Identification of Two Mutations (F758W and F758Y) in the *N*-methyl-D-aspartate Receptor Glycine-binding Site that Selectively Prevent Competitive Inhibition by Xenon without Affecting Glycine Binding

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ABSTRACT

Background: Xenon is a general anesthetic with neuroprotective properties. Xenon inhibition at the glycine-binding site of the *N*-Methyl-D-aspartate (NMDA) receptor mediates xenon neuroprotection against ischemic injury *in vitro*. Here we identify specific amino acids important for xenon binding to the NMDA receptor, with the aim of finding silent mutations that eliminate xenon binding but leave normal receptor function intact.

Methods: Site-directed mutagenesis was used to mutate specific amino-acids in the GluN1 subunit of rat NMDA receptors. Mutant GluN1/GluN2A receptors were expressed in HEK 293 cells and were assessed functionally using patch-clamp electrophysiology. The responses of the mutant receptors to glycine and anesthetics were determined.

Results: Mutation of phenylalanine 758 to an aromatic tryptophan or tyrosine left glycine affinity unchanged, but eliminated xenon binding without affecting the binding of sevoflurane or isoflurane.

Conclusions: These findings confirm xenon binds to the glycine site of the GluN1 subunit of the NMDA receptor and indicate that interactions between xenon and the aromatic ring of the phenylalanine 758 residue are important for xenon binding. Our most important finding is that we have identified two mutations, F758W and F758Y, that eliminate xenon binding to the NMDA receptor glycine site without changing the glycine affinity of the receptor or the binding of volatile anesthetics. The identification of these selective mutations will allow knock-in animals to be used to dissect the mechanism(s) of xenon's neuroprotective and anesthetic properties *in vivo*.

What We Already Know about This Topic

- Xenon is an anesthetic with neuroprotective properties, the latter because of actions on the glycine binding site to the *N*-methyl-D-aspartate (NMDA) receptor

What This Article Tells Us That Is New

- Using site-targeted mutagenesis of the rat NMDA receptor, two mutations eliminated xenon binding at the glycine site without altering glycine affinity or the binding of sevoflurane or isoflurane
- The identification of these mutations will allow better understanding of the mechanisms of xenon-induced anesthesia and neuroprotection

GENERAL anesthetics are thought to act at only a small number of molecular targets at critical loci in the brain.[1–6] The noble gas xenon was first used clinically as a general anesthetic in the 1950s,[7,8] but until recently, the targets underlying xenon's biologic activity were unknown. Work in our laboratory first identified the *N*-Methyl-D-aspartate (NMDA) subtype of glutamate receptor as a molecular target for xenon,[9,10] and we subsequently showed that xenon inhibits NMDA receptors by competing with the co-

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agonist glycine at the glycine-binding site on the GluN1 subunit.[11] The finding that xenon is an NMDA receptor antagonist led to the idea that xenon could be used as a neuroprotectant, as overactivation of NMDA receptors plays a role in various pathologic conditions, such as ischemia, stroke, and traumatic brain injury.[12–14] Xenon has now been shown to be neuroprotective in a variety of *in vitro* and *in vivo* models of ischemia and stroke.[15–26] We have recently shown that xenon neuroprotection against hypoxic/ischemic injury *in vitro* is mediated by xenon inhibition at the glycine site of the NMDA receptor.[27] Inhibition of the NMDA receptor is a plausible mechanism for xenon neuroprotection *in vivo*. Nevertheless, a few other targets have emerged that could also play a role in xenon's neuroprotective effects. The two-pore domain potassium channel TREK-1[28] and the adenosine triphosphate-sensitive potassium channel[29] are activated by xenon. Activation of TREK-1 has been implicated in the neuroprotective actions of the fatty acid linolenate,[30] and activation of the adenosine triphosphate-sensitive potassium channel is protective against ischemic injury.[31] Determining which of these targets are important for xenon neuroprotection is timely, as xenon is now beginning clinical trials as a neuroprotectant. Advances in molecular genetics have opened up the possibility of generating mouse models that can be used to determine the role of specific targets in endpoints such as general anesthesia and neuroprotection. Because of the importance of NMDA receptors in normal physiologic function, global knock-out animals lacking NMDA receptors die soon after birth.[32] A better strategy is based on a knock-in mutation in which the sensitivity to a drug is eliminated but the normal functioning of the receptor is preserved. Such an approach using knock-in mice with a point mutation in the γ-aminobutyric acid receptor type A β subunit has been used to understand the pathways involved in propofol and etomidate anesthesia.[33–37]

The aim of this study is to dissect the molecular interactions xenon makes with the NMDA receptor, to identify mutations in the NMDA receptor that prevent xenon binding at the glycine site, while at the same time not affecting the sensitivity of the receptor to glycine. Although the specific molecular interactions that xenon makes at the NMDA receptor are not known, our modeling simulations identified a small number of amino acids close to the predicted xenon binding site and therefore most likely to interact with the xenon atoms.[11] The current study is aimed at determining which of these amino acids are critical for the binding of xenon. Isoflurane competes with glycine at the NMDA receptor glycine site.[11] Here we test the hypothesis that sevoflurane and the gaseous anesthetics nitrous oxide and cyclopropane act at the glycine site of the NMDA receptor.

Materials and Methods

Cell Culture and Transfection

HEK-293 cells (tsA201) were obtained from the European Collection of Cell Cultures (Salisbury, United Kingdom) and cultured using standard procedures.[11] For electrophysiology the cells were plated onto glass coverslips coated with poly-D-lysine. The culture medium was Dulbecco's modified Eagle's medium without glutamine, containing 400 μM DL-2-Amino-5-phosphonopentanoic acid (DL-AP5) and 1 mM $MgCl_2$ to minimize excitotoxicity. Cells were transfected with complimentary DNA using the calcium phosphate technique. The complimentary DNA clones for rat NMDA receptor GluN1–1a and GluN2A subunits were provided by Professor Stephen Heinemann, Ph.D., of the Salk Institute, Laboratory of Molecular Neurobiology, La Jolla, California. Mutants of the GluN1 subunit were made using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by DNA sequencing (MWG Biotech, Ebersberg, Germany). Cells were cotransfected with green fluorescent protein for identification. Cells were used for electrophysiology 24–48 h after transfection.

Electrophysiology

Whole cell recordings were made using an Axoclamp 200B amplifier (Axon Instruments, Foster City, CA). Pipettes (3–5 MΩ) were fabricated from borosilicate glass and filled with internal solution containing (in mM): 110 K gluconate, 2.5 NaCl, 10 HEPES, 10 1,2-Bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA), and titrated to pH 7.3 using KOH. The extracellular solution contained (in mM): 150 NaCl, 2.5 KCl, 2 $CaCl_2$, 10 HEPES, and titrated to pH 7.35 using sodium hydroxide. Cells were voltage-clamped at −60 mV, currents were filtered at 100 Hz (−3 dB) using an 8-pole Bessel filter (model 900 Frequency Devices Inc., Ottawa, IL), digitized (Digidata 1332A, Axon Instruments), and stored on a computer. Series resistance was compensated 75–90%. Data were acquired and peak currents measured using pClamp software (Axon Instruments). Solutions containing xenon, nitrous oxide, and cyclopropane were prepared by bubbling gases through scintered glass bubblers in Dreschel bottles containing extracellular saline, as described previously.[9,28] Solutions of isoflurane and sevoflurane were prepared from saturated aqueous solutions, as described previously.[9,11] Cells were exposed to NMDA and anesthetics using a rapid perfusion system.[38] In order to minimize variation because of pipetting errors, our experimental protocol used preexposure to glycine that is present at the same concentrations in all solutions in a given experiment. As a result the experiments at different glycine concentrations were performed on different groups of cells. For each cell at a given glycine concentration, control measurements are made before and after each anesthetic exposure. We aimed to have the same group size for each glycine concentration (typically 7 or 8 cells), although in some cases they were not identical.

Data Analysis

Concentration-response curves for NMDA and glycine were fitted to the Hill equation: $y = I_{max} \times [agonist]^{n_H} / \{ [EC_{50}]^{n_H} +$

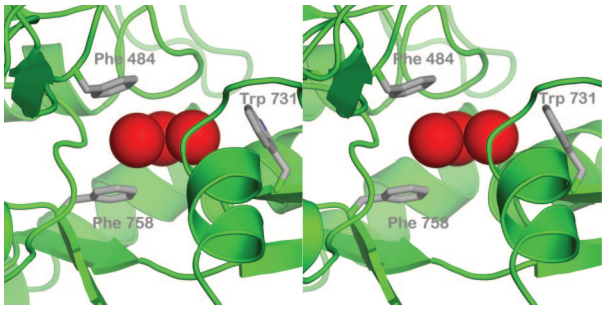

Fig. 1. Aromatic residues are important for the binding of xenon and glycine at the glycine binding site of the *N*-methyl-D-aspartate receptor. Stereo view showing the predicted position of xenon atoms (*red spheres*) in the glycine site together with the aromatic residues phenylalanine 758, phenylalanine 484, and tryptophan 731. The crystallographic structure, 1PBQ,[51] was obtained from the Protein Data Bank. The positions of xenon atoms were predicted using Grand Canonical Monte Carlo modeling simulations.[11] Images were created using the PyMOL Molecular Graphics System (see http://www.pymol.org; accessed May 21, 2012). Phe 484 = phenylalanine 484; Phe 758 = phenylalanine 758; Trp 731 = tryptophan 731.

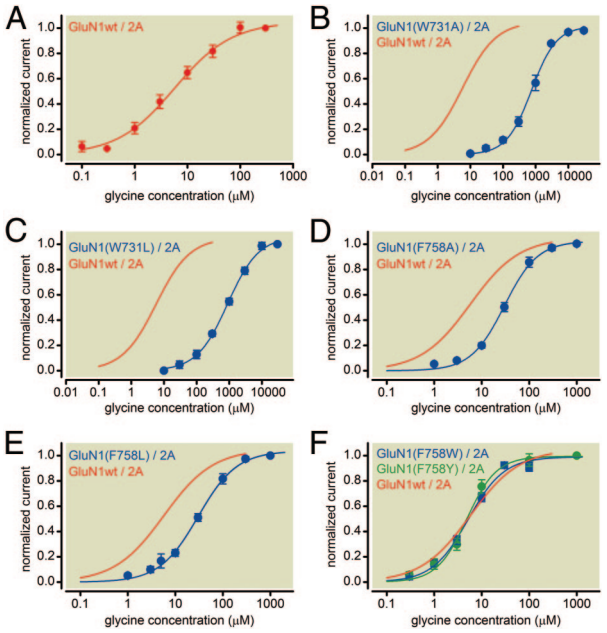

Fig. 2. Glycine concentration-response curves of wild-type and GluN1 mutant receptors: (*A*) wild-type GluN1/2A receptors, (*B*) GluN1 (W731A)/2A mutant receptors, (*C*) GluN1(W731L)/2A mutant receptors, (*D*) GluN1(F758A)/2A mutant receptors, (*E*) GluN1(F758L)/2A mutant receptors, and (*F*) GluN1(F758W)/2A (*blue squares*) and GluN1(F758Y)/2A (*green circles*) mutant receptors. The curves shown are fit to the Hill equation. The points are mean values from an average of nine cells; the error bars are standard errors. Data have been normalized to the highest saturating glycine concentration for each receptor.

[agonist]^{n_H} }, where n_H is the Hill coefficient. Values of I_{max} were not constrained and these are quoted together with the other parameters. Error bars are the SEM. We compared anesthetic inhibition at different glycine concentrations, using ANOVA with Tukey *post hoc* test. As described above, data were not paired. $P < 0.05$ was considered to indicate a significant difference between groups. Statistical tests were implemented using the SigmaPlot (Systat Inc., Point Richmond, CA) or Origin (OriginLab Corp, Northampton MA) software packages.

Results

Glycine Affinity of Mutant NMDA Receptors

We chose to study NMDA receptors consisting of GluN1/GluN2A subunits, the most common subunit combination in adult hippocampus and neocortex.[39,40] We were looking for a mutation that would disrupt xenon binding while having a minimal effect on glycine binding. We decided to focus our attention on the aromatic amino acids closest to the xenon binding site identified by our modeling (fig. 1). As the amino acids being mutated are in the glycine binding site, it is important to know the glycine concentration-response of each receptor so that we can compare the mutant and wild-type receptors at equivalent glycine concentrations. We first characterized the response of the receptors to glycine in the absence of anesthetic. The concentration response curve for glycine for the wild-type and mutant GluN1/GluN2A receptors is shown in figure 2 and typical currents evoked are shown in figure 3. For the wild-type GluN1/GluN2A receptors the EC_{50}, n_H, and I_{max} for glycine were 5.7 ± 1.5 μM, 0.8 ± 0.2, and 1.05 ± 0.11 respectively (fig. 2A). Next we determined the glycine response of the GluN1 mutant receptors. The first amino acid that we mutated was tryptophan 731. We made two mutants of the tryptophan residue, GluN1(W731A)/GluN2A, where we mutated it to an alanine, and W731L, where we mutated it to a leucine. Although both of these mutants formed functional receptors, the apparent glycine affinity was greatly reduced, by more than 130-fold. The EC_{50}, n_H, and I_{max} for glycine were 744 ± 66 μM, 1.1 ± 0.1, and 1.01 ± 0.02, respectively, for the W731A mutant (fig. 2B) and 895 ± 78 μM, 0.92 ± 0.06, and 1.06 ± 0.02 respectively for the W731L mutant (fig. 2C). In both W731 mutants, the maximum currents evoked were greatly attenuated compared with wild-type receptors (fig. 3B) and were so small (approximately 30pA) that it would not have been possible to accurately determine the degree of xenon inhibition. For this reason these mutants were not investigated further. We then went on to mutate the phenylalanine 758 residue, which was first mutated to alanine. The glycine concentration-response curve for the F758A mutant is shown in figure 2D and typical currents are shown in figure 3C. The effect of the F758A mutation caused only a modest reduction (approximately sixfold) in the apparent glycine affinity compared with wild-type receptors, with the EC_{50} for glycine being 30.0 ± 2.5 μM, n_H 1.2 ± 0.1, and I_{max} 1.03 ± 0.02. We next mutated the phenylalanine 758 residue to a leucine. The concentration-response curve for glycine for the F758L

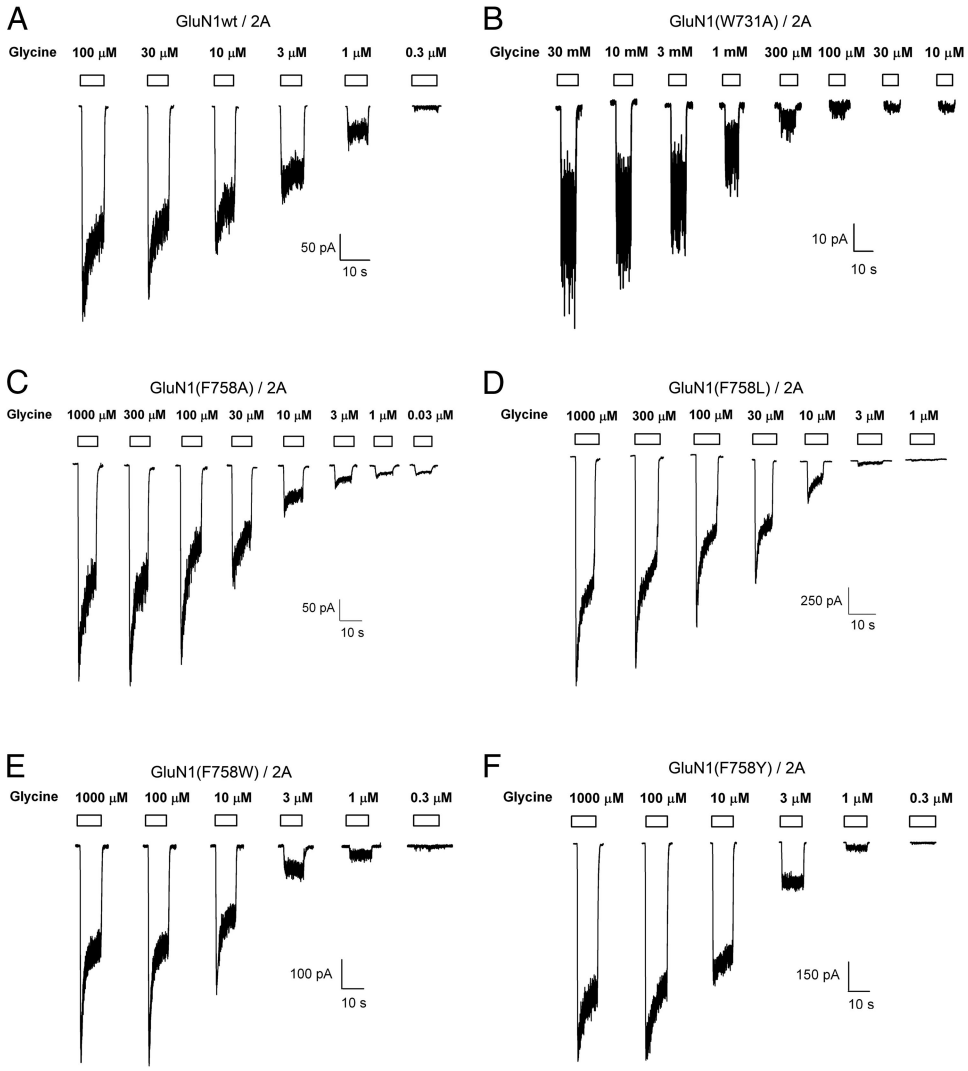

Fig. 3. Typical currents evoked by 100 μM *N*-methyl-D-aspartate at different concentrations of glycine for (*A*) wild-type GluN1/2A, (*B*) GluN1(W731A)/2A, (*C*) GluN1(F758A)/2A, (*D*) GluN1(F758L)/2A, (*E*) GluN1(F758W)/2A, and (*F*) GluN1(F758Y)/2A receptors.

mutant is shown in figure 2E and typical currents from the F758L receptors are shown in figure 3D. The effect of the F758L mutation on apparent glycine affinity was also modest with a sixfold reduction in apparent glycine affinity compared with the wild-type receptors. The EC_{50}, n_H, and I_{max} for glycine were 30.1 ± 2.6 μM, 1.1 ± 0.1, and 1.04 ± 0.03, respectively, for the F758L mutant. We then mutated phenylalanine 758 to an aromatic tryptophan residue. The glycine concentration-response curve is shown in figure 2F and typical currents for the GluN1(F758W)/GluN2A mutant are shown in figure 3E. Interestingly, the F758W receptors did not have significantly different apparent glycine affinity compared with wild-type. In the F758W mutant receptors, the EC_{50} for glycine was 5.2 ± 0.2 μM, n_H was 1.1 ± 0.1, and I_{max} was 0.99 ± 0.02 compared with an EC_{50} for glycine of 5.7 ± 1.5 μM and n_H of 0.8 ± 0.2 in the wild-type receptors. Finally we mutated phenylalanine 758 to an aromatic tyrosine residue. The glycine concentration-response curve is shown in figure 2F and typical currents for the GluN1(F758Y)/GluN2A mutant are shown in figure 3F. The F758Y receptors did not have a significantly different apparent glycine affinity compared with wild-type. In the F758Y mutant receptors, the EC_{50} for glycine was 4.8 ± 0.5 μM, n_H was 1.4 ± 0.2, and I_{max} was 0.99 ± 0.03. The F758W and F758Y mutants have an apparent glycine affinity unchanged compared with the wild-type receptors. We investigated whether the mutations affected the apparent affinity of the receptors to NMDA. We measured the NMDA dose-response of the F758W mutant and found that the EC_{50} was 44.7 ± 4.7 μM, n_H was 1.1 ± 0.1, and I_{max} was 1.00 ± 0.04 (n = 12 cells, data not shown). For the F578Y mutant the values were EC_{50} 65 ± 14 μM, n_H 1.0 ± 0.2, and I_{max} 1.11 ± 0.09 (n = 11 cells, data not shown). These values compare with values for the wild-type GluN1/2A receptor of 21 ± 3 μM, n_H 1.1 ± 0.1, and I_{max} 1.02 ± 0.05 (n = 6 cells, data not shown).

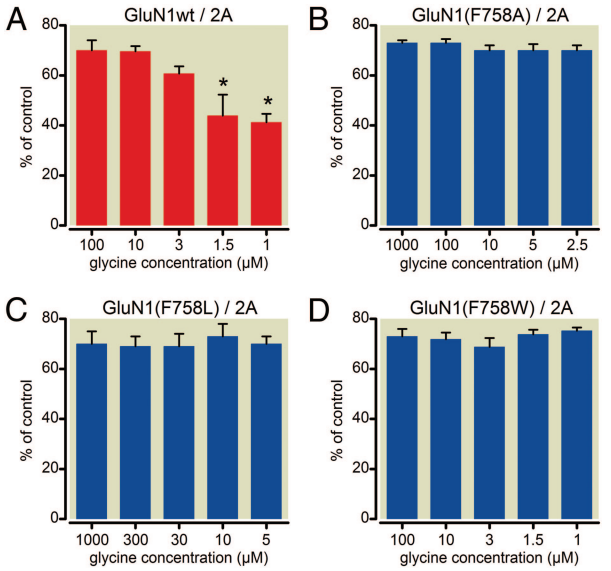

Fig. 4. Glycine dependence of xenon (80%) inhibition of wild-type and GluN1mutant receptors. (*A*) Xenon inhibition of wild-type GluN1/2A receptors increases as glycine concentration is reduced. For the GluN1 F758 mutants, glycine dependence of inhibition is abolished. (*B*) GluN1(F758A)/2A receptors, (*C*) GluN1(F758L)/2A, and (*D*) GluN1(F758W)/2A. The % of control represents the ratio of the current in the presence of anesthetic to that in the absence of anesthetic at each glycine concentration. The bars are mean values from an average of eight cells at each glycine concentration; the error bars are standard errors. $^*P < 0.025$ is significantly different from inhibition at 100 μM glycine.

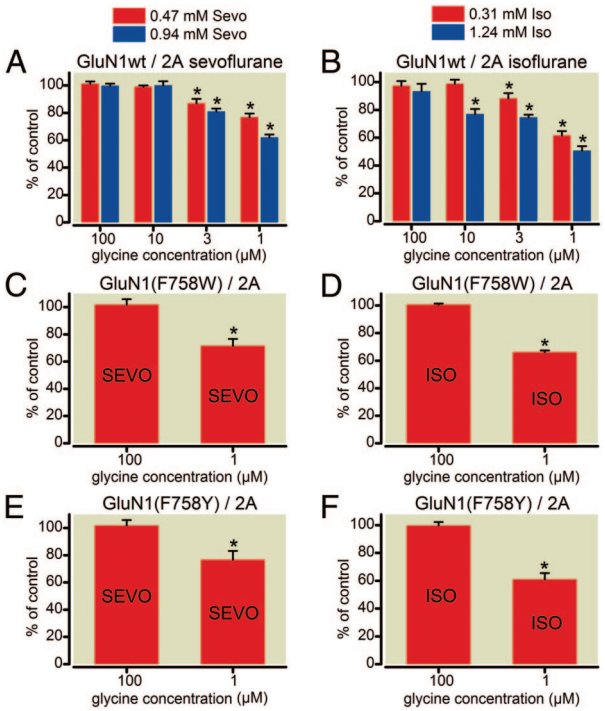

Fig. 5. Glycine dependence of sevoflurane and isoflurane inhibition. (*A*) Inhibition of wild-type GluN1/2A receptors by sevoflurane increases as glycine concentration decreases; *red bars* are inhibition by 0.47 mM sevoflurane and *blue bars* 0.94 mM sevoflurane. (*B*) Inhibition of wild-type GluN1/2A receptors by isoflurane increases as glycine concentration decreases; *red bars* are inhibition by 0.31 mM isoflurane and *blue bars* 1.24 mM isoflurane. (*C*) Inhibition of GluN1(F758W)/2A receptors by sevoflurane (0.47 mM) increases as glycine concentration decreases. (*D*) Inhibition of GluN1(F758W)/2A receptors by isoflurane (0.61 mM) increases as glycine concentration decreases. (*E*) Inhibition of GluN1(F758Y)/2A receptors by sevoflurane (0.47 mM) increases as glycine concentration decreases. (*F*) Inhibition of GluN1(F758Y)/2A receptors by isoflurane (0.61 mM) increases as glycine concentration decreases. The % of control represents the ratio of the current in the presence of anesthetic to that in the absence of anesthetic at each glycine concentration. The bars are mean values from an average of six cells at each glycine concentration; the error bars are standard errors. $^*P < 0.025$ is significantly different from inhibition at 100 μM glycine. Iso = isoflurane; Sevo = sevoflurane.

Xenon Inhibition of Wild-type and Mutant NMDA Receptors

To test whether the mutations have affected anesthetic binding at the glycine site, we performed experiments to measure the degree of xenon inhibition of the mutant receptors at different concentrations of glycine (figs. 4, 5, and 6). If xenon binds at the glycine site and competes with glycine, then the degree of inhibition by xenon will depend on the concentration of glycine, with xenon inhibiting the receptors more at low glycine concentrations. If the mutation has disrupted xenon binding then the glycine dependence of the inhibition should be attenuated or abolished. We measured the degree of xenon inhibition of the wild-type and mutant NMDA receptors at a range of glycine concentrations spanning the glycine dose-response curve for each receptor. Figure 4A shows the inhibition of the wild-type GluN1/GluN2A receptors by 80% xenon. For the wild-type receptors the degree of xenon inhibition is dependent on the glycine concentration, increasing from 29 ± 1% (n = 11) inhibition at 100 μM glycine to 59 ± 5% (n = 10) inhibition at 1 μM glycine. This increase in inhibition at a low glycine concentration of 1 μM compared with a saturating concentration of 100 μM glycine is significant ($P < 0.001$, ANOVA with Tukey *post hoc* test) and is consistent with xenon binding at the glycine site and competing with the binding of glycine. We next investigated xenon inhibition of the Phenylalanine 758 mutants. The inhibition of the F758A mutant by xenon is shown in figure 4B. Unlike the wild-type receptors, there was no glycine dependence to the xenon inhibition of F758A receptors. Xenon (80%) inhibited F758A receptors by 27 ± 1% (n = 7) at a saturating glycine concentration of 1,000 μM and by 30 ± 2% (n = 14) at a low glycine concentration of 2.5 μM. Figure 4C shows the inhibition of the F758L mutant by xenon. Similar to the phenylalanine to alanine mutant, the glycine dependence of xenon inhibition was absent in the GluN1(F758L)/GluN2A receptors, with 80% xenon inhibiting by 30 ± 5% (n = 11) at a saturating concentration of 1,000 μM glycine and by the same amount (30 ± 3%; n =

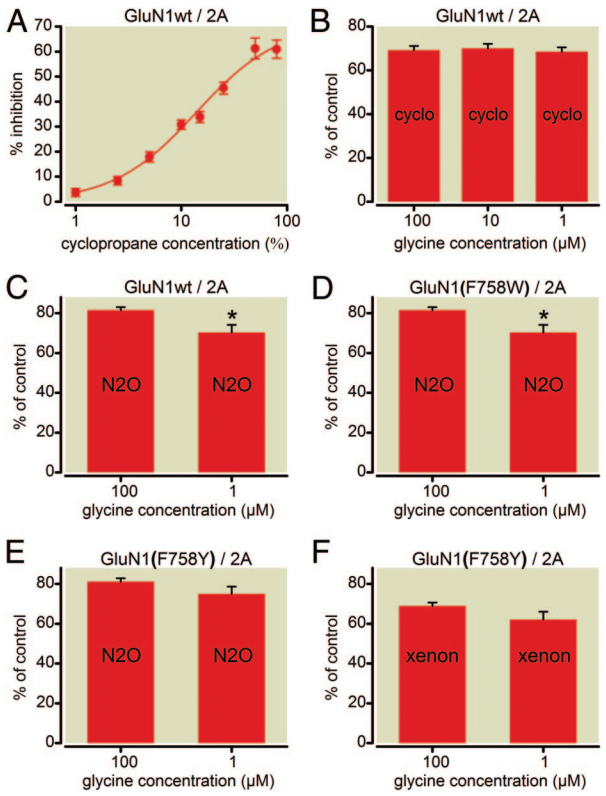

Fig. 6. Inhibition related to glycine concentration. (*A*) Cyclopropane inhibition of wild-type GluN1/2A receptors is concentration dependent. The curve is fitted to a Hill equation. The points are mean values of 6–14 cells at each cyclopropane concentration. (*B*) Cyclopropane (10%) inhibition of wild-type GluN1/2A receptors is not glycine dependent. The points are mean values of an average of 13 cells at each glycine concentration. (*C*) Inhibition of wild-type GluN1/2A receptors by N_2O (80%) is glycine dependent. (*D*) Inhibition of GluN1(F758W)/2A receptors by N_2O (80%) is glycine dependent. (*E*) Inhibition of GluN1(F758Y)/2A receptors by N_2O (80%) is not glycine dependent. (*F*) Inhibition of GluN1 (F758Y)/2A receptors by xenon (80%) is not glycine dependent. The % of control represents the ratio of the current in the presence of anesthetic to that in the absence of anesthetic at each glycine concentration. The points are mean values of an average of eight cells at each glycine concentration. *$P < 0.025$ is significantly different from inhibition at 100 μM glycine. Cyclo = cyclopropane. N_2O = nitrous oxide.

10) at a low glycine concentration of 5 μM. We then investigated the inhibition of the F758W mutant by xenon (fig. 4D). We found that for this mutant, the glycine dependence of the inhibition was also abolished, with 80% xenon inhibiting by 27 ± 3% (n = 10) at a saturating concentration of 100 μM glycine and 25 ± 1% (n = 6) at a low glycine concentration of 1 μM. Finally we investigated the xenon inhibition of the F758Y mutation (fig. 6F). We found that this mutation also eliminated the glycine dependence of the inhibition, with 80% xenon inhibiting by 31 ± 2% (n = 5) at a saturating concentration of 100 μM glycine and 38 ± 4% (n = 6) at a low glycine concentration of 1 μM.

Inhibition of Wild-type and Mutant NMDA Receptors by Sevoflurane, Isoflurane, Nitrous Oxide, and Cyclopropane

We wished to determine whether sevoflurane inhibition was glycine-dependent. At high glycine concentrations, wild-type GluN1/2A receptors were insensitive to sevoflurane. We found that sevoflurane inhibition increases significantly at low glycine concentrations compared with saturating glycine concentration. Figure 5A shows the inhibition by sevoflurane in wild-type GluN1/2A receptors; sevoflurane (0.47 mM and 0.91 mM) does not inhibit at 100 μM glycine (101 ± 2%, n = 6; and 100 ± 2% of control, n = 9), but inhibition increases significantly ($P < 0.001$ ANOVA with Tukey *post hoc* test) to 23 ± 3% (n = 10) and 28 ± 3% (n = 10) at 1 μM glycine. Similarly at high glycine, the wild-type receptors were insensitive to isoflurane. Figure 5B shows that isoflurane (0.31 mM and 1.24 mM) does not inhibit at 100 μM glycine (97 ± 3%, n = 6; and 93 ± 5% of control, n = 4) but that inhibition increases to 38 ± 2% (n = 11) and 49 ± 3% (n = 7), respectively, at 1 μM glycine. We found that nitrous oxide (80%) inhibited wild-type receptors less than the same concentration of xenon, but that there was still a glycine dependence to the inhibition (see fig. 6C). Inhibition by nitrous oxide (80%) increased significantly ($P < 0.05$) from 19.2 ± 2.6% (n = 8) at 100 μM glycine to 29.8 ± 3.7% (n = 7) at 1 μM glycine. These findings are consistent with sevoflurane, isoflurane, xenon, and nitrous oxide all competing with glycine at the glycine binding site of the GluN1 subunit. We then investigated the inhibition of wild-type GluN1/GluN2A receptors by the gaseous anesthetic cyclopropane at concentrations ranging from 1% to 80%. Figure 6A shows a concentration-response curve for inhibition of wild-type receptors by cyclopropane. Cyclopropane inhibition of the wild-type receptors is dose-dependent, increasing from 4 ± 2% to 61% ± 4% as the cyclopropane concentration is increased from 1% to 80%. The EC_{50} for cyclopropane inhibition was 14 ± 3% and n_H was 1.1 ± 0.1. We investigated whether cyclopropane inhibition of wild-type receptors was competitive with glycine. Figure 6B shows that the inhibition of the wild-type GluN1/GluN2A receptors by 10% cyclopropane is not glycine-dependent, with 10% cyclopropane inhibiting wild-type NMDA receptors by 31 ± 2% (n = 14) at 100 μM glycine and by the same amount (32 ± 2%, n = 14) at 1 μM glycine. This lack of glycine dependence indicates that cyclopropane, unlike the other anesthetics tested, does not act at the glycine site of the NMDA receptor.

Having identified two mutations (F758W and F758Y) that eliminate the binding of xenon at the glycine site without changing the apparent glycine affinity of the GluN1/2A receptors, we wished to determine whether these mutations were specific for xenon or whether they would disrupt the binding of other anesthetics acting at the glycine site. First we tested the F758W mutant with sevoflurane and isoflurane (figs. 5C and 5D). Interestingly we found that there was still

a glycine dependence to the inhibition of the F758W mutant receptors. At 100 μM glycine, the receptors were insensitive to 0.47 mM sevoflurane (102 ± 4% of control, n = 5) but inhibition increased significantly ($P < 0.001$) to 28.4 ± 5.1% (n = 4) at 1 μM glycine. Similarly, the F758W mutant was insensitive to isoflurane (0.62 mM) at 100 μM glycine (100 ± 1% of control, n = 5) but inhibition increased significantly ($P < 0.001$) to 33.9 ± 1.3% (n = 4) at 1 μM glycine. The behavior of sevoflurane and isoflurane in the F758Y mutant was similar (figs. 5E and 5F). At 100 μM glycine the F758Y mutant was insensitive to both sevoflurane (0.47 mM) and isoflurane (0.62 mM), with values of 102 ± 4% (n = 4) and 100 ± 2% (n = 5) of control, respectively, but inhibition increased significantly ($P < 0.001$) to 23.4 ± 6.6% (n = 5) and 39.0 ± 4.5% (n = 6), respectively, at 1 μM glycine. Finally we investigated the inhibition of the F758W and F758Y mutants by nitrous oxide (figs. 6D and 6E). We found that for the F758W mutant there was a significant difference ($P < 0.05$) in the inhibition by nitrous oxide (80%) at 100 μM glycine (18.5 ± 1.5% inhibition; n = 8), compared with 1 μM glycine (29.8 ± 3.9% inhibition; n = 7). However, with the F758Y mutant, there was no significant difference in the inhibition by nitrous oxide (80%) at high glycine (100 μM) compared with low glycine (1 μM).

Discussion

Effect of GluN1 Mutations on Glycine Binding

We concentrated our mutational studies on amino acids likely to interact with xenon, but less likely to be critical for glycine binding. X-ray crystallographic studies have characterized xenon binding sites on a number of proteins.[41–46] One feature that these xenon binding cavities have in common is that they contain aromatic amino acids in close proximity to xenon atoms. The earliest study by Schoenborn *et al.*[44] identified a phenylalanine and a histidine within 5 Å of the xenon site in myoglobin. More recent studies have identified aromatic phenylalanine residues close to xenon atoms in urate oxidase, β-endotoxin CytB, and elastase.[41,45] The binding site on the NMDA receptor predicted by our modeling has xenon atoms coordinated by three aromatic residues, W731, F758, and F484 (fig. 1). A modeling study by Seto *et al.*[47] identified a xenon site on human serum albumin where xenon is coordinated by three phenylalanines. Interestingly, a strikingly similar arrangement with xenon coordinated by three aromatic phenylalanine residues has been observed in the X-ray crystallographic structure of xenon bound to PsbO protein (a component of the Photosystem II complex).[46]

Our mutational strategy focused on these aromatic residues to test the hypothesis that they are important for xenon binding to the NMDA receptor. A study by Hirai *et al.*[48] reported that mutating phenylalanine 758 to alanine had little effect on glycine binding. When we mutated phenylalanine 758 to nonaromatic alanine or leucine residues, we found that this had only a modest effect on apparent glycine affinity. The finding that the F758A and F758L mutants had reduced glycine sensitivity suggests that glycine interacts with the aromatic ring of phenylalanine. In order to test this prediction we mutated phenylalanine 758 to the aromatic amino acids, tryptophan and tyrosine. We found that the apparent glycine affinity of the F758W and F758Y mutants was unchanged compared with wild-type receptors. In terms of sensitivity to glycine, the F758W and F758Y mutations are silent, consistent with the aromatic ring of tryptophan and tyrosine making similar interactions with glycine, as does the aromatic ring of phenylalanine in the wild-type receptors. Nevertheless, an alternative explanation is that the similar glycine affinity of these mutants might be explained by the similar molecular volumes of the aromatic residues compared with the smaller leucine and alanine.

Effect of GluN1 Mutations on Xenon Binding

In order to test whether the mutations have disrupted xenon binding at the glycine site, we measured the degree of xenon inhibition at different glycine concentrations. Because the xenon inhibition is competitive with glycine, if a suitably wide range of concentrations is not used, there is the possibility of being misled into thinking the mutation has eliminated xenon binding when, in fact, all it has done is change the glycine affinity of the receptor. We previously showed that the F639A mutation in the second transmembrane region of the NR1 subunit of the NMDA receptor that attenuates xenon inhibition of NMDA receptors[49] simply changes the affinity of the receptor to glycine.[11] The F758 mutations either had no effect or only a very modest effect on glycine affinity. Although the inhibition of wild-type receptors increased at low glycine concentrations (fig. 4A), we found that in the F758A, F758L, F758W, and F758Y mutants, there was no glycine dependence to the inhibition by xenon consistent with these mutations preventing xenon from binding at the glycine site. This is consistent with our hypothesis that xenon prefers to interact with aromatic residues. In terms of their unchanged glycine affinity, the F758W and F758Y mutants behave like the wild-type receptor. However, unlike the wild-type receptors, the xenon inhibition of the F758W and F758Y mutants was not glycine-dependent. This indicates that xenon cannot interact with tryptophan or tyrosine in the same manner as it does with phenylalanine. The reason for xenon's preference for phenylalanine may be because of the particular stoichiometry of its aromatic ring and/or differences in molecular volume and polarity compared with the larger tryptophan or polar tyrosine residues.

Effect of GluN1 Mutations on Sevoflurane, Isoflurane, and Nitrous Oxide Binding. Sevoflurane and isoflurane inhibition of wild-type GluN1/2A receptors appears to be entirely competitive, in contrast to the inhibition by xenon and nitrous oxide that exhibit a residual inhibition at saturating glycine concentrations, presumably representing an action at

the noncompetitive site we previously observed.[11] Having shown that the F758W and F758Y mutations eliminate the competitive inhibition by xenon at the glycine site without changing the apparent glycine affinity, we determined whether these mutations would prevent the binding of other anesthetics that act at the GluN1 glycine-binding site. Surprisingly, we found that the glycine dependence of inhibition by sevoflurane and isoflurane was unaffected, indicating that phenylalanine 758 is necessary for xenon to bind at the glycine site but that, unlike xenon, sevoflurane and isoflurane can interact equally well with tryptophan and tyrosine residues in the mutant receptors. In the case of nitrous oxide, although it binds rather weakly, it appears that in the F758W mutant it can bind at the glycine site, but in the F758Y mutant it cannot bind at the glycine site.

Interactions of Xenon at the Glycine Binding Site and Implications for *In Vivo* Xenon Anesthesia and Neuroprotection Studies. The idea that general anesthetics might act by competitive inhibition of receptors was first suggested more than 25 yr ago,[50] when little was known about the effects of anesthetics on central nervous system receptors. However, studies on model systems supported the idea that anesthetics bind in protein pockets of circumscribed size.[51,52] That xenon can bind in preformed cavities in proteins had been known since the pioneering x-ray crystallographic work of Schoenborn *et al.*[44] on myoglobin. Trudell *et al.*[53,54] concluded the myoglobin site is a good model for the sites underlying general anesthesia. Neuroprotective NMDA receptor glycine site antagonists, such as gavestinel, have been shown to be well tolerated in patients,[55] and some of these glycine-site antagonists have also been shown to have antitoxic and anticonvulsant activity in models of cocaine withdrawal.[56,57] One of the reasons for our interest in xenon's molecular interactions at the glycine site is the idea that we could identify a silent or near-silent mutation that prevents xenon binding without affecting glycine affinity. As xenon and glycine are structurally distinct, it is plausible that they occupy partially overlapping sites and interact with different amino acids at the glycine site. Figure 7A shows the position of the xenon atoms in the glycine site. The aromatic rings of F758, F484, and W731 coordinate the xenon atoms in the binding site, most likely making London dispersion or induced-dipole interactions with the xenon atoms. Xenon appears to stabilize the open conformation of the ligand binding domain of the GluN1 subunit, in a similar manner to the competitive antagonist dichloro-kynurenic acid.[58] Figure 7B shows that when glycine binds the conformation changes and W731 moves to maintain close interaction with glycine (F484 also moves to maintain contact with glycine, but F758 moves only slightly, undergoing a rotation of the plane of the aromatic ring). Our studies confirm the importance of aromatic amino acids in the binding of glycine, showing that mutating phenylalanine 758 to nonaromatic residues reduces glycine binding but mutating it to aromatic tryptophan or tyrosine leaves the glycine affinity unchanged. However, mutating

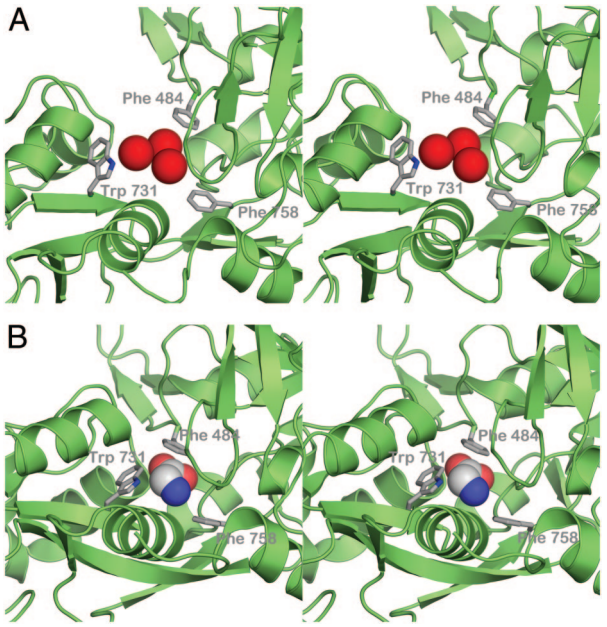

Fig. 7. Interactions with aromatic amino acids are important for xenon and glycine binding. Stereo views showing (*A*) Xenon atoms (*red spheres*) are predicted to bind in the *N*-methyl-D-aspartate receptor glycine site and stabilize the open conformation of the ligand binding domain. Interactions with phenylalanine 758 are important for xenon binding. Mutating F758 to a tryptophan, leucine, or alanine eliminates xenon binding. (*B*) With glycine bound the ligand binding domain adopts a closed conformation. The W731 moves as the cleft closes and maintains close contact with the glycine molecule. Mutation of W731 to alanine or leucine greatly reduces glycine binding. The F758 residue is also involved in glycine binding; mutating it to an aromatic tryptophan has no effect on glycine binding but eliminates xenon binding. Crystallographic structures (1PBQ and 1PB7)[51] were obtained from the Protein Data Bank. The positions of xenon atoms shown were predicted using Grand Canonical Monte Carlo modeling simulations.[11] Images were created using the PyMOL Molecular Graphics System (see http://www.pymol.org; accessed May 21, 2012). Phe 484 = phenylalanine 484; Phe 758 = phenylalanine 758; Trp 731 = tryptophan 731.

phenylalanine 758 to a tryptophan or tyrosine eliminated xenon binding. It appears that the xenon atoms make particularly favorable interactions with the aromatic ring of the phenylalanine, but that xenon cannot make equivalent interactions with the aromatic ring of tryptophan or tyrosine. The F758W and F758Y mutants have the same glycine sensitivity as the wild-type receptor but can no longer bind xenon in the glycine site, opening up the possibility of making knock-in animals with NMDA receptors that should behave normally at physiologic glycine concentrations, but with reduced sensitivity to the anesthetic and neuroprotective effects of xenon. There are two caveats. Although the F758W and F758Y mutants are silent in terms of glycine affinity, they both show a small decrease in affinity for NMDA. Second, the mutations eliminate the competitive component of xenon inhibition, but there is still a residual noncompetitive component

of the inhibition. There is *in vitro* evidence that the competitive inhibition by xenon at the NMDA receptor glycine site mediates xenon neuroprotection. It would therefore be of great interest to be able to abolish the competitive component of the inhibition *in vivo*. Xenon has been shown to affect only a limited number of targets, notably NMDA receptors, 2-pore domain potassium channels, and the adenosine triphosphate-sensitive potassium channel.[9,28,29,59,60] Knock-in mice with NMDA receptors with the F758W and F758Y mutations will be of great use in dissecting out the roles played by the NMDA receptor in mediating the anesthetic, analgesic and neuroprotective properties of xenon.

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