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Effects of Anesthetics on Mutant *N*-Methyl-D-Aspartate Receptors Expressed in *Xenopus* Oocytes

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ABSTRACT

Alcohols, inhaled anesthetics, and some injectable anesthetics inhibit the function of *N*-methyl-D-aspartate (NMDA) receptors, but the mechanisms responsible for this inhibition are not fully understood. Recently, it was shown that ethanol inhibition of NMDA receptors was reduced by mutation of residues in the transmembrane (TM) segment 3 of the NR1 subunit (F639A) or in TM4 of the NR2A subunit (A825W), suggesting putative ethanol binding sites. We hypothesized that the actions of other anesthetics might also require these amino acids and evaluated the effects of anesthetics on the NMDA receptors expressed in *Xenopus* oocytes with two-electrode voltage-clamp recording. Effects of hexanol, octanol, isoflurane, halothane, chloroform, cyclopropane, 1-chloro-1,2,2-trifluorocyclobutane, and xenon were reduced or eliminated in the mutant NMDA receptors, whereas the inhibitory effects of nitrous oxide, ketamine, and benzene were not affected by these mutations. Rapid applications of glutamate and glycine by a T-tube device provided activation time constants, which suggested different properties of ketamine and isoflurane inhibition. Thus, amino acids in TM3 and TM4 are important for the actions of many anesthetics, but nitrous oxide, benzene, and ketamine seem to have distinct mechanisms for inhibition of the NMDA receptors.

NMDA receptors compose a major class of excitatory ligand-gated ion channels. These receptors are one subtype of excitatory glutamate receptors, which use the coagonists glycine and glutamate for activation (Johnson and Ascher, 1987). Recent molecular cloning studies have revealed that NMDA receptor subunits consist of NR1, NR2A-D, and NR3A-B (Meguro et al., 1992; Monyer et al., 1992; Mascia et al., 2000; Chatterton et al., 2002) and that a functional receptor is a heterotetramer containing NR1 and other subunit(s) (Laube et al., 1998).

Several lines of evidence indicate that many anesthetics, including inhaled anesthetics, affect excitatory synaptic transmission by inhibiting NMDA receptors. The inhibition by halothane of excitatory synaptic transmission in the central nervous system was first reported by Richards (1973). Later, studies in hippocampal slices and neurons showed that volatile anesthetics depress activation of neuronal responses by NMDA (Yang and Zorumski, 1991; Perouansky et al., 1995). Moreover, various volatile anesthetics inhibit glutamate-stimulated activation of NMDA receptors, measured by the binding of MK-801, a use-dependent NMDA receptor blocker, to rat cerebral cortex (Martin et al., 1995). The role of NMDA receptors in spinal cord in the immobilizing action of anesthetics is also supported by in vivo studies (Stabernack et al., 2003).

Studies of recombinant receptors also provide evidence for modulation of NMDA receptors by volatile and gaseous anesthetics. Hollmann et al. (2001) studied the effects of isoflurane, sevoflurane, and desflurane on recombinant NR1/NR2A and NR1/NR2B glutamate receptors. Their findings show that clinically relevant concentrations of these anesthetics inhibit NMDA receptors in a reversible, dose-dependent, and voltage-insensitive manner (Hollmann et al., 2001). Likewise, enflurane, urethane, cyclopropane, and butane inhibit NMDA-stimulated currents in oocytes expressing NMDA receptors (Lin et al., 1993; Hara and Harris, 2002; Hara et al., 2002). Another study showed that xenon reduces NMDA-activated currents in a noncompetitive manner in rat

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; MK-801, dizocilpine hydrogen maleate; TM, transmembrane; AP5, d-(−)-2-amino-5-phosphonopentanoic acid; F3, 1-chloro-1,2,2-trifluorocyclobutane; F6, 1,2-dichlorohexafluorocyclobutane; MAC, minimum alveolar concentration; ANOVA, analysis of variance; atm, atmosphere.

hippocampal neurons (Franks et al., 1998), and both nitrous oxide and xenon moderately inhibit recombinant NMDA receptors (Yamakura and Harris, 2000). Recently, it has been reported that ethanol inhibition of NMDA receptors is reduced by mutation of phenylalanine residue to alanine in the NR1 subunit (F639A) using oocytes and HEK293 cells (Ronald et al., 2001). In addition, Honse et al. (2004) revealed that a tryptophan substitution at alanine 825 in TM4 of the NR2A subunit (A825W) increased the IC_{50} of ethanol over 2-fold. Taken together, these results suggest that both the NR1 and NR2A subunits have ethanol-sensitive sites.

Ketamine is an injectable anesthetic that produces immobility and amnesia by blocking NMDA receptors without influencing GABA or glycine receptors. It is considered to be an open-channel blocker, although other mechanisms of block may also occur (Orser et al., 1997). To gain insight into the characteristics of anesthetic and *n*-alcohol actions on NMDA receptors, we investigated the effects of a wide range of anesthetics, as well as a nonanesthetic compound, on recombinant NR1/NR2A (wild type) and mutant NR1(F639A)/NR2A, NR1/NR2A(A825W), and NR1(F639A)/NR2A(A825W) receptors expressed in *Xenopus* oocytes using a two-electrode voltage-clamp recording system. We tested NMDA receptors composed by NR1/NR2A subunits because these were most sensitive to ethanol (Mirshahi and Woodward, 1995). We also examined the mechanism of anesthetic block using a rapid application system in oocytes, which allowed us to distinguish changes in activation kinetics produced by competitive and open-channel blockers.

Materials and Methods

Materials. *Xenopus laevis* female frogs were purchased from Xenopus Express (Homosassa, FL). Isoflurane was from Marsam Pharmaceutical Inc. (Cherry Hill, NJ). Halothane, chloroform, cyclopropane, ketamine, *n*-hexanol, *n*-octanol, glycine, L-glutamate, benzene, and other chemicals were obtained from Sigma-Aldrich Inc. (St. Louis, MO). Nitrous oxide and xenon were purchased from Airgas Southwest (San Antonio, TX). d-(−)-2-Amino-5-phosphonopentanoic acid (AP5) was from Tocris Cookson Inc. (Ellisville, MO). 1-Chloro-1,2,2-trifluorocyclobutane (F3) and 1,2-dichlorohexafluorocyclobutane (F6) were obtained from Lancaster Synthesis Inc. (Windham, NH).

mRNA Synthesis. The cDNAs encoding the human NR1 and NR2A subunits were kindly provided by Dr. Thomas Kuner (University of Heidelberg, Germany). The cDNAs of the NR1(F639A) and NR2A(A825W) mutants were made by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). These mutants were reported by Ronald et al. (2001) and Honse et al. (2004), respectively. These cDNAs were used for cRNA synthesis using either T7 or SP6 RNA polymerase. In vitro transcripts were prepared using the mMESSAGE mMACHINE Kit (Ambion, Austin, TX). The quality and quantity of these RNAs were determined by using the RNA 6000 Nano assay with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Oocyte Expression. *X. laevis* female frogs were anesthetized, and ovarian tissue was dissected according to the guidelines of the Animal Care and Use Committees of the University of Texas. Oocytes were mechanically isolated from the ovary and placed in modified Barth's solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.91 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, and 10 mM HEPES adjusted to pH 7.5. Isolated oocytes are enveloped in a follicle cell layer. The layer was first removed manually with fine forceps. To complete defolliculation, oocytes were then exposed to collagenase type 1A solution containing 0.5 mg/ml collagenase, 83 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES adjusted to pH 7.5 for 10 min. Oocytes were injected with 40 nl of a 1:1 mixture of NR1 and NR2A subunit mRNA, with a concentration of approximately 1 ng/nl. The injected oocytes were singly placed in Corning cell wells (Corning Glass Works, Corning, NY) containing incubation medium (sterile modified Barth's solution supplemented with 10 mg/l streptomycin, 100,000 units/l penicillin, 50 mg/l gentamicin, 90 mg/l theophylline, and 220 mg/l pyruvate) and incubated at 15 to 19°C. Two to four days after injection, the oocytes were used in electrophysiological recording.

Two-Electrode Voltage-Clamp Recording. Oocytes injected with the cRNA were placed in a rectangular chamber (~100-μl volume) and continuously perfused (2 ml/min) with Mg^{2+}- and Ca^{2+} free Ringer solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $BaCl_2$, 10 mM HEPES, pH 7.2) using a peristaltic pump (Cole-Parmer Instrument Co., Chicago, IL). To minimize interference by the Ca^{2+}-activated Cl^- current, barium chloride was used instead of calcium chloride in the Ringer solution. Two-electrode voltage clamp recordings were obtained from injected oocytes using a Warner Instruments model OC-725C (Hamden, CT), and oocytes were clamped at −70 mV. The amplitude of expressed NR1/NR2A NMDA receptor-mediated currents was typically 1 to 8 μA, whereas oocytes injected with NR1 subunit alone produced at most 50-nA currents. Recording electrodes were made with a puller (P-97; Sutter Instruments Co., Novato, CA) from borosilicate capillary glass (Frederick Haer and Co., Bowdoinham, ME) and had a final resistance of 1 to 5 MΩ when filled with 3 M KCl. All electrophysiological recordings were carried out at a room temperature of around 23°C.

The bath concentrations of the anesthetics (isoflurane, halothane, chloroform, cyclopropane, *n*-hexanol, *n*-octanol, nitrous oxide, xenon, F3, ketamine, and benzene) and nonanesthetic (F6) were determined by gas chromatography. Isoflurane, halothane, chloroform, *n*-hexanol, benzene, and ketamine were directly dissolved in the Ba^{2+} Ringer's solution. The *n*-octanol was first dissolved in dimethyl sulfoxide, then diluted in the Ba^{2+} Ringer's solution to a final dimethyl sulfoxide concentration not exceeding 0.05%. The Ba^{2+} Ringer's solution (20 ml) in a sealed glass vial was bubbled with 100% gaseous anesthetics (cyclopropane, nitrous oxide, and xenon) at a flow rate of approximately 250 ml/min for at least 10 min to provide a saturated solution of anesthetic. The saturated solution was introduced into the bath through an 18-gauge polyethylene tube. The bath concentrations of cyclopropane, nitrous oxide, and xenon were quantified by gas chromatography as described previously, and those were 0.88 atm (133 mM), 0.58 atm (12.2 mM), and 0.46 atm (2.0 mM), respectively. Saturated solutions of F3 and F6 were made by equilibrating 100 μl of each compound in 20 ml of the Ba^{2+} Ringer's solution in a sealed glass syringe, protected from light, for at least 24 h before use. The saturated solutions of F3 and F6 were approximately 15 MAC (or predicted MAC for F6). MAC is the minimum alveolar concentration of anesthetic required to eliminate movement in response to a noxious stimulus in 50% of subjects. Final solutions of these compounds were prepared from the saturated solutions immediately before application. All compounds were tested at a concentration corresponding to MAC (or the predicted MAC for F6) in the final bath concentration. Calculated 1 MAC for isoflurane, halothane, chloroform, *n*-hexanol, *n*-octanol, F3, F6, and ketamine were 0.32 mM, 0.25 mM, 1 mM, 0.57 mM, 57 μM, 0.8 mM, 17.8 μM, and 2 μM, respectively (Saidman et al., 1967; Idvall et al., 1979; Dayton et al., 1983; Mihic et al., 1994; Dildy-Mayfield et al., 1996). The bath concentration of cyclopropane (0.57 atm, 12.2 mM) corresponded to approximately 1 MAC (Hara et al., 2002). Those of nitrous oxide and xenon (0.57 atm, 12 mM and 0.46 atm, 2.0 mM, respectively) were comparable with 0.5 MAC (Yamakura and Harris, 2000). Oocytes were perfused with the anesthetics for 1 min before being coapplied with agonists to allow for complete equilibration of the oocytes with the compounds. A 15-min washout period was allowed after application of the anesthetic/agonist solutions.

T-Tube Application System. Anesthetic compounds were applied by a rapid application system (T-tube), a modification of a Y-tube system (Murase et al., 1990). A T-shaped connector was held 1 mm above an oocyte facing toward the membrane. The internal diameter of the open side of the T was 1.2 mm (larger than the oocytes tested). The drugs flowed into one arm of the tube connector by a peristaltic pump at 4 ml/min and flowed out from the other hand of the connector by another peristaltic pump at a rate little faster that the flow of pump, e.g., 4.2 ml/min. At the time of drug application, a stopcock between the T-connector and the pump was closed so that the drug would replace the oocyte bath solution rapidly. Current were low-pass filtered at 50 Hz and digitized with a sampling rate of 100 Hz, using the Digidata 1200 interface and pClamp7 software (Axon Instruments, Foster City, CA) and were also recorded by PowerLab 2/20 and Chart 5 software (ADInstruments Pty Ltd., Castle Hill, Australia). The current kinetics was analyzed by fitting the curve to a single exponential association or dissociation curve using a Chebyshev fit method on the pClamp7 software.

Data Analysis. Concentration-response data for agonists were fitted to the equation $I/I_{\max} = F/[1 + (EC_{50}/A)^{n_H}]$, where I represents the current, $I_{\max}$, F is the maximal current in the absence of anesthetic compound, EC_{50} is the maximal relative response in the presence of anesthetic compound, the agonist concentration for half-maximal response is A, and n_H is the Hill coefficient.

The activation or inhibition time constants (τ) for the currents were measured using pClamp7 software. Data were digitized at 100 Hz and low-pass filtered at 50-Hz currents. The τs were derived from a Chebyshev fit method to the activation or inhibition currents of the equation,

$$y = A_0 + A_1 e^{-t/\tau} \quad (1)$$

Data were obtained from five to 20 oocytes taken from at least three different frogs for each experiment. Control responses to glutamate/glycine were measured before and after applications of anesthetic compounds to minimize possible shifts in the control currents as a recording proceeded. Data are shown as means ± S.E.s. Statistical analysis was conducted by one-way analysis of variance (ANOVA) for multiple comparisons and Student's t test for comparisons between two groups, using GraphPad Prism version 4.0a (GraphPad Software Inc., San Diego, CA). Differences were considered as statistically significant at $p < 0.05$.

Molecular Volumes of Anesthetic Compounds. Dr. James R. Trudell (Stanford University, Stanford, CA) provided the molecular volumes of anesthetic agents. They were built in Spartan version 5.1 (Wavefunction, San Diego, CA) and then optimized with the Merck MMFF94 force field. The volumes are van der Waals volumes in Ångstrom units cubed per molecule, ~30% less than obtained by dividing megawatts by density (milliliters per mole).

Results

The glutamate concentration-response relation was determined for each receptor with the glycine concentration fixed at 10 μM (Fig. 1A). Calculated EC_{50} values of glutamate for NR1/NR2A (wild type), mutant NR1(F639A)/NR2A (F639A), NR1/NR2A(A825W) (A825W), and NR1(A639F)/NR2A(A825W) (F639A/A825W) were 1.9 ($n_H = 1.26$), 1.9 ($n_H = 1.02$), 1.8 ($n_H = 1.04$), and 1.9 ($n_H = 1.20$) μM, respectively. This demonstrates that these mutations of the NMDA receptors did not alter the ability of the physiological agonist glutamate to activate the receptor in our experimental conditions. Although the concentration-response relations of glycine for the receptors were not explored in detail, the 10 μM concentration provided a maximal response for all receptors (data not shown). Isoflurane inhibited the NMDA receptor function noncompetitively (Fig. 1B). The glutamate EC_{50} values of the wild-type receptor with and without isoflurane were 1.9 ± 0.6 and 1.8 ± 0.5 μM, respectively ($n = 4$). For ketamine and isoflurane, the IC_{50}s were 1.4 ± 0.4 μM for ketamine and 0.56 ± 0.03 mM for isoflurane (Fig. 1C).

Isoflurane, halothane, and chloroform significantly inhibited the currents produced by wild-type receptors (Fig. 2A).

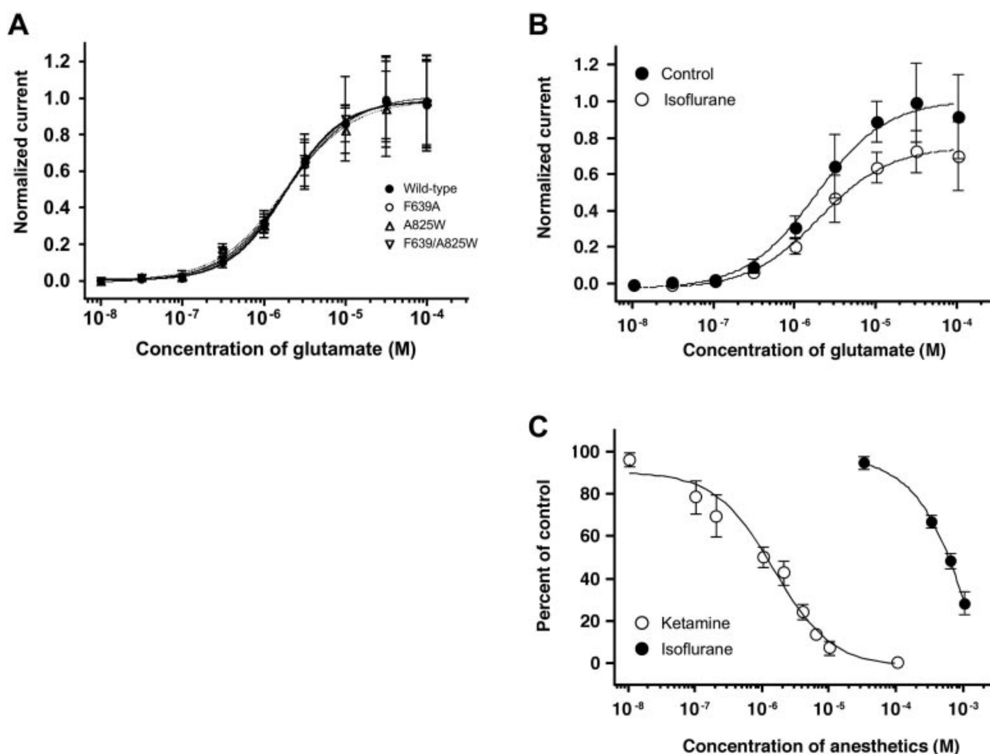

Fig. 1. A, glutamate concentration-current response curves for the wild-type and mutant NMDA receptors (NR1/NR2A); filled circles, wild type ($n = 4$); open circles, F639A ($n = 5$); triangles, A825W ($n = 6$); inverted triangles, F639A/A825W ($n = 4$). Normalized currents and glutamate concentrations (M) are shown in vertical and horizontal axes, respectively. B, glutamate concentration current-response curves for wild-type NMDA receptors (with 10 μM glycine) with (open circles) and without (filled circles) 1 MAC isoflurane ($n = 4$). Normalized currents and glutamate concentrations (molar) are shown in vertical and horizontal axes, respectively. C, concentration current-response curves for ketamine (open circles) and isoflurane (filled circles) inhibition of wild-type NMDA receptors. Percentage of control responses and anesthetic concentrations (molar) are shown in vertical and horizontal axes, respectively. The IC_{50} was 1.4 ± 0.4 μM for ketamine and 0.56 ± 0.03 mM for isoflurane. The currents were evoked by a maximal glycine concentration (10 μM), and EC_{50} glutamate was applied for 30 s. Anesthetic compounds were applied for 1 min and then applied with agonists for 30 s. All data are expressed as mean ± S. E.

For isoflurane, the inhibition was 68 ± 3% (n = 12), 49 ± 4% (n = 18), and 29 ± 5% (n = 8) of control responses at the concentrations corresponding to values of 1, 2, and 3 MAC ($p < 0.001$; Fig. 2B). This is consistent with a report showing approximately 35% inhibition by 1 MAC isoflurane (Hollmann et al., 2001). Halothane also inhibited the currents to 73 ± 2%, 61 ± 6%, and 50 ± 5% of control at 1, 2, and 3 MAC, respectively ($p < 0.001$; Fig. 2B). Chloroform had similar effects and inhibited the wild type to 79 ± 2% of control at 1 MAC, 64 ± 10% of control at 2 MAC, and 49 ± 5% at 3 MAC ($p < 0.001$; Fig. 2B). On the other hand, the F639A mutation markedly reduced the sensitivity to isoflurane (at 96 ± 3% of control), and abolished the effects of halothane (99 ± 3%) and chloroform (97 ± 4%) on the currents at 1 MAC (Fig. 2B). Likewise, A825W eliminated the inhibition of isoflurane (at 98 ± 4% of control), halothane (99 ± 3%), and chloroform (98 ± 4%) at 1 MAC (Fig. 2B).

Both n-hexanol and n-octanol suppressed the currents of the wild-type receptors in a concentration-dependent manner (Fig. 3). At a concentration corresponding to 1 MAC, n-hexanol and n-octanol significantly inhibited the currents to 70 ± 3% ($p < 0.001$) and 81 ± 2% ($p < 0.01$), respectively (Fig. 3). The F639A mutation reduced the inhibition of n-hexanol (98 ± 1.12%) and abolished the effects of n-octanol (99.8 ± 1.12%) on the currents at 1 MAC (Fig. 3). In addition, A825W also eliminated the inhibition of n-hexanol (101.2 ± 1.2%) and n-octanol (100 ± 1.24%) at 1 MAC (Fig. 3).

Nitrous oxide, xenon, and cyclopropane reduced the currents mediated by the wild-type receptors to 77 ± 10% ($p <$ 0.01), 58 ± 3% ($p < 0.001$), and 63 ± 2% ($p < 0.001$) of control,

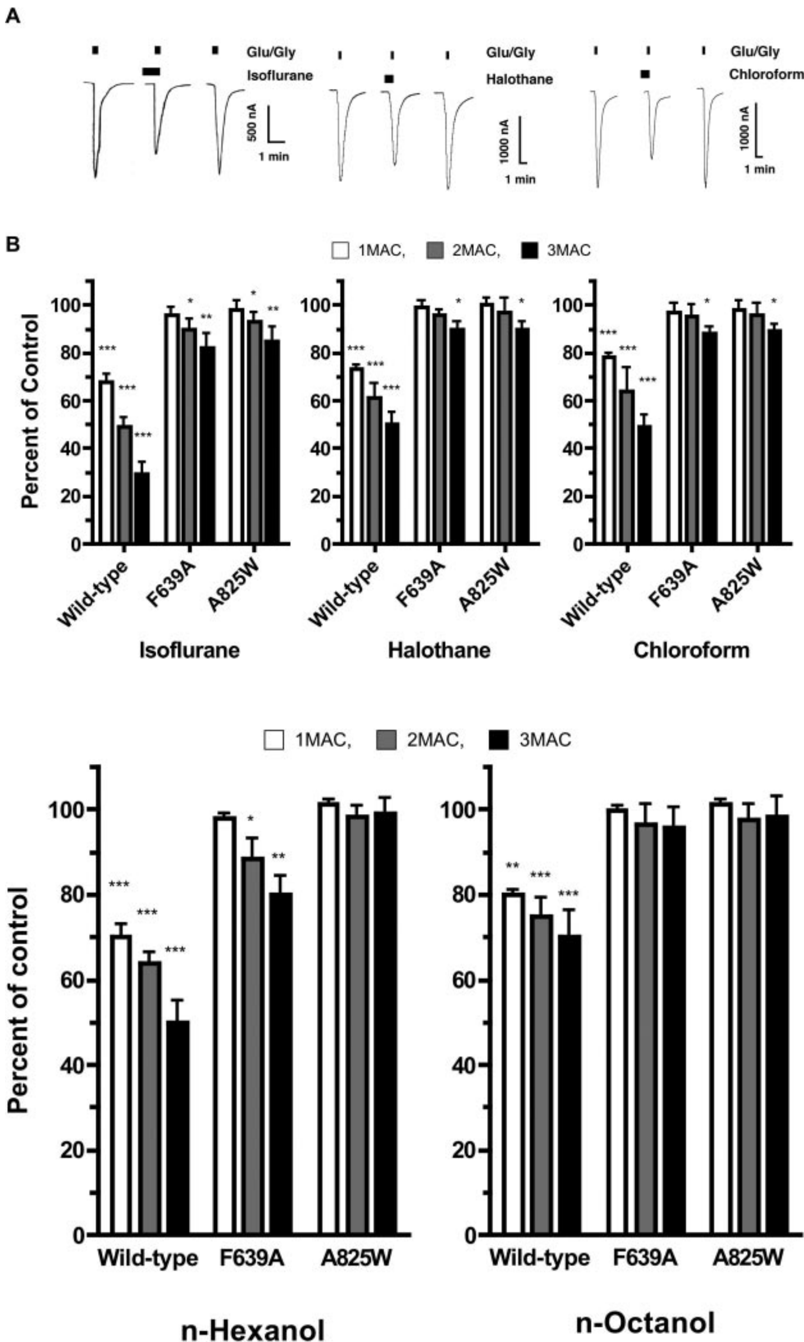

Fig. 2. A. Representative tracings of current responses of the wild-type NMDA receptors (NR1/NR2A) before, during, and after perfusion of 1 MAC isoflurane, halothane, or chloroform. Currents were stimulated with a maximal glycine concentration and EC_{50} glutamate for 30 s (Glu/Gly). Filled bars, applications of anesthetic agents (1.5 min.). B, effects of isoflurane, halothane, or chloroform on the wild-type and mutant (F639A and A825W) NMDA receptors at concentrations of 1 (open bars), 2 (gray bars), or 3 (closed bars) MAC. Bars, mean ± S.E. (n = 5–15) as percentage of controls. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with control responses, using one-way ANOVA.

Fig. 3. Effects of n-alcohols on wild-type and mutant (F639A and A825W) NMDA receptors. Effects of n-hexanol (left) and n-octanol (right) are given at concentrations of 1 (open bar), 2 (gray bar), or 3 (closed bar) MAC. Bars, mean ± S. E. (n = 5–15) as percentage of controls. *, $p <$ 0.05; **, $p < 0.01$; ***, $p < 0.001$, compared with control responses, using one-way ANOVA.

respectively (Fig. 4). The F639A and A825W mutations reduced the xenon inhibition to 87 ± 2% and 69.4 ± 1.2% of control, respectively, and eliminated the inhibitory effect of cyclopropane (Fig. 4). Surprisingly, nitrous oxide inhibition of the mutant receptors was similar to that seen with wild type (Fig. 4).

Both F3 and F6 are predicted by their lipid solubilities to be anesthetics and are structurally similar. However, F3 produces anesthesia in vivo, whereas F6 does not (Koblin et al., 1994). We found that F3 significantly inhibited the wild-type to 80 ± 3% of control at 2 MAC ($p < 0.05$), whereas F6 had little effect on the receptor (Fig. 5). The inhibition by F3 at 2 MAC was relatively weak compared with that of other anesthetics (Fig. 2B), and even at the saturated concentration (approximately 15 MAC), F3 showed only approximately 30% inhibition (Fig. 5). For the F639A and A825W receptors, F3 and F6 had no effects (Fig. 5).

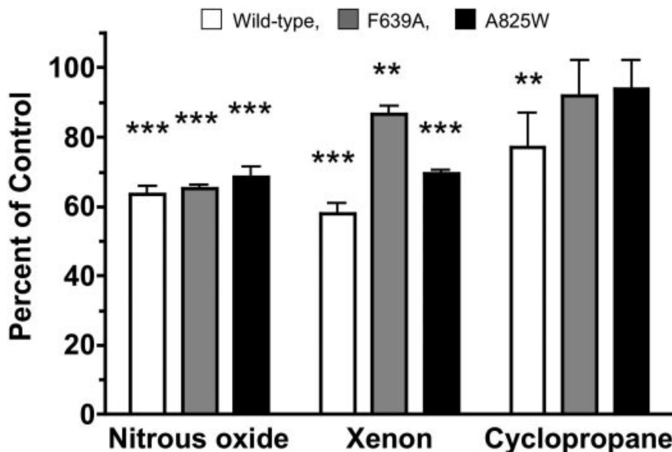

Fig. 4. Effects of nitrous oxide, xenon, and cyclopropane on the wild-type (open bar), F639A (gray bar) ,and A825W (closed bar) NMDA receptors. The bath concentrations of cyclopropane, nitrous oxide, and xenon corresponded to approximately 1, 0.5, and 0.5 MAC, respectively. Bars and errors, mean ± S.E. (n = 5–15) as percentage of controls. **, $p < 0.01$; ***, $p < 0.001$, compared with control responses, using one-way ANOVA.

We next tested five anesthetics on receptors containing both the NR1 and the NR2A mutant subunits (F639A/A825W). F639A/A825W was not affected by isoflurane or chloroform and reduced the xenon inhibition, although these compounds significantly suppressed the wild-type-mediated currents (Fig. 6). The A825W mutation attenuated xenon inhibition (69% of control) compared with the wild type (58% of control); xenon decreased both the wild-type- and A825W-mediated currents ($p < 0.001$) compared with control responses. Xenon caused a statistically ($p < 0.05$), but likely unimportant, decrease in the F639A/A825W-mediated currents (93% of control). Interestingly, nitrous oxide and ketamine inhibited all of the mutant NMDA receptors to the same extent as the wild-type NMDA receptors (Figs. 4 and 6).

Benzene has been shown to inhibit function of the wild-type NMDA receptors (Raines et al., 2004), and we tested this compound on the mutant receptors. The inhibitory action of benzene was not affected by mutation of NR1, NR2A, or the combined mutations (Fig. 7).

Although all of the anesthetics inhibited the NMDA receptor functions, the effects of mutation suggested different mechanisms of inhibition. We asked if more rapid application of glutamate might reveal differences in the features of the inhibition produced by two of the anesthetics, isoflurane and

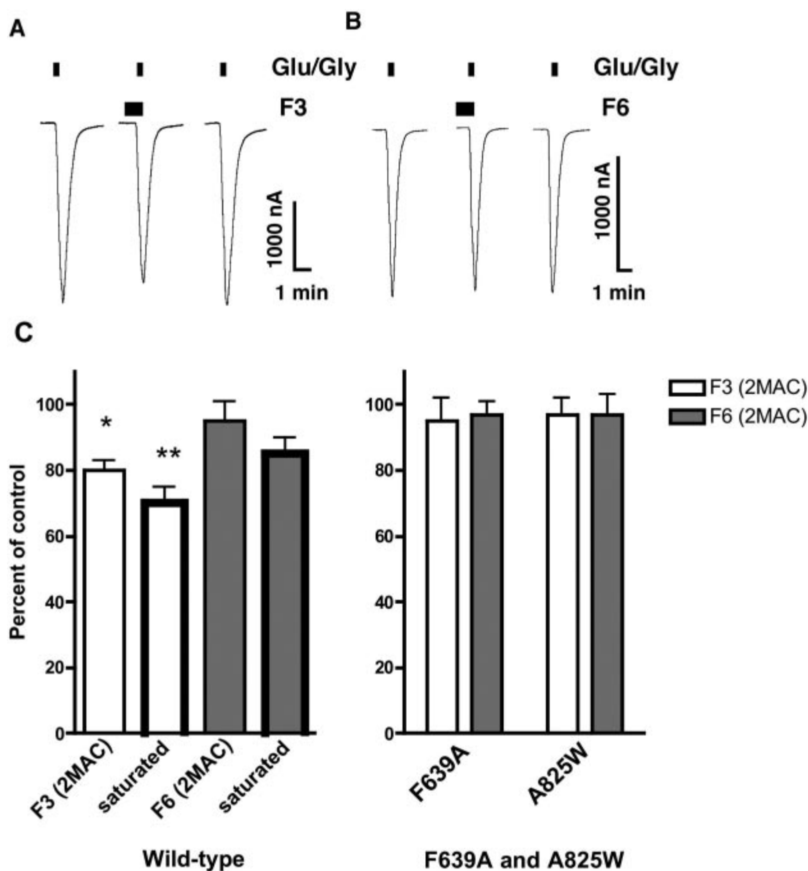

Fig. 5. Inhibition of the NMDA receptors by an anesthetic (F3) but not by a nonanesthetic (F6). Currents stimulated with a glycine concentration of maximal response (10 μM) and EC_{50} glutamate for 30 s (Glu/Gly) are shown. A, tracings obtained from a single oocyte expressing the wild-type NMDA receptors show the effects of 2 MAC F3. Agonists were applied for 30 s after 1-min pretreatment of F3. B, tracings show the effect of 2 MAC (predicted) F6 on the responses of wild-type NMDA receptors. C, effects of F3 (white bar) and F6 (gray bar) on wild-type NMDA receptors (left). Saturated solutions of both F3 (striped bar) and F6 (striped gray bar) correspond to approximately 15 MAC. Effects of F3 or F6 at 2 MAC on mutant receptors are shown in the right. Values are expressed as mean ± S.E. (n = 9–19) as percentage of controls. *, $p < 0.05$; **, $p < 0.01$, compared with control using one-way ANOVA.

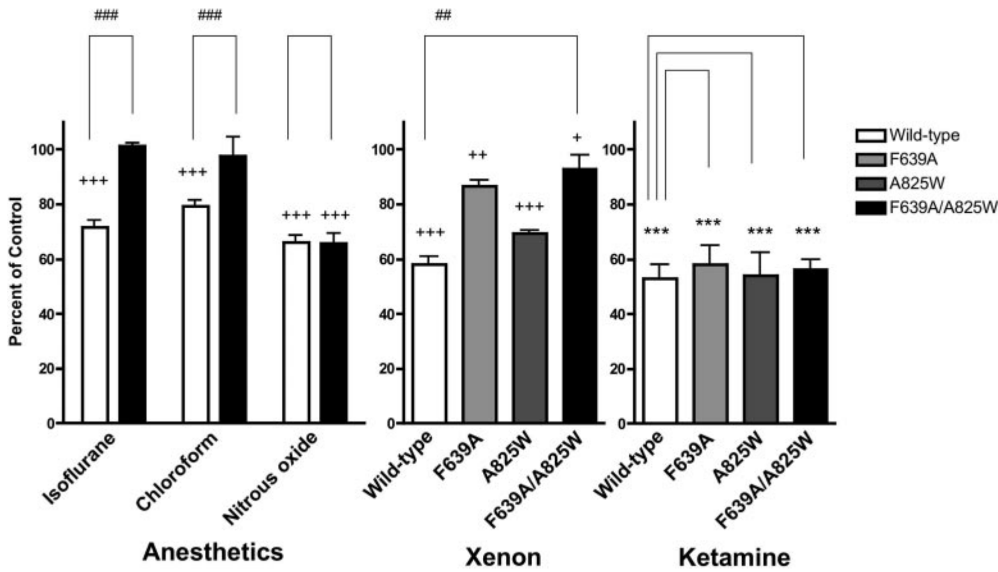

Fig. 6. Effects of anesthetics on wild-type (open bars) and F639A/A825W mutant (lattice bars) receptors are shown on the left. Isoflurane and chloroform were applied at 1 MAC, nitrous oxide and xenon at 0.5 MAC. A paired Student's t test was used to determine significance of differences in the responses before (control) and after treatment of anesthetics (+, $p < 0.05$; ++, $p < 0.01$; +++, $p < 0.001$). An unpaired Student's t test was conducted for comparison of wild-type and F639A/A825W (##, $p < 0.01$; ###, $p < 0.001$). Effects of 1 MAC ketamine on the wild-type (open bar), F639A (gray bar), A825W (dark gray bar), and F639A/A825W (closed bar) are also shown at right. Ketamine (2 μM) was used as 1 MAC in our experiments. Bars and errors, mean ± S.E. (n = 5–15) as percentage of controls. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with control responses, using one-way ANOVA.

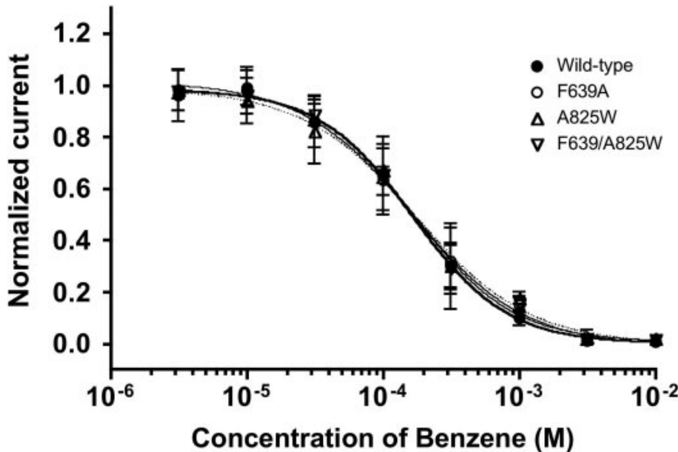

Fig. 7. Concentration current-response curves for benzene inhibition of wild-type and mutant NMDA receptors. Filled circles, wild-type (n = 5); open circles, F639A (n = 5); open triangle, A825W (n = 5); inverted triangle, F639A/A825W (n = 5). Currents were evoked by a maximal glycine concentration (10 μM) and EC_{50} glutamate for 30 s. Benzene was applied for 1 min and then applied with agonists for 30 s. All data are expressed as mean ± S.E. Percentage of control values and benzene concentrations (molar) are shown. IC_{50} and Hill coefficients of benzene on wild-type, F639A, A825W, and F639A/A825W receptors were 0.17, 0.16, 0.18, and 0.17 mM and 1.26, 1.06, 1.04, and 1.20, respectively.

ketamine. We employed a T-tube system to provide a rapid application of drugs to the oocytes. Measurements of junction potential with an open-tip pipette showed that the solution exchange took place within 50 ms (Fig. 8A). In comparison, bath application of drugs took ~15 s to obtain maximal currents (Fig. 8B), whereas currents reached their peak within 1 s when agonists were applied through the T-tube system (Fig. 8C), indicating that whole-oocyte exchange time with the T-tube system would be much faster than with bath application. To determine whether this application system could distinguish the blocking effects of ketamine, an open-channel blocker, and AP5, a competitive blocker at the glutamate site, activation kinetics of the NMDA receptors were examined in the presence of these blockers. Current kinetics of NMDA receptors were analyzed by fitting the curves to a single exponential association or dissociation curve, and the time constant was given as τ. For the control time constant (control τ), currents were elicited by 10 μM glycine and 2 μM glutamate applied for 10 s via the T-tube (Fig. 8, D and E); this was 0.85 ± 0.11 s (n = 53). The effect of coapplication of ketamine (2 μM) is shown in Fig. 8, D and E. This concentration of ketamine (2 μM) did not change the time constant of the response (0.88 ± 0.22 s, n = 8), and the inhibition by ketamine (Fig. 8E, a/b) gradually increased over 6 s. The maximal inhibition by ketamine was 55 ± 3%, and this was consistent with results obtained from the bath application system in our experiments. AP5 (2 μM) was also coapplied with agonists through the T-tube, and this concentration of AP5 significantly delayed the time constant of control (4.47 ± 1.2 s, n = 8), and the inhibition by AP5 reached a maximum within 500 ms (Fig. 8, F and G). Thus, by use of the rapid application T-tube system, the ketamine and AP5 effects could be distinguished.

To study isoflurane action, we applied glutamate and glycine with 1 or 2 MAC isoflurane through the T-tube application system with or without a 1-min isoflurane pretreatment. Without pretreatment, 1 MAC isoflurane applied together with agonists slightly, but not significantly, increased the activation time constant from 0.85 ± 0.1 to 1.36 ± 0.2 s, whereas 2 MAC isoflurane significantly slowed activation (1.77 ± 0.35 s, n = 15) (Fig. 9, A and B). Inhibition by 1 and 2 MAC isoflurane without pretreatment were 88 ± 1% and 72 ± 3% of control, respectively (Fig. 9A). Including a 1-min preapplication of isoflurane increased inhibition: 2 MAC isoflurane increased the activation time constant significantly (5.9 ± 1.9 s, n = 17) and inhibited function by 50 ± 4% (Fig. 9, A and B). Inhibition by isoflurane reached a maximum immediately (Fig. 9B). Similar results were observed with 1 MAC isoflurane (data not shown).

We also evaluated the effect of 2 MAC isoflurane on the activation time constant of F639A. The control time constant of Phe639 was determined to be 0.94 ± 0.06 s, and this was not significantly different from that of the wild-type NMDA receptors (0.85 ± 0.1 s). The 2 MAC isoflurane with 1-min pretreatment did not show significant effect on the time constant (0.96 ± 0.14 s).

AP5 (2 μM), ketamine (2 μM, 1 MAC), and isoflurane (1 and 2 MAC) were applied during activation to obtain the

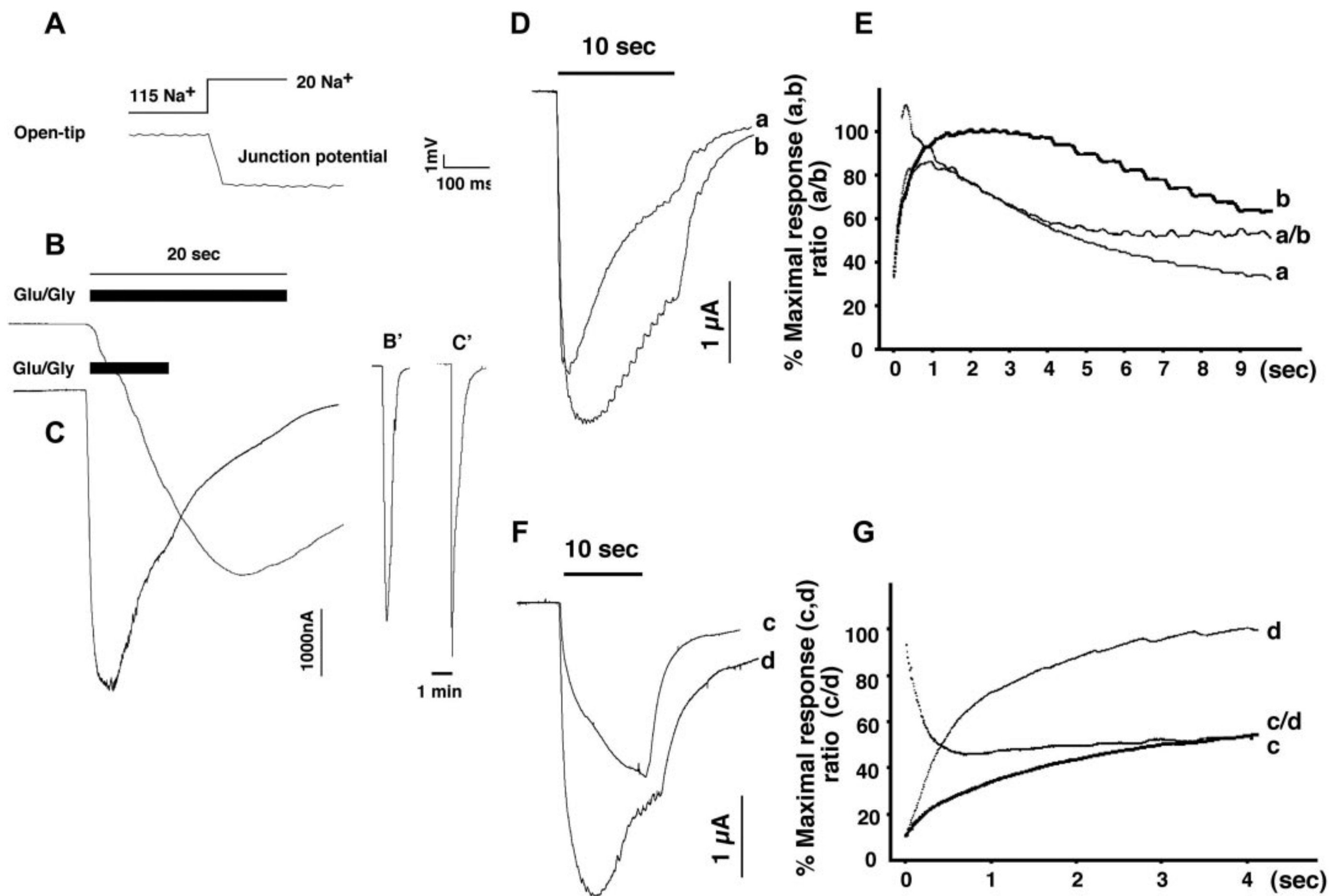

Fig. 8. A to C, examination of the speed of solution exchange with the T-tube drug application system. Ringer solution containing 115 mM NaCl was continuously perfused at a rate of 2 ml/min to the oocyte bath, and a solution containing 20 mM NaCl was perfused at a rate of 4 ml/min though the T-tube. When the stopcock in the downstream of the T-connector was closed, 20 mM NaCl containing solution rapidly came to the bath. The time course of changes in the voltage is taken as an indicator of the speed of solution change. A, measurements of junction potential with an open tip pipette indicates that the solution exchange took place within 50 ms. Representative tracings of current responses of the wild-type NMDA receptors induced by the maximal concentration of glutamate and glycine applied using the bath application system (B) and the T-tube application system (C). Closed bars, duration of drug application time. For comparison, the same tracings are shown in the reduced time scale (B′ and C′), which is similar to the scales shown in Figs. 2 and 5. D to G, effects of ketamine and AP5 on activation kinetics using the T-tube application system. D, superimposed representative tracings obtained from a single oocyte. The currents were evoked by 10-s application of 10 μM glycine and 2 μM glutamate together with (a) or without (b) 2 μM ketamine. E, Time course of ketamine induced suppression on the currents (as percentage of maximal response) and ratio (a/b) of the control and ketamine currents. Currents obtained from D were normalized to the maximal control response and plotted every 10 ms. This result is representative of the six oocytes studied. F, superimposed representative tracings obtained from a single oocyte. The currents were evoked by 10-s application of 10 μM glycine and 2 μM glutamate together with (c) or without (d) 2 μM AP5. G, time course of AP5-induced suppression on the currents and percent ratio (c/d) of the AP5 effect. Currents obtained from F were normalized to the maximal control response and plotted every 10 ms. This result is representative of the six oocytes studied. Curves were fitted with a single exponential function, and the resulting K values were used for comparing the activation kinetics.

inhibition time constants of these drugs (Fig. 9C). To compare the inhibition rates, currents were obtained in every 10 ms until they leveled off and fitted by single exponential function. The inhibition time constants of AP5, ketamine, 1 MAC isoflurane, and 2 MAC isoflurane were 5.1 ± 0.9, 20.5 ± 3.5, 5.9 ± 1.4, and 5.8 ± 1.3 s, respectively (Fig. 9D).

Discussion

The main aim of this study was to compare the action of a diverse class of anesthetic agents on wild-type and mutant NMDA receptors to determine whether single amino acid mutations could abolish the actions of these drugs. Furthermore, we hoped that these mutations could distinguish among the anesthetics to delineate different functional groups based on their mechanism of inhibition of NMDA receptors.

We found that isoflurane, halothane, and chloroform suppressed NMDA receptor function by 22 to 32% at concentrations corresponding to 1 MAC. This is consistent with a report showing ~35% inhibition by 1 MAC isoflurane (Hollmann et al., 2001). Isoflurane inhibited the receptor noncompetitively in our study, and Hollmann et al. (2001) also showed that glutamate, glycine, and magnesium concentrations did not influence anesthetic inhibition of NMDA receptors. In this study, a wide range of inhaled anesthetics, n-alcohols, and ketamine inhibited NMDA receptor-mediated currents in oocytes expressing NMDA receptors, confirming and extending the work of others (Yamakura and Harris, 2000; Hollmann et al., 2001; Hara et al., 2002). Koblin et al. (1994) define a nonimmobilizer as a compound that lacks any anesthetic (immobilizing) effect, even though it was predicted to be an anesthetic by its lipid solubility (Meyer-Overton relationship). It is important to note that the lipid

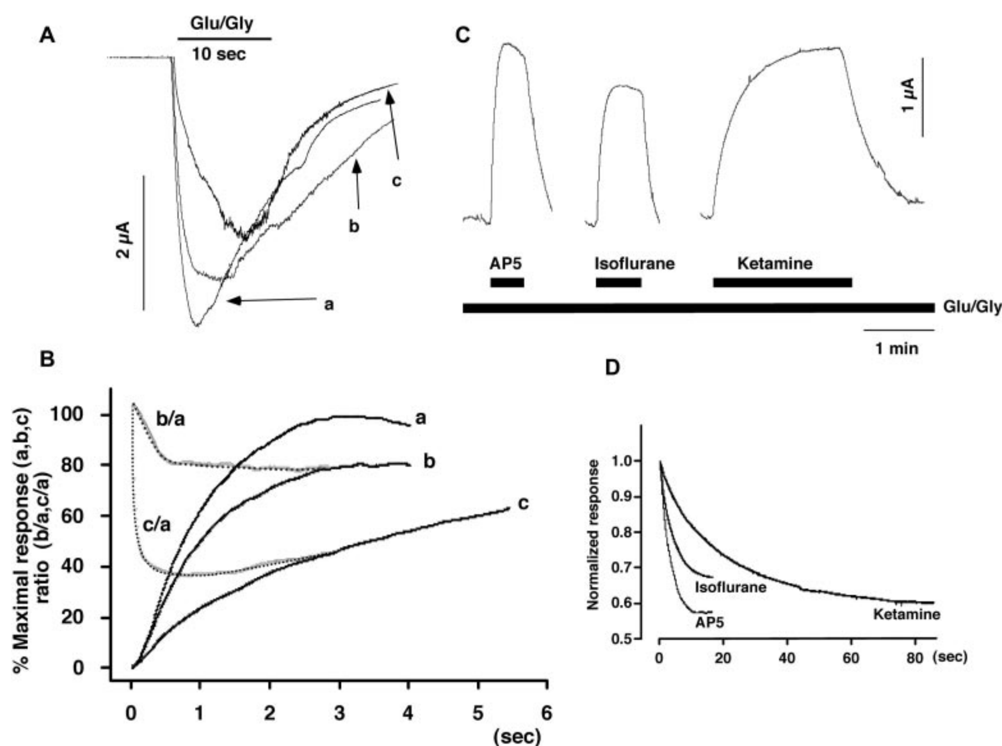

Fig. 9. A and B, effects of isoflurane on the activation kinetics of the NMDA receptors. A, superimposed representative tracings obtained from a single oocyte using the T-tube drug application system. The currents were evoked by a 10-s application of 10 μM glycine and 2 μM glutamate (a), coapplication of glycine and glutamate with 2 MAC isoflurane (b), or coapplication of glycine, glutamate, and 2 MAC isoflurane after a 1-min preincubation of 2 MAC isoflurane (c). B, time course of 2 MAC isoflurane (b and c)-induced suppression on the currents and percent ratio (b/a, c/a) of the isoflurane effects. Currents obtained from A were normalized to the maximal control response and plotted every 10 ms. The average responses (from 15–17 oocytes) are shown in this figure. C and D, kinetics of block by AP5, ketamine, and isoflurane on the NMDA receptors. C, representative tracings from a single oocyte showing the blockade of currents (10 μM glycine, 2 μM glutamate) by 2 μM AP5, 2 MAC isoflurane, or 2 μM ketamine. Closed bars, duration of drug applications. Inhibitors were applied until their effects leveled off. D, time course of block by AP5, isoflurane, and ketamine. Data obtained from C were normalized to the initial points of the block and plotted every 10 s. Plotted data were fitted with single exponential function until they reach maximal inhibition and used to determine the inhibition time constant. The average of results (from five oocytes) is shown in this figure.

TABLE 1

Summary of effects of anesthetics on the wild-type and mutant NMDA receptors

Drugs were tested at concentrations corresponding to 1 MAC, except nitrous oxide and xenon were tested at approximately 0.5 MAC and F3 and F6 were tested at 2 MAC. Molecular volumes (in Ångstrom units cubed) were provided by Dr. James R. Trudell (Stanford University) and calculated as described under *Materials and Methods*. Compared with the wild-type, the mutant NMDA receptors were suppressed by nitrous oxide, ketamine, and benzene at same extent, whereas xenon varied the effects.

	Wild Type	F639A	A825W	F639A/A825W	Molecular Volume
Isoflurane	↓↓↓	0	0	0	140
Halothane	↓↓↓	0	0	NT	110
Chloroform	↓↓↓	0	0	0	94
Cyclopropane	↓↓	0	0	NT	56
F3 (2 MAC)	↓	0	0	NT	123
F6 (2 MAC)	0	0	0	NT	166
Octanol	↓	0	0	NT	193
Hexanol	↓↓↓	0	0	NT	152
N_2O (0.5 MAC)	↓↓↓	↓↓↓	↓↓↓	↓↓↓	49
Xenon (0.5 MAC)	↓↓↓	↓↓	***	*	45
Ketamine	↓↓↓	↓↓↓	↓↓↓	↓↓↓	267
Benzene	↓↓↓	↓↓↓	↓↓↓	↓↓↓	106

NT, not tested.
↓ $p < 0.05$; ↓↓ $p < 0.01$; ↓↓↓ $p < 0.001$, compared with control responses, using one-way ANOVA and Student's *t* test. 0, no significant difference from control.
*** A825W attenuated xenon inhibition compared with the wild type; * the effects of xenon on the F639A/A825W are very slight.

soluble nonimmobilizer, F6, does not inhibit NMDA receptor function. Thus, the effects of the 11 compounds tested in the present work are consistent with inhibition of NMDA receptor function playing a role in anesthesia.

A key question is whether actions of this diverse group of compounds can be reduced or prevented by mutation of NMDA receptor subunits. We selected the F639A mutation in the NR1 subunit and the A825W mutation in the NR2A subunit based on reports by others (Ronald et al., 2001; Honse et al., 2004) that these mutations reduce the ability of ethanol to inhibit channel function. Ronald et al. (2001) constructed 11 mutations in the NR1 subunit and found that the F639A was most effective at reducing actions of ethanol. Honse et al. (2004) tested five mutations in the TM4 region of the NR2A subunit (positions 821, 822, 824, 825, and 826) and found that the A825A mutation reduced the action of ethanol on the channel yet did not affect the NMDA EC_{50}. The studies found that the F639A or A825W did not completely block the actions of ethanol but produced a partial reduction. For example, 100 mM ethanol inhibited the wild-type receptor by

~30% and the F639A mutant by 15% (Ronald et al., 2001). Thus, previous results with ethanol are similar to our present results with xenon, where the mutants only partially prevented the inhibitory action (Table 1). However, for most of the anesthetics, the results were much more striking than for ethanol because the mutations produced almost complete blockade of the actions of even high concentrations of anesthetic. For example, 3 MAC isoflurane inhibited the wild-type receptor by ~70% but only inhibited receptors containing F639A or A825W by 15 to 20%. Complete, or almost complete, removal of drug action was also obtained with halothane, chloroform, hexanol, octanol, cyclopropane, and F3. Equally striking was the complete lack of effect of the mutations on the actions of nitrous oxide and benzene. Recently, Raines et al. (2004) reported that aromatic anesthetic compounds, such as benzene, inhibited NR1/NR2B receptors and demonstrated that the inhibitory potency was independent of molecular volume, but it correlated strongly with the ability to engage in cation-π interactions. This interaction between π electrons in aromatic compounds and cationic charges in protein residues is recognized as an important molecular force for binding of ligands to receptor targets. Thus, it is possible that some, but not all, of these agents require a positively charged amino acid in the binding site.

A critical question is whether these mutations reduce anesthetic action because they decrease binding of the drugs to the NMDA receptor subunits. Mutations of other ligand-gated ion channels suggest such a mechanism (Yamakura et al., 2001; Lobo and Harris, 2005), but the present results are not sufficient to delineate a binding site. For example, in the $GABA_A$ and glycine receptors, introduction of larger amino acids reduced the actions of anesthetics and changed the alcohol "cut-off" in a manner consistent with a binding cavity (Wick et al., 1998; Jenkins et al., 2001), but for the 639 site, introduction of a smaller amino acid (F639A) reduced the anesthetic action. In studies with the glycine receptor, the two sites that were shown to be effective in preventing anesthetic action are known to face each other (Lobo and Harris, 2005). However, the Phe639 and Ala825 sites that reduced anesthetic inhibition of NMDA receptors in the present study are on different subunits. These sites probably do not contribute to a common binding pocket because NMDA receptors are thought to exist as a dimer of dimers with NR1s and NR2s associating with one another (Schorge and Colquhoun, 2003; Furukawa et al., 2005). In addition, tryptophan substitution at positions in the NR1 TM4 domain that are analogous to Ala825 in the NR2A subunit do not, by themselves, reduce ethanol inhibition of NMDA receptors (Thetford Smothers and Woodward, 2006). However, these substitutions restore normal ethanol inhibition in receptors carrying the F639A mutation (Thetford Smothers and Woodward, 2006). Although similar studies have not yet been done with the NR2A subunit, these results suggest that both NR1 and NR2 subunits may contain complementary binding pockets for anesthetics that, when occupied, disrupt channel opening. Thus, it is likely that at least one of these sites is involved in "transduction" rather than binding and is able to reduce anesthetic action without decreasing binding of drugs to the receptor. Studies with methanethiosulfonates or other covalent labeling reagents, as used with other ligand-gated ion channels (Mascia et al., 2000; Lobo et al., 2004), will be required to determine whether either of these amino acids forms part of an anesthetic binding site. Regardless of whether either of these sites are critical for anesthetic binding, the mutations are clearly able to differentiate nitrous oxide, benzene, and ketamine from most of the other anesthetic agents, indicating multiple mechanisms of action on the NMDA receptor. These differences are not related to the molecular volumes of these drugs (Table 1).

To define differences in drug action on the NMDA receptors, we established a rapid application system using a T-tube connector. Our results of 50 ms for solution exchange and the activation time constant of 850 ms for the control NMDA current indicate that our system is useful for oocyte studies. We were able to distinguish differences in the NMDA receptor activation time constant produced by coapplication of AP5 (4.47 s) or ketamine (0.88 s). These results indicate a rapid inhibition by AP5. Ketamine did not change the time constant in comparison with the control (0.85 s), and the inhibition gradually increased over 6 s, suggesting an open channel blockade. Our results also showed more rapid inhibition by isoflurane than by ketamine when these drugs were coapplied with glutamate/glycine. From these results, isoflurane does not seem to be a use-dependent (open channel) blocker. Because the inhibition time constant of isoflurane was faster than that of ketamine (20.5 s) but slower than that of AP5 (5.1 s), our results indicate that the mechanism of isoflurane inhibition is different from that of ketamine and AP5. This is consistent with previous studies showing that the relatively slow onset of isoflurane may result from "soaking" in the receptor (Criswell et al., 2004). Our use of a nonsaturating concentration of glutamate means that the activation rates should primarily reflect the binding rate of glutamate to the receptor, rather than the rate of opening of fully bound receptor (thus, the slowing of the activation rate by AP5). The unexpected effect of isoflurane on this process may indicate that the allosteric action of isoflurane that changes channel gating also changes glutamate binding. Such a "backward" connection between gating and binding has often been proposed (Colquhoun, 1998).

In conclusion, we found that amino acid substitutions in the TM3 of NR1 and TM4 of NR2A subunits of the NMDA receptor reduce the action of many, but not all, anesthetic agents. A question for future studies is whether these amino acids participate in the binding of anesthetics to the protein as has been suggested for transmembrane residues in other ligand-gated ion channels (Mascia et al., 2000).

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References

Chatterton JE, Awobuluyi M, Premkumar LS, Takahashi H, Talantova M, Shin Y, Cui J, Tu S, Sevarino KA, Nakanishi N, et al. (2002) Excitatory glycine receptors containing the NR3 family of NMDA receptor subunits. *Nature (Lond)* **415:**793–798.

Colquhoun D (1998) Binding, gating, affinity and efficacy: the interpretation of structure-activity relationships for agonists and of the effects of mutating receptors. *Br J Pharmacol* **125:**924–947.

Criswell HE, Ming Z, Pleasant N, Griffith BL, Mueller RA, and Breese GR (2004) Macrokinetic analysis of blockade of NMDA-gated currents by substituted alcohols, alkanes and ethers. *Brain Res* **1015:**107–113.

Dayton PG, Stiller RL, Cook DR, and Perel JM (1983) The binding of ketamine to plasma proteins: emphasis on human plasma. *Eur J Clin Pharmacol* **24:**825–831.

Dildy-Mayfield JE, Mihic SJ, Liu Y, Deitrich RA, and Harris RA (1996) Actions of long chain alcohols on $GABA_A$ and glutamate receptors: relation to in vivo effects. *Br J Pharmacol* **118:**378–384.

Franks NP, Dickinson R, de Sousa SL, Hall AC, and Lieb WR (1998) How does xenon produce anaesthesia? *Nature (Lond)* **396:**324.

Furukawa H, Singh SK, Mancusso R, and Gouaux E (2005) Subunit arrangement and function in NMDA receptors. *Nature (Lond)* **438:**185–192.

Hara K, Eger EI 2nd, Laster MJ, and Harris RA (2002) Nonhalogenated alkanes cyclopropane and butane affect neurotransmitter-gated ion channel and G-protein-coupled receptors: differential actions on $GABA_A$ and glycine receptors. *Anesthesiology* **97:**1512–1520.

Hara K and Harris RA (2002) The anesthetic mechanism of urethane: the effects on neurotransmitter-gated ion channels. *Anesth Analg* **94:**313–318.

Hollmann MW, Liu HT, Hoenemann CW, Liu WH, and Durieux ME (2001) Modulation of NMDA receptor function by ketamine and magnesium: II. Interactions with volatile anesthetics. *Anesth Analg* **92:**1182–1191.

Honse Y, Ren H, Lipsky RH, and Peoples RW (2004) Sites in the fourth membrane-associated domain regulate alcohol sensitivity of the NMDA receptor. *Neuropharmacology* **46:**647–654.

Idvall J, Ahlgren I, Aronsen KR, and Stenberg P (1979) Ketamine infusions: pharmacokinetics and clinical effects. *Br J Anaesth* **51:**1167–1173.

Jenkins A, Greenblatt EP, Faulkner HJ, Bertaccini E, Light A, Lin A, Andreasen A, Viner A, Trudell JR, and Harrison NL (2001) Evidence for a common binding cavity for three general anesthetics within the $GABA_A$ receptor. *J Neurosci* **21:** RC136.

Johnson JW and Ascher P (1987) Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature (Lond)* **325:**529–531.

Koblin DD, Chortkoff BS, Laster MJ, Eger EI 2nd, Halsey MJ, and Ionescu P (1994) Polyhalogenated and perfluorinated compounds that disobey the Meyer-Overton hypothesis. *Anesth Analg* **79:**1043–1048.

Laube B, Kuhse J, and Betz H (1998) Evidence for a tetrameric structure of recombinant NMDA receptors. *J Neurosci* **18:**2954–2961.

Lin LH, Chen LL, and Harris RA (1993) Enflurane inhibits NMDA, AMPA and kainate-induced currents in *Xenopus* oocytes expressing mouse and human brain mRNA. *FASEB J* **7:**479–485.

Lobo IA and Harris RA (2005) Sites of alcohol and volatile anesthetic action on glycine receptors. *Int Rev Neurobiol* **65:**53–87.

Lobo IA, Mascia MP, Trudell JR, and Harris RA (2004) Channel gating of the glycine receptor changes accessibility to residues implicated in receptor potentiation by alcohols and anesthetics. *J Biol Chem* **279:**33919–33927.

Martin DC, Plagenhoef M, Abraham J, Dennison RL, and Aronstam RS (1995) Volatile anesthetics and glutamate activation of *N*-methyl-D-aspartate receptors. *Biochem Pharmacol* **49:**809–817.

Mascia MP, Trudell JR, and Harris RA (2000) Specific binding sites for alcohols and anesthetics on ligand-gated ion channels. *Proc Natl Acad Sci USA* **97:**9305–9310.

Meguro H, Mori H, Araki K, Kushiya E, Kutsuwada T, Yamazaki M, Kumanishi T, Arakawa M, Sakimura K, and Mishina M (1992) Functional characterization of a heteromeric NMDA receptor channel expressed from cloned cDNAs. *Nature (Lond)* **357:**70–74.

Mihic SJ, McQuilkin SJ, Eger EI 2nd, Ionescu P, and Harris RA (1994) Potentiation of gamma-aminobutyric acid type A receptor-mediated chloride currents by novel halogenated compounds correlates with their abilities to induce general anesthesia. *Mol Pharmacol* **46:**851–857.

Mirshahi T and Woodward JJ (1995) Ethanol sensitivity of heteromeric NMDA receptors: effects of subunit assembly, glycine and NMDAR1 Mg^{2+}-insensitive mutants. *Neuropharmacology* **34:**347–355.

Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B, and Seeburg PH (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science (Wash DC)* **256:**1217–1221.

Murase K, Randic M, Shirasaki T, Nakagawa T, and Akaike N (1990) Serotonin suppresses *N*-methyl-D-aspartate responses in acutely isolated spinal dorsal horn neurons of the rat. *Brain Res* **525:**84–91.

Orser BA, Pennefather PS, and MacDonald JF (1997) Multiple mechanisms of ketamine blockade of *N*-methyl-D-aspartate receptors. *Anesthesiology* **86:**903–917.

Perouansky M, Baranov D, Salman M, and Yaari Y (1995) Effects of halothane on glutamate receptor-mediated excitatory postsynaptic currents: a patch-clamp study in adult mouse hippocampal slices. *Anesthesiology* **83:**109–119.

Raines DE, Gioia F, Claycomb RJ, and Stevens RJ (2004) The *N*-methyl-D-aspartate receptor inhibitory potencies of aromatic inhaled drugs of abuse: evidence for modulation by cation-pi interactions. *J Pharmacol Exp Ther* **311:**14–21.

Richards CD (1973) On the mechanism of halothane anaesthesia. *J Physiol (Lond)* **233:**439–456.

Ronald KM, Mirshahi T, and Woodward JJ (2001) Ethanol inhibition of *N*-methyl-D-aspartate receptors is reduced by site-directed mutagenesis of a transmembrane domain phenylalanine residue. *J Biol Chem* **276:**44729–44735.

Saidman LJ, Eger EI 2nd, Munson ES, Babad AA, and Muallem M (1967) Minimum alveolar concentrations of methoxyflurane, halothane, ether and cyclopropane in man: correlation with theories of anesthesia. *Anesthesiology* **28:**994–1002.

Schorge S and Colquhoun D (2003) Studies of NMDA receptor function and stoichiometry with truncated and tandem subunits. *J Neurosci* **23:**1151–1158.

Stabernack C, Sonner JM, Laster M, Zhang Y, Xing Y, Sharma M, and Eger EI 2nd (2003) Spinal *N*-methyl-D-aspartate receptors may contribute to the immobilizing action of isoflurane. *Anesth Analg* **96:**102–107.

Thetford Smothers C and Woodward JJ (2006) Effects of amino acid substitutions in transmembrane domains of the NR1 subunit on the ethanol inhibition of recombinant *N*-methyl-D-aspartate receptors. *Alcohol Clin Exp Res* **30:**523–530.

Wick MJ, Mihic SJ, Ueno S, Mascia MP, Trudell JR, Brozowski SJ, Ye Q, Harrison NL, and Harris RA (1998) Mutations of gamma-aminobutyric acid and glycine receptors change alcohol cutoff: evidence for an alcohol receptor? *Proc Natl Acad Sci USA* **95:**6504–6509.

Yamakura T, Bertaccini E, Trudell JR, and Harris RA (2001) Anesthetics and ion channels: molecular models and sites of action. *Annu Rev Pharmacol Toxicol* **41:**23–51.

Yamakura T and Harris RA (2000) Effects of gaseous anesthetics nitrous oxide and xenon on ligand-gated ion channels: comparison with isoflurane and ethanol. *Anesthesiology* **93:**1095–1101.

Yang J and Zorumski CF (1991) Effects of isoflurane on *N*-methyl-D-aspartate gated ion channels in cultured rat hippocampal neurons. *Ann NY Acad Sci* **625:**287–289.

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