane	17 ± 5%	26 ± 4%
Sevoflurane	14 ± 2%	22 ± 5%

Data are presented as mean ± SD.
* Inhibition at 5.4 mM (1.6 atm) xenon (i.e., 1 MAC for rats) was extrapolated from data using lower xenon concentrations (see Methods).

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Table 3. Percentage Inhibition of NMDA-evoked Currents by Aromatic Anesthetics at 1 MAC in Rats

Anesthetic	Inhibition
Benzene	74 ± 6%
Fluorobenzene	60 ± 8%
1,2-difluorobenzene	58 ± 6%
1,4-difluorobenzene	21 ± 8%
1,2,4-trifluorobenzene	25 ± 7%
1,3,5-trifluorobenzene	15 ± 10%
Pentafluorobenzene (PFB)	16 ± 6%
Hexafluorobenzene (HFB)	12 ± 6%

Data presented as mean ± SD.

engage in hydrogen-bonding interactions. Thus, for the clinical anesthetics, hydrophobicity may play a relatively more important role. Because multiple anesthetic effects may contribute to MAC (including, but not limited to, NMDA receptor inhibition), it is difficult to draw conclusions regarding the anesthetic binding site for the NMDA receptor with inhibitory potency data at equal anesthetizing concentrations. However, the results of this *in vitro* study may be useful for future investigations of whether NMDA receptor inhibition contributes to MAC, potentially complementing *in vivo* data with other NMDA receptor antagonists, for example, to better understand the role this receptor plays in general anesthesia.

Halothane and sevoflurane provide neuroprotection during focal ischemia in rats (15), and overstimulation of NMDA receptors may play a critical role in the pathophysiology of neuronal injury and death (1). In an *in vivo* rat model of NMDA-mediated neuronal injury, Harada et al. (16) reported that 2 MAC but not 1 MAC isoflurane reduced the volume of cortical infarction, selective neuronal necrosis, and total volume of tissue injury after intracortical NMDA injection. In contrast, using a similar *in vivo* neuronal injury model, Wilhelm et al. (3) showed that 40% xenon (a dose considerably smaller than 1 MAC) significantly decreased the number of degenerated neurons, and even larger reductions were obtained at larger xenon concentrations. Our data suggest that xenon may be a more potent neuroprotectant than isoflurane because it produces a greater degree of NMDA receptor inhibition at equianesthetic concentrations.

In summary, our data show that at equianesthetic concentrations general anesthetics inhibit NMDA receptors to different extents. Such differences may be useful for defining the role that this receptor plays in producing the *in vivo* actions of general anesthetics.

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