

# Mutations M287L and Q266I in the Glycine Receptor $\alpha$ 1 Subunit Change Sensitivity to Volatile Anesthetics in Oocytes and Neurons, but Not the Minimal Alveolar Concentration in Knockin Mice

Cecilia M. Borghese, Ph.D.,\* Wei Xiong, Ph.D.,† S. Irene Oh, B.S.,‡ Angel Ho, B.S.,§ S. John Mihic, Ph.D.,|| Li Zhang, M.D.,# David M. Lovinger, Ph.D.,\*\* Gregg E. Homanics, Ph.D.,†† Edmond I. Eger 2nd, M.D.,‡‡ R. Adron Harris, Ph.D.§§

## ABSTRACT

**Background:** Volatile anesthetics (VAs) alter the function of key central nervous system proteins but it is not clear which, if any, of these targets mediates the immobility produced by VAs in the face of noxious stimulation. A leading candidate is the glycine receptor, a ligand-gated ion channel important for spinal physiology. VAs variously enhance such function, and blockade of spinal glycine receptors with strychnine affects the minimal alveolar concentration (an anesthetic  $EC_{50}$ ) in proportion to the degree of enhancement.

**Methods:** We produced single amino acid mutations into the glycine receptor  $\alpha$ 1 subunit that increased (M287L, third transmembrane region) or decreased (Q266I, second transmembrane region) sensitivity to isoflurane in recombinant receptors, and introduced such receptors into mice. The resulting knockin mice presented impaired glycinergic trans-

### What We Already Know about This Topic

- Inhibitory spinal glycine receptor function is enhanced by volatile anesthetics, making this a leading candidate for their immobilizing effect
- Point mutations in the  $\alpha$ 1 subunit of glycine receptors have been identified that increase or decrease receptor potentiation by volatile anesthetics

### What This Article Tells Us That Is New

- Mice harboring specific mutations in their glycine receptors that increased or decreased potentiation by volatile anesthetic *in vitro* did not have significantly altered changes in anesthetic potency *in vivo*
- These findings indicate that this glycine receptor does not mediate anesthetic immobility, and that other targets must be considered

\* Research Associate, || Associate Professor, §§ Professor, The University of Texas at Austin, Waggoner Center for Alcohol and Addiction Research, Austin, Texas. † Research Fellow, # Medical Officer, \*\* Laboratory Chief, Laboratory for Integrative Neuroscience, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Rockville, Maryland. ‡ Staff Research Associate, ‡‡ Professor Emeritus, Department of Anesthesia and Perioperative Care, University of California San Francisco, San Francisco, California. § Staff Research Assistant, Diabetes Center, University of California San Francisco. †† Professor, Departments of Anesthesiology and Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, Pennsylvania.

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Address correspondence to Dr. Harris: The University of Texas at Austin, Waggoner Center for Alcohol and Addiction Research, 1 University Station A4800, Austin, Texas 78712-0159. harris@mail.utexas.edu. Information on purchasing reprints may be found at [www.anesthesiology.org](http://www.anesthesiology.org) or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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mission, but heterozygous animals survived to adulthood, and we determined the effect of isoflurane on glycine-evoked responses of brainstem neurons from the knockin mice, and the minimal alveolar concentration for isoflurane and other VAs in the immature and mature knockin mice.

**Results:** Studies of glycine-evoked currents in brainstem neurons from knockin mice confirmed the changes seen with recombinant receptors. No increases in the minimal alveolar concentration were found in knockin mice, but the minimal alveolar concentration for isoflurane and enflurane (but not halothane) decreased in 2-week-old Q266I mice. This change is opposite to the one expected for a mutation that decreases the sensitivity to volatile anesthetics.

**Conclusion:** Taken together, these results indicate that glycine receptors containing the  $\alpha$ 1 subunit are not likely to be crucial for the action of isoflurane and other VAs.

**A** PRESENT consensus suggests that volatile anesthetics (VAs) bind to proteins to produce their effects, including immobility.<sup>1</sup> The key question is, "Which proteins?" The list of candidates is short.<sup>1-3</sup> A top candidate is the glycine receptor (GlyR).

Evidence for the GlyR as a target for VA includes their localization and sensitivity to VAs. The RNAs encoding the GlyR subunits are mostly in the spinal cord and brainstem,<sup>4</sup>

and the spinal cord mediates the immobility effect of VA.<sup>5</sup> Moreover, anesthetic concentrations of VAs substantially enhance inhibitory currents through the GlyR, both in heterologous systems<sup>6–8</sup> and neurons.<sup>8</sup> More direct evidence arises from the use of strychnine, a competitive GlyR-specific antagonist, both at the neuronal and whole animal level. The application of halothane to decerebrate rats depressed the spinal cord sensory neuronal activity to noxious stimuli; the concurrent application of strychnine partially reversed the halothane-induced depression.<sup>9</sup> Sevoflurane reduced spontaneous action potential firing of ventral horn interneurons in cultured spinal cord tissue slices; application of strychnine decreased the sevoflurane effect.<sup>10</sup> Intrathecal administration of strychnine increased the minimal alveolar concentration (MAC) of isoflurane.<sup>11</sup> The magnitude of GlyR potentiation expressed in oocytes varies according to the VA used.<sup>12</sup> Intrathecal administration of strychnine to rats anesthetized with halothane, isoflurane, or cyclopropane increased MAC in proportion to the enhancement of GlyRs observed *in vitro*.<sup>13</sup> Furthermore, intrathecal glycine reduced the isoflurane MAC in rats.<sup>14</sup> These results are compatible with the hypothesis that GlyRs partly mediate the capacity of VAs to produce immobility.

Construction of chimeric glycine- $\gamma$ -aminobutyric acid type A receptors (GABA<sub>A</sub>-Rs) allowed the identification of transmembrane amino acids crucial to the VAs' action, namely S267 and A288 in the second and third GlyR transmembrane domains.<sup>15</sup> Mutation of S267 to cysteine and subsequent labeling with thiol-specific reagents blocked the VA potentiation of GlyRs.<sup>16,17</sup> Furthermore, a crystal structure of a prokaryotic protein closely related to GlyR was obtained in the presence of VAs and showed selective binding of the anesthetics to the regions proposed in the earlier studies of GlyR.<sup>18</sup>

The identification of a mutation in a receptor that renders it insensitive or hypersensitive to modulation by VAs would allow construction of a knockin mouse that could be tested for changes in the sensitivity to VAs. This approach was successful in showing that specific GABA<sub>A</sub>-Rs are responsible for at least some of the actions of certain injectable anesthetics.<sup>19,20</sup> In our studies of alcohol action, we found two mutations in the  $\alpha$ 1 GlyR subunit, one in the second (Q266I), and another in the third transmembrane domain (M287L) that decreased ethanol modulation of GlyR.<sup>21</sup> Mutant mice were constructed in which each of these mutant  $\alpha$ 1 GlyR subunits replaced the endogenous GlyR subunit (knockin mice).<sup>21</sup> The goal of the present study was to determine if these mutations altered the action of VA on GlyR and if such alterations would affect the immobility produced by VA. This was accomplished by studying glycine responses of recombinant receptors expressed in *Xenopus* oocytes and of neurons isolated from brainstem of wild-type and knockin mice, and the immobilizing potency (the MAC of anesthetic that abolished movement in response to noxious stimulation

in 50% of test subjects) of anesthetics in wild-type and knockin mice.

## Materials and Methods

Knockin mouse production and the electrophysiological techniques were described in detail in Borghese *et al.*<sup>21</sup> We briefly describe them here, and add pertinent details. The experimental work on animals conformed to the guidelines laid out in the Guide for the Care and Use of Laboratory Animals, and approval of the corresponding institutional Animal Care Committees was obtained.

### Electrophysiology in *Xenopus* Oocytes

Chloroform was purchased from Sigma-Aldrich (Saint Louis, MO), and isoflurane was purchased from Marsam Pharmaceuticals (Cherry Hill, NJ). Isoflurane and chloroform solutions were prepared in buffer immediately before application.

Manually isolated oocytes from *Xenopus laevis* frogs were injected in the nuclei with complementary DNA encoding wild-type and mutated human  $\alpha$ 1 GlyR subunits. After 1–5 days, recordings were carried out using the whole cell two-electrode voltage clamp configuration. All drugs were applied by bath-perfusion and solutions were prepared the day of the experiment. To study the chloroform (1 mM) and isoflurane (0.3 mM) modulation of glycine currents, the glycine concentration equivalent to 5% of the maximal glycine-evoked current (EC<sub>5</sub>) was determined after applying the glycine concentration that produced maximal current (3 mM for wild-type, 10 mM for mutant). Glycine was removed with a washout of 5 min between all applications, except after application of maximal glycine concentrations (15 min). After two applications of EC<sub>5</sub> glycine, each of the modulators was preapplied for 1 min and then coapplied with glycine for 30 s. EC<sub>5</sub> glycine was applied between coapplication of glycine and modulator. The concentrations of isoflurane and chloroform were chosen as equivalent to 1 x MAC.<sup>22</sup> All experiments shown include data obtained from oocytes taken from at least two different frogs, and oocytes that presented a maximal current more than 20  $\mu$ A were not included in the data collection.

### Knockin Mouse Production

Breeding colonies were established in the Lovinger and Eger laboratories, and mice were produced by heterozygous breeding, resulting in litters with wild-type, heterozygous mutant, and homozygous mutant mice. Homozygous mice for either mutation appear normal at birth, but display tremors and reduced growth by 2 weeks of age with lethality occurring by 2–3 weeks of age for Q266I mice.<sup>21</sup> Thus, studies with mice older than 3 weeks of age were carried out with only heterozygous (*vs.* wild-type) mice.

### Isolation of Neurons and Electrophysiological Recording

Postnatal day 14–21 mice were sacrificed by decapitation during halothane (Sigma-Aldrich) anesthesia and their

brains were rapidly removed and placed in ice-cold buffer. Brainstem slices were cut using a vibrating blade microtome (Leica VT1200S; Leica Microsystems, Wetzlar, Germany), incubated with pronase, and then with thermolysin. The slices were then transferred to a culture dish, and mildly triturated using pipettes to dissociate single neurons.

Membrane currents were recorded in the whole cell configuration at 20–22°C. Cells were held at –60 mV unless otherwise indicated. Data were acquired using pClamp 9.2 software (Molecular Devices, Sunnyvale, CA). Isoflurane was purchased from Baxter (Deerfield, IL). Isoflurane solution was prepared in the extracellular recording solution immediately before beginning experiments each day, and kept in a sealed bottle. Solutions were applied through 3-barrel square glass tubing (Warner Instrument, Hamden, CT) with a tip diameter of approximately 700  $\mu\text{m}$ , using the Warner Fast-Step stepper-motor driven system. The solution exchange time constants were approximately 4 ms for an open pipette tip and 4–12 ms for whole cell recording. The solution in the wells feeding into the solution exchange system was changed after recording from 1 or 2 cells to replenish isoflurane in the line. The drug application system was sealed at all times except when applying isoflurane to a cell, to reduce loss of the volatile anesthetic. In neurons that were recorded for at least 30 min, the isoflurane-induced potentiation did not change over time, indicating limited, if any, loss of the volatile anesthetic from our system.

### MAC Determination

Animals were held in rooms with 12-h on-off light cycles. The offspring were observed daily for signs of central nervous system irritability, namely tremors. When tremors were observed in a given litter, the capacity of VA to induce immobility in 50% of individual littermates in the face of noxious stimulation (*i.e.*, MAC) was tested within 2–4 days for all mice using techniques described previously.<sup>23</sup> Rectal temperatures were monitored and usually maintained between 35.5 and 38.5°C. Anesthetic concentrations were monitored with an infrared analyzer (Datascop, Helsinki, Finland). The initial anesthetic concentration was imposed for a 40-min equilibration period. A low initial target concentration was chosen to ensure movement in response to up to a 1-min application of an alligator clip to the base of the tail. We then increased the anesthetic concentration in steps of 15–20% of the preceding concentration, holding each step for a minimum of 20 to 30 min before again applying the alligator clip. This continued until all mice ceased moving in response to the stimulation. At the end of each step (after application of the tail clip), a sample of gas was taken for anesthetic analysis by gas chromatography. On completion of these studies, snips of tail tips were sent to the Harris laboratory for genotyping. Two groups that were not genotyped (2-week-old Q266I mice tested with enflurane and halothane) were grouped according to the phenotype: tremoring (homozygous) and non-tremoring (wild-type and heterozygous)

mice. Mice then were returned to their home cages and in 2 days tested with another anesthetic. The test anesthetics were isoflurane and enflurane for M287L mice, and isoflurane, halothane, and enflurane for Q266I mice. Surviving mice (*i.e.*, wild-type and heterozygous mice) then were allowed to grow to 8–12 weeks of age and were tested for MAC for isoflurane, enflurane, and/or cyclopropane.

The gas chromatograph (Gow-Mac 750 flame ionization detector gas chromatograph; Gow-Mac Instrument Corp., Bridgewater, NJ) was calibrated with secondary standards from tanks containing volatile anesthetic or from concentrations obtained by serial dilution from 100% cyclopropane. MAC for each mouse was calculated as the mean of the concentrations that bracketed movement-nonmovement. The group MAC and standard errors were calculated from these individual values.

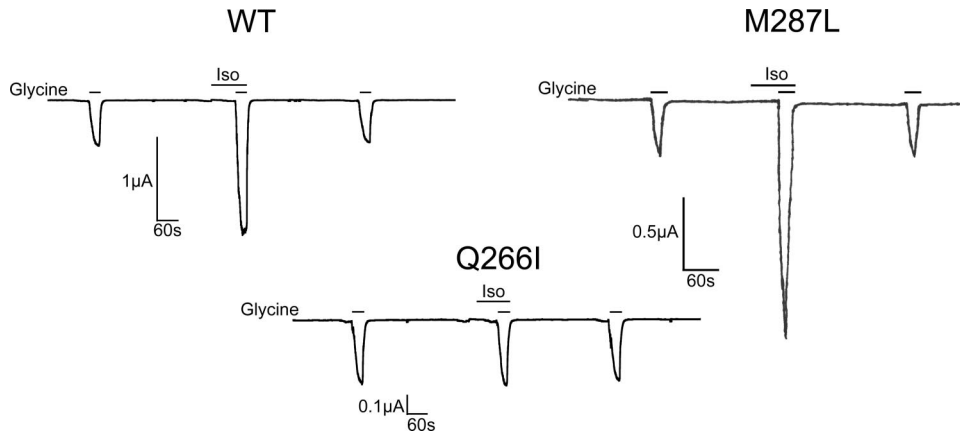
### Statistical Analysis

Prism (GraphPad Software Inc., La Jolla, CA) was used to conduct the statistical analysis. In all cases, pooled data are presented as mean  $\pm$  SEM. Differences were assessed with two-tailed Student *t* test in the oocyte recordings. In the neuronal recordings, we used one-way ANOVA followed by Dunnett multiple comparison test, where the objective is to identify groups whose means are significantly different from the mean of a selected “reference group,” in our case, the wild-type mice. We accepted  $P < 0.05$  as significant.

### Results

We first asked if the M287L or Q266I mutations altered the sensitivity of GlyR to modulation by anesthetics. The application of isoflurane or chloroform produced different effects on the glycine-evoked currents through the mutant receptors expressed in *Xenopus* oocytes (representative tracings in the presence of isoflurane in fig. 1). When applied to the  $\alpha 1$ (M287L) GlyR, 0.3 mM isoflurane showed an increased potentiation compared with  $\alpha 1$  wild-type, whereas the chloroform (1 mM) potentiation was unchanged (fig. 2A). In line with the observed lack of alcohol effects on Q266I,<sup>21</sup> isoflurane potentiation was almost abolished in  $\alpha 1$ (Q266I) GlyR, and chloroform potentiation was severely decreased (fig. 2B).

Previous studies had shown that although the glycine affinity is not modified in neurons from homozygous mice, the maximal glycine current is decreased in homozygous mice.<sup>21</sup> We studied the isoflurane effect on submaximal (10  $\mu\text{M}$ ) glycine-activated currents in isolated neurons from the knockin mice (fig. 3 shows representative tracings). The GlyR modulation by isoflurane (1 mM) in isolated neurons from knockin mice showed changes that were similar to those observed in *Xenopus* oocytes. In neurons from M287L mice, the isoflurane effect on glycine-evoked currents was increased, and this depended on gene “dosage”: it was greater in neurons from homozygous than in heterozygous mice, and greater in heterozygous than in wild-type mice (figs. 3A and fig. 4). In neurons from Q266I mice, the isoflurane



**Fig. 1.** Representative tracings of isoflurane (0.3 mM) effect on currents induced by glycine (with a concentration that induced a current that is 5% of the maximal current, effective concentration 5, or  $EC_{50}$ ) in homomeric  $\alpha 1$  wild-type,  $\alpha 1$  (M287L) and  $\alpha 1$  (Q266I) GlyRs expressed in *Xenopus* oocytes. Data from multiple recordings are presented in fig. 2. WT = wild-type.

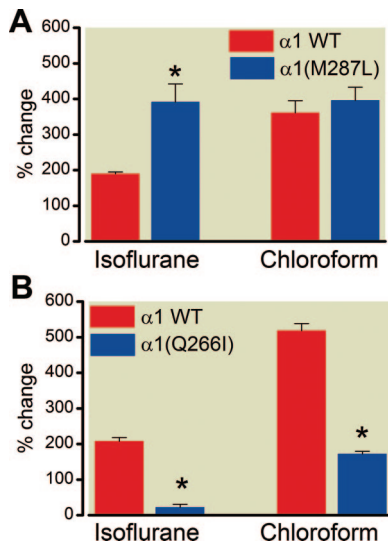
effect was decreased according to the presence of the mutation, and essentially absent in homozygous and smaller in heterozygous than in wild-type (figs. 3B and 4B).

Anesthetic sensitivity *in vivo* was evaluated by determining the MAC values for M287L and Q266I mice of different ages: 14–21 days of age and adult (more than 8 weeks old), in homozygous, heterozygous, and wild-type mice (tables 1 and 2). Cyclopropane was tested as a negative control; it has a small effect on GlyRs expressed in heterologous systems.<sup>12</sup> The MAC values for isoflurane and enflurane were unchanged in M287L knockin mice compared with wild-type mice. In adult Q266I heterozygous mice, the MAC values

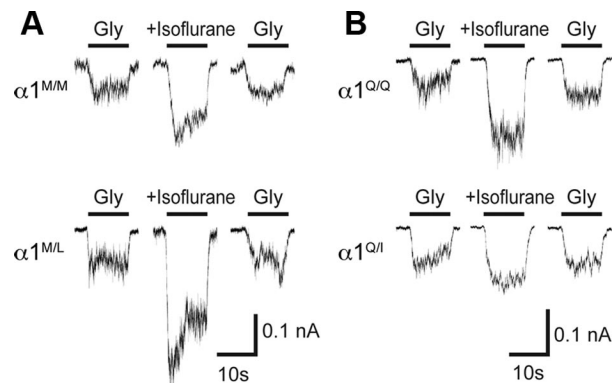
for isoflurane and cyclopropane were not modified compared with wild-type mice. In 2-week-old Q266I mice, tremoring (homozygous) mice showed a decrease in isoflurane and enflurane MAC values compared with non-tremoring (heterozygous and wild-type), whereas halothane showed no differences between these two groups.

## Discussion

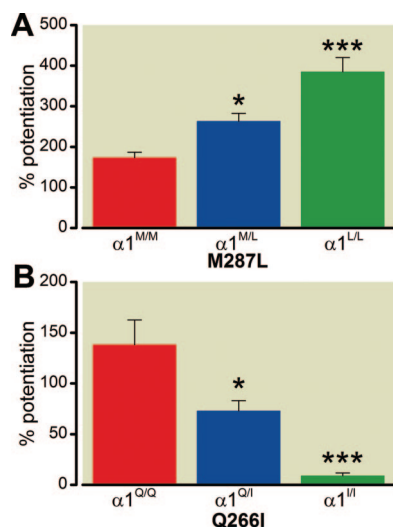
Defining protein sites of VA action has proven remarkably difficult.<sup>1,3</sup> It is useful to consider the criteria that must be satisfied for a protein to be considered a plausible target for VA action. VAs have many similarities to ethanol (small molecules, low potency, multiple potential sites of action), and ethanol might be considered as a VA. Four criteria were proposed for defining a molecular target of alcohol action,<sup>24</sup> and they are equally applicable to other VA. In brief, they are: (1) function of the protein must be altered by pharmacology



**Fig. 2.** Volatile anesthetics' effect on glycine responses in homomeric  $\alpha 1$  wild-type (WT),  $\alpha 1$  (M287L), and  $\alpha 1$  (Q266I) expressed in *Xenopus* oocytes. Isoflurane (0.3 mM) and chloroform (1 mM) potentiation of glycine responses (with a concentration that induced a current that is 5% of the maximal current, effective concentration 5, or  $EC_{50}$ ) in (A)  $\alpha 1$  wild-type ( $n = 6$ ) and  $\alpha 1$  (M287L) ( $n = 5$ ), and (B)  $\alpha 1$  wild-type ( $n = 4$ ) and  $\alpha 1$  (Q266I) ( $n = 4$ ). Values are means  $\pm$  SEM; \* $P < 0.01$  versus corresponding wild-type, Student  $t$  test analysis.



**Fig. 3.** Representative tracings of isoflurane (1 mM) on (10  $\mu$ M) glycine-evoked currents in neurons from wild-type or heterozygous mutant mice. (A) Currents in neurons from  $\alpha 1$  (M287L) mice:  $\alpha 1^{M/M}$ , wild-type (upper panel) and  $\alpha 1^{M/L}$ , heterozygous (lower panel). (B) Currents in neurons from  $\alpha 1$  (Q266I) mice:  $\alpha 1^{Q/Q}$ , wild-type (upper panel) and  $\alpha 1^{Q/I}$ , heterozygous (lower panel). Data from multiple tracings are presented in Fig. 4. Gly = glycine.



**Fig. 4.** Isoflurane (1 mM) effect on glycine-evoked (10  $\mu$ M) currents in isolated neurons from wild-type and knockin mice. (A) Neurons from M287L knockin mice:  $\alpha 1^{M/M}$ , wild-type;  $\alpha 1^{M/L}$ , heterozygous;  $\alpha 1^{L/L}$ , homozygous. (B) Neurons from Q266I knockin mice:  $\alpha 1^{Q/Q}$ , wild-type;  $\alpha 1^{Q/I}$ , heterozygous;  $\alpha 1^{I/I}$ , homozygous. Values are means  $\pm$  SEM; n = 6 for each group; \* $P < 0.05$ , \*\*\* $P < 0.001$  versus corresponding wild-type, one-way ANOVA, Dunnett *post hoc* test.

ically reasonable concentrations of the drug; (2) mutation of specific amino acids should provide evidence of a binding site; (3) manipulation of the protein *in vivo* (e.g., knockin mice) should produce expected changes in behavioral actions of the drug; and (4) physical structural studies should demonstrate the presence of the drug at the proposed sites in the protein. Both GABA<sub>A</sub>-Rs and GlyRs fulfill several of these criteria. Pharmacological concentrations of most (but not all) VAs enhance the function of these receptors,<sup>2</sup> and extensive mutation and thiol-labeling studies provide evidence for a binding site within the transmembrane regions.<sup>15–17</sup> In addition, a recent structural study of GLIC, a ligand-gated ion channel closely related to GlyR and GABA<sub>A</sub>-R, shows the binding of anesthetics in the site proposed based on mutations.<sup>18</sup> The requirement to introduce a VA-resistant protein into a mouse and reduce the VA immobilizing effect *in vivo* has proven to be the most difficult criterion to satisfy. Thus far, knockin mice constructed with VA resistant GABA<sub>A</sub>-Rs have not shown any increase in MAC.<sup>25–28</sup> This is in contrast to injectable anesthetics such as propofol and etomidate,

where a knockin mouse with a GABA<sub>A</sub>-R resistant subunit [ $\beta 3(N265M)$ ] displayed remarkable resistance to anesthetics<sup>20</sup> with little change in the action of VAs.<sup>28</sup> With the lack of support for GABA<sub>A</sub>-R as a mechanism for the immobility produced by VA, GlyR emerged as one of the “last best hopes,”<sup>29</sup> making the construction and study of knockin mice for this receptor a critical step. To accomplish this, we constructed single amino acid mutations in the  $\alpha 1$  GlyR subunit that would increase or decrease the actions of VA and constructed knockin mice with each of the mutations.<sup>21</sup> We chose the  $\alpha 1$  subunit because there are two kinds of GlyR subunits,  $\alpha$  ( $\alpha 1$ –4) and  $\beta$ . The  $\alpha 2$  subunit predominates perinatally, whereas the heteromeric  $\alpha 1\beta$  GlyRs replace them during the first 3 weeks of the postnatal stage, and are the main GlyR present in spinal cord and brainstem in adulthood.<sup>4</sup> The  $\alpha 1\beta$  GlyR is difficult to express in *Xenopus* oocytes, so we decided to use the homomeric  $\alpha 1$  GlyR in our heterologous experiments.

The striking finding from the present experiments is that introduction of GlyR  $\alpha 1$  subunits with either enhanced or decreased modulation by VA into mice has almost no effect on the ability of these agents to produce immobility, and none in the direction expected if the immobility effect was mediated by enhancement of currents mediated by GlyRs. Electrophysiological studies of recombinant receptors as well as neurons from the mutant mice showed that the M287L and Q266I mutations in transmembrane regions of the GlyR consistently altered anesthetic actions, yet the MAC for immobility was not altered for any anesthetic tested, with two exceptions. The 2-week-old homozygous Q266I mice showed a small decrease in the MAC value for isoflurane and enflurane (17% and 9%, respectively). If the immobility effect were in part mediated by GlyRs, the presence of a mutation in the GlyRs that made them insensitive to a VA would be predicted to increase, not decrease, the concentration of anesthetic necessary to achieve immobility in the face of a noxious stimulus. In this particular case, the MAC was reduced. In 2-week-old mice, the perinatal  $\alpha 2$  GlyRs are being replaced by the  $\alpha 1\beta$  GlyRs,<sup>44</sup> which carry the Q266I mutation in  $\alpha 1$ . These mutated GlyRs have a reduced functionality<sup>21</sup> that translates in modification in the behavior and response to nonglycinergic drugs.<sup>30</sup> Some of these changes could be attributed to the development of compensatory

**Table 1.** Minimal Alveolar Concentration in M287L Knockin Mice

Volatile Anesthetic	Age	$\alpha 1^{M/M}$	$\alpha 1^{M/L}$	$\alpha 1^{L/L}$
		(Wild-type)	(Heterozygous)	(Homozygous)
Isoflurane	2–4 weeks	1.65 $\pm$ 0.07 (5)	1.77 $\pm$ 0.02 (4)	1.65 $\pm$ 0.06 (7)
	Adult*	1.54 $\pm$ 0.07 (6)	1.50 $\pm$ 0.06 (10)	—
Enflurane	2–4 weeks	2.45 $\pm$ 0.07 (5)	2.42 $\pm$ 0.08 (8)	2.33 $\pm$ 0.03 (9)
	Adult	2.28 $\pm$ 0.07 (6)	2.38 $\pm$ 0.05 (7)	—

Values are in % atmosphere (average  $\pm$  SEM, n between parenthesis).

\* Mice were 8 weeks old or older.

**Table 2.** Minimal Alveolar Concentration in Q266I Knockin Mice

Volatile Anesthetic	Age	$\alpha 1^{Q/Q}$ (Wild-type)	$\alpha 1^{Q/I}$ (Heterozygous)	$\alpha 1^{I/I}$ (Homozygous)
Isoflurane	2 weeks	1.79 ± 0.07 (10)		1.48 ± 0.03 (8)*
Enflurane	2 weeks	3.55 ± 0.09 (8)		3.23 ± 0.11 (5)*
Halothane	2 weeks	1.93 ± 0.05 (9)		1.86 ± 0.09 (9)
Isoflurane	Adult	1.69 ± 0.06 (7)	1.73 ± 0.07 (5)	—
Cyclopropane	Adult	18.7 ± 1.1 (7)	16.4 ± 1.0 (5)	—

Values are in % atmosphere (average ± SEM, n between parenthesis). Two-week-old mice were grouped according to their phenotype (tremoring mice: homozygous; non-tremoring mice: wild-type or heterozygous). Adult mice (8 weeks old or older) were grouped according to genotype.

\*  $P < 0.05$  vs. corresponding control group, Student  $t$  test.

changes. Furthermore, the adult heterozygous Q266I did not differ from wild-type in their response to isoflurane.

Because strychnine increased MAC for VAs in proportion to their ability to modulate GlyR (halothane more than isoflurane more than cyclopropane), it appeared that actions of VAs on GlyR likely mediated at least part of immobility, making the GlyR a leading candidate target for VAs.<sup>13,29</sup> However, the current findings provide strong evidence that the  $\alpha 1$  GlyR is not important for VA immobility. One caveat is that the effects of the mutations we studied were not limited to alterations in VA actions on the receptors, because they also impaired the function of GlyRs. It is possible that reduced glycinergic neurotransmission in the mutants could lead to compensatory changes in other neurotransmitters. Indeed, we found that the sedative effects of several drugs that do not affect GlyRs, such as flurazepam and ketamine, were increased in both mutants.<sup>30</sup> In addition, the changes in the loss of righting reflex induced by flurazepam and ketamine were more pronounced in Q266I than in M287L, as could be expected considering that the impairment in glycinergic transmission, as assessed by the acoustic startle reflex and the mortality of homozygous knockin mice, was more marked in Q266I than in M287L.<sup>21,30</sup> However, we need to keep in mind that immobility (assessed through MAC) and sedation (assessed through loss of righting reflex) are mediated by different areas in the central nervous system.<sup>31</sup> Furthermore, the data do not support the simple explanation that main systems, like the GABAergic and glutamatergic transmission, have undergone changes to compensate for a decrease in the glycinergic transmission, because the *N*-methyl-D-aspartic acid-induced tonic convulsions were the same in heterozygous knockin mice *versus* wild-type,<sup>30</sup> and the flunitrazepam binding was unchanged in Q266I heterozygous mice in brainstem and spinal cord.<sup>21</sup> So, even if other signaling systems changed, we would have to propose that these changes exactly offset the altered sensitivity of the GlyR to VAs in exactly the same structures, when the compensatory changes would presumably offset the decreased glycinergic transmission, not the changes in the sensitivity to isoflurane. This seems unlikely because both the M287L and Q266I mutations reduce GlyR function, but they do it to a different degree,<sup>21</sup> and there is no evidence for specific com-

pensatory changes in the structures mediating immobility. We conclude that actions of VAs on the  $\alpha 1$  GlyR have little or no role in the production of immobility by VAs. As Thomas Henry Huxley said,<sup>32</sup> we face once again “the great tragedy of Science – the slaying of a beautiful hypothesis by an ugly fact.” We conclude that the known molecular targets of VAs are not able to account for the immobilizing actions of VAs, and a search for new targets is required.

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## References

1. Franks NP: Molecular targets underlying general anaesthesia. *Br J Pharmacol* 2006; 147(Suppl 1):S72–81
2. Yamakura T, Bertaccini E, Trudell JR, Harris RA: Anesthetics and ion channels: Molecular models and sites of action. *Annu Rev Pharmacol Toxicol* 2001; 41:23–51
3. Eger EI 2nd, Raines DE, Shafer SL, Hemmings HC Jr, Sonner JM: Is a new paradigm needed to explain how inhaled anesthetics produce immobility? *Anesth Analg* 2008; 107:832–48
4. Lynch JW: Native glycine receptor subtypes and their physiological roles. *Neuropharmacology* 2009; 56:303–9
5. Antognini JF, Carstens E: *In vivo* characterization of clinical anaesthesia and its components. *Br J Anaesth* 2002; 89: 156–66
6. Mascia MP, Machu TK, Harris RA: Enhancement of homomeric glycine receptor function by long-chain alcohols and anaesthetics. *Br J Pharmacol* 1996; 119:1331–6
7. Harrison NL, Kugler JL, Jones MV, Greenblatt EP, Pritchett DB: Positive modulation of human  $\gamma$ -aminobutyric acid type A and glycine receptors by the inhalation anesthetic isoflurane. *Mol Pharmacol* 1993; 44:628–32
8. Downie DL, Hall AC, Lieb WR, Franks NP: Effects of inhalational general anaesthetics on native glycine receptors in rat medullary neurons and recombinant glycine receptors in *Xenopus* oocytes. *Br J Pharmacol* 1996; 118:493–502
9. Yamauchi M, Sekiyama H, Shimada SG, Collins JG: Halothane suppression of spinal sensory neuronal responses to noxious peripheral stimuli is mediated, in part, by both GABA(A) and glycine receptor systems. *ANESTHESIOLOGY* 2002; 97:412–7
10. Grasshoff C, Antkowiak B: Propofol and sevoflurane depress spinal neurons *in vitro via* different molecular targets. *ANESTHESIOLOGY* 2004; 101:1167–76

11. Zhang Y, Wu S, Eger EI 2nd, Sonner JM: Neither GABA(A) nor strychnine-sensitive glycine receptors are the sole mediators of MAC for isoflurane. *Anesth Analg* 2001; 92:123-7
12. Hara K, Eger EI 2nd, Laster MJ, Harris RA: Nonhalogenated alkanes cyclopropane and butane affect neurotransmitter-gated ion channel and G-protein-coupled receptors: Differential actions on GABA<sub>A</sub> and glycine receptors. *ANESTHESIOLOGY* 2002; 97:1512-20
13. Zhang Y, Laster MJ, Hara K, Harris RA, Eger EI 2nd, Stabernack CR, Sonner JM: Glycine receptors mediate part of the immobility produced by inhaled anesthetics. *Anesth Analg* 2003; 96:97-101
14. Zhao J, Zhang Y, Eger EI 2nd, Sonner J: Intrathecal glycine significantly decreases the minimum alveolar concentration of isoflurane in rats. *Chin Med Sci J* 2008; 23:16-8
15. Mihic SJ, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, Mascia MP, Valenzuela CF, Hanson KK, Greenblatt EP, Harris RA, Harrison NL: Sites of alcohol and volatile anaesthetic action on GABA(A) and glycine receptors. *Nature* 1997; 389:385-9
16. Mascia MP, Trudell JR, Harris RA: Specific binding sites for alcohols and anesthetics on ligand-gated ion channels. *Proc Natl Acad Sci U S A* 2000; 97:9305-10
17. Lobo IA, Mascia MP, Trudell JR, Harris RA: Channel gating of the glycine receptor changes accessibility to residues implicated in receptor potentiation by alcohols and anesthetics. *J Biol Chem* 2004; 279:33919-27
18. Nury H, Van Renterghem C, Weng Y, Tran A, Baaden M, Dufresne V, Changeux JP, Sonner JM, Delarue M, Corringer PJ: X-ray structures of general anaesthetics bound to a pentameric ligand-gated ion channel. *Nature* 2011; 469:428-31
19. Zeller A, Arras M, Jurd R, Rudolph U: Identification of a molecular target mediating the general anesthetic actions of pentobarbital. *Mol Pharmacol* 2007; 71:852-9
20. Jurd R, Arras M, Lambert S, Drexler B, Siegwart R, Crestani F, Zaugg M, Vogt KE, Ledermann B, Antkowiak B, Rudolph U: General anesthetic actions *in vivo* strongly attenuated by a point mutation in the GABA(A) receptor  $\beta 3$  subunit. *FASEB J* 2003; 17:250-2
21. Borghese CM, Blednov YA, Quan Y, Iyer SV, Xiong W, Mihic SJ, Zhang L, Lovinger DM, Trudell JR, Homanics GE, Harris RA: Characterization of two mutations, M287L and Q266I, in the  $\alpha 1$  glycine receptor subunit that modify sensitivity to alcohols. *J Pharmacol Exp Ther* 2012; 340:304-16
22. Franks NP, Lieb WR: Molecular and cellular mechanisms of general anaesthesia. *Nature* 1994; 367:607-14
23. Sonner JM, Gong D, Eger EI 2nd: Naturally occurring variability in anesthetic potency among inbred mouse strains. *Anesth Analg* 2000; 91:720-6
24. Harris RA, Trudell JR, Mihic SJ: Ethanol's molecular targets. *Sci Signal* 2008; 1:re7
25. Werner DF, Swihart A, Rau V, Jia F, Borghese CM, McCracken ML, Iyer S, Fanselow MS, Oh I, Sonner JM, Eger EI 2nd, Harrison NL, Harris RA, Homanics GE: Inhaled anesthetic responses of recombinant receptors and knockin mice harboring  $\alpha 2$ (S270H/L277A) GABA(A) receptor subunits that are resistant to isoflurane. *J Pharmacol Exp Ther* 2011; 336:134-44
26. Kim J, Atherley R, Werner DF, Homanics GE, Carstens E, Antognini JF: Isoflurane depression of spinal nociceptive processing and minimum alveolar anesthetic concentration are not attenuated in mice expressing isoflurane resistant  $\gamma$ -aminobutyric acid type-A receptors. *Neurosci Lett* 2007; 420:209-12
27. Sonner JM, Werner DF, Elsen FP, Xing Y, Liao M, Harris RA, Harrison NL, Fanselow MS, Eger EI 2nd, Homanics GE: Effect of isoflurane and other potent inhaled anesthetics on minimum alveolar concentration, learning, and the righting reflex in mice engineered to express  $\alpha 1$   $\gamma$ -aminobutyric acid type A receptors unresponsive to isoflurane. *ANESTHESIOLOGY* 2007; 106:107-13
28. Liao M, Sonner JM, Jurd R, Rudolph U, Borghese CM, Harris RA, Laster MJ, Eger EI 2nd:  $\beta 3$ -containing  $\gamma$ -aminobutyric acidA receptors are not major targets for the amnesic and immobilizing actions of isoflurane. *Anesth Analg* 2005; 101:412-8
29. Sonner JM, Antognini JF, Dutton RC, Flood P, Gray AT, Harris RA, Homanics GE, Kendig J, Orser B, Raines DE, Rampil IJ, Trudell J, Vissel B, Eger EI 2nd: Inhaled anesthetics and immobility: Mechanisms, mysteries, and minimum alveolar anesthetic concentration. *Anesth Analg* 2003; 97:718-40
30. Blednov YA, Benavidez JM, Homanics GE, Harris RA: Behavioral characterization of knockin mice with mutations M287L and Q266I in the glycine receptor  $\alpha 1$  subunit. *J Pharmacol Exp Ther* 2012; 340:317-29
31. Rudolph U, Antkowiak B: Molecular and neuronal substrates for general anaesthetics. *Nat Rev Neurosci* 2004; 5:709-20
32. Huxley TH: *Collected Essays, First Edition*. London: Macmillan; 1908:244