

Blockade of AMPA Receptors and Volatile Anesthetics

Reduced Anesthetic Requirements in GluR2 Null Mutant Mice for Loss of the Righting Reflex and Antinociception but Not Minimum Alveolar Concentration

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Background: The α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype of glutamate receptor mediates fast excitatory neurotransmission in the central nervous system. Many general anesthetics inhibit AMPA receptors *in vitro*; however, it is not certain if this inhibition contributes to the behavioral properties of these drugs. AMPA receptors lacking the GluR2 subunit are resistant to blockade by barbiturates *in vitro*. Paradoxically, GluR2 null mutant (–/–) mice are more sensitive to barbiturate-induced loss of the righting reflex (LORR) compared with wild-type (+/+) littermates. To determine if interactions between anesthetics and AMPA receptors account for the increased sensitivity of (–/–) mice, the effects of volatile anesthetics that do not directly inhibit AMPA receptors were examined.

Methods: Isoflurane, halothane, desflurane, or sevoflurane were administered to (–/–) and (+/+) littermate controls. Anesthetic requirements for LORR, movement to tail clamp (minimum alveolar concentration [MAC]), and hind-paw withdrawal latency (HPWL) were determined. Electrophysiologic methods examined the inhibition of AMPA receptors by isoflurane and halothane.

Results: Anesthetic requirements for LORR and HPWL were decreased, whereas MAC values were unchanged in (–/–) mice. Isoflurane and halothane caused minimal inhibition of AMPA receptors at clinically relevant concentrations.

Conclusions: Direct blockade of AMPA receptors did not account for the increased sensitivity to volatile anesthetics in GluR2 null mutant mice for HPWL or LORR. Thus, the deficiency of GluR2-containing AMPA receptors increases the sensitivity of neuronal circuitry mediating these end points, but not MAC.

GluR2-containing receptors do not contribute appreciably to MAC in this mouse model. These results illustrate the difficulties in attributing behavioral responses to drug–receptor interactions in genetically engineered animals.

INTRAVENOUS and volatile anesthetics influence a multitude of protein receptors present in neurons. A current research priority is to determine which drug–receptor interactions underlie the clinical properties of anesthetics and elucidate how changes in receptor function influence behavior. Substantial evidence indicates that anesthetics enhance inhibitory neurotransmission mediated by γ -aminobutyric acid type A (GABA_A) receptors^{1–3} (for review see Franks and Lieb⁴). However, a smaller body of literature suggests that some volatile and intravenous anesthetics inhibit glutamate-mediated excitatory transmission primarily by attenuating transmitter release or, alternatively, by inhibiting postsynaptic receptors.⁵ Although anesthetics impair glutamatergic neurotransmission in a variety of experimental preparations *in vitro*, the importance of this action to the behavioral properties of these drugs has not been determined.

Glutamate, the major excitatory neurotransmitter in the mammalian central nervous system, activates *N*-methyl-D-aspartate (NMDA) and non-NMDA ionotropic receptors. The α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor is a non-NMDA receptor that mediates fast excitatory neurotransmission.^{6,7} The AMPA subtype of glutamate receptor is a cation-selective ligand-gated ion channel composed of multiple subunit subtypes. Four distinct genes encoding the glutamate receptor subunits—GluR1, 2, 3, and 4—have been identified, and subunit composition determines the physiologic and pharmacologic properties of AMPA receptors.^{8,9} In particular, the properties of the GluR2 subunit that is present in most native AMPA receptors dominate the characteristics of heteromeric receptor complexes.⁹ The GluR2 subunit imparts calcium permeability and sensitivity to polyamines and influences the localization of receptors to the synapse. GluR2-containing AMPA receptors are inhibited by barbiturates at clinically relevant concentrations, whereas GluR2-deficient receptors are relatively insensitive to barbiturate blockade.^{10–12} Previously, the authors demonstrated that barbiturate inhibition of GluR2-deficient receptors *in vitro* was reduced compared with wild-type AMPA receptors. In contrast, GluR2 null mutant (–/–) mice were more sensitive to

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the neurodepression by pentobarbital, as indicated by the loss of righting reflex (LORR).¹² Thus, inhibition of postsynaptic AMPA receptors by barbiturates did not correlate with the *in vivo* behavioral sensitivity of GluR2 (–/–) mice. We hypothesized that GluR2 (–/–) mice would demonstrate an increased sensitivity to general anesthetics because of alterations in excitatory neurotransmission. Consequently, it was predicted that GluR2 (–/–) mice would be more sensitive to anesthetics, regardless of the potency of these drugs for blockade of AMPA receptors. In this study, the authors examine the sensitivity of GluR2 (–/–) mice to anesthetics that do not directly inhibit AMPA receptors. The results demonstrate that GluR2 (–/–) mice are more sensitive to volatile anesthetics for the behavioral end points of hypnosis and antinociception but not immobility in response to a painful stimulus. Electrophysiologic findings indicate that the increased anesthetic sensitivity of the GluR2 (–/–) mice cannot be attributed to differences in the blockade of AMPA receptors. Yet unidentified mechanism(s) account for the enhanced sensitivity of these mice to volatile anesthetics.

Materials and Methods

Null Mutant GluR2 Mice

The Animal Care Committee at the University of Toronto approved the electrophysiologic studies. The Committee on Animal Research at University of California-San Francisco approved the behavioral studies. Jia *et al.*¹³ developed (–/–) mice lacking the GluR2 subunit protein. Gene targeting in embryonic stem cells permitted the disruption of the M1 and pore loop regions of the gene encoding the GluR2 subunit, thereby preventing the expression of the entire GluR2 protein. GluR2 null allele mutants are fertile but show poor parenting ability, are smaller during early development, and have a higher postnatal mortality. However, their weight and size are similar to normal littermates by 5–7 weeks, and, subsequently, they have a normal life expectancy.¹³ Adult GluR2 (–/–) mice demonstrate decreased exploratory behaviors, reduced rearing and locomotion scores, and impaired performance on the rotarod and water maze tests.¹⁴ Nevertheless, the mice demonstrated a brisk righting reflex when positioned supine with no apparent impairment of gross motor abilities. The overall strength of the mice was normal, and no gross alterations were detected in neuroanatomic loci or fiber pathways.¹³ Mice used for the behavioral studies were 9–30 weeks of age.

Behavioral Studies of Sensitivity to Volatile Anesthetics

The Loss of Righting Reflex (Hypnosis) Study. In these studies, the behavioral end point of LORR was used as a surrogate measure for hypnosis. Thirty-two sex-

and age-matched (–/–) and wild-type (+/+) mice were individually placed in large airtight acrylic cylinders. Isoflurane, halothane, or sevoflurane was administered in 2 l/min oxygen through a modified circle circuit at initial concentrations of 1.0%, 1.2%, and 1.8% atm, respectively. All the mice experienced LORR at these concentrations. Rectal temperature probes (YSI Inc., Yellow Springs, OH) were then inserted, and core temperature was actively maintained between 36 and 39°C by active warming with hot water-filled plastic bags placed around the cylinders. The initial concentrations of desflurane, isoflurane, and halothane were continually measured using an infrared gas analyzer (Datex Ohmeda model #5250, Louisville, CO). The concentration of anesthetic was maintained for a minimum equilibration period of 20 min. At the end of the equilibration period, a gas sample was obtained from the cylinder and analyzed using a calibrated gas chromatograph (Gow-Mac 750, Bethlehem, PA). The concentration of the anesthetic was then repeatedly decreased in 10–20% increments and allowed to reequilibrate at each concentration. Mice were observed continuously for recovery of the righting reflex. The concentration reported for LORR was calculated by averaging the two concentrations at which the mouse either retained or lost the righting reflex.

The Loss of Movement to Tail Clamp (Immobility) Study. To investigate the immobilizing properties of volatile anesthetics in (–/–) and (+/+) mice, minimum alveolar concentration (MAC) values were determined using the classical tail-clamp paradigm. As described above, 11–12 sex- and age-matched (–/–) or (+/+) mice were restrained in acrylic cylinders (one mouse per cylinder, eight cylinders per circuit). Rectal temperature probes were inserted into the conscious mouse, and cylinders were placed into an open circle circuit. Throughout the experiment, rectal temperature of the mice were maintained between 36 and 39°C. Isoflurane (0.6%), halothane (0.4%), or desflurane (5.6%) was administered for 20 min. After the 20-min equilibration period, movement to tail clamp was tested by the placement of an alligator clip on the tail. If no movement was observed, the clamp was gently twisted for a 1-min period. If any movement to tail clamp was detected, the concentration of volatile anesthetic was increased by 20% for another 20-min equilibration period, and the response was tested again. The concentration at which the mouse lost its tail-clamp reflex was noted. The concentration of anesthetic was confirmed by gas chromatographic analysis of samples drawn from the cylinders.

Antinociceptive Properties of Volatile Anesthetics

Analgesic properties of the volatile anesthetics on thermal nociception were tested using the hind-paw withdrawal latency (HPWL) test, as previously described.¹⁵ The HPWL testing apparatus (Plantar Test, Ugo Basile

Biological Research Apparatus, Comerio, Italy) was modified to allow for the administration of volatile anesthetics. Up to five unrestrained mice were individually placed in clear plastic enclosures that sat on top of the glass floor of the testing apparatus. Each enclosure was covered with a porous plastic lid that permitted ventilation but prevented the mice from escaping. An additional plastic lid, large enough to cover all five enclosures, created the closed environment into which anesthetics were introduced. This box-shaped plastic lid was sealed with a silicone rubber gasket to the surface of the glass floor. A circuit for the delivery and scavenging of anesthetics was connected to the enclosure using gas-tight fittings. The mice were allowed to acclimate to the test environment for approximately 20 min to minimize the exploratory activity during the testing of the plantar withdrawal reflex. A movable radiant heat source (Radium tungsten halogen lamp, model EJY, 19V, 80 W; General Electric, Glen Allen, VA) on the Ugo Basile plantar heating device (Plantar Test, Ugo Basile Biological Research Apparatus) was used at intensities set at 25, 30, or 40 infrared intensity (IR; arbitrary units). Heat emanating from a 7-mm-diameter aperture was aimed at the plantar surface of one hind paw, through the glass floor. A photocell capable of detecting any movement of the paw would automatically turn off the heat source and record the latency time from the start of the stimulus. This method provides a sensitive measure of noxious heat response using an automated end point. Baseline measurements of HPWL (5 latency times per paw, total of 10 measurements per mouse) for each hind paw were made for (−/−) and (+/+) mice before the exposure to anesthetics using 25, 30, and 40 IR. After testing nociceptive measurements at 30 IR for (−/−) and (+/+) mice, isoflurane was administered and the HPWL was determined. The intensity of 30 IR was selected for testing anesthetic sensitivity as this intensity is commonly used for studying drug actions. Isoflurane was delivered in a stepwise manner at 0.1, 0.2, 0.4, and 0.6 MAC. Each concentration step was sustained for a minimum of 30 min, at which time isoflurane equilibrium in the mice was presumed to occur, and HPWL measurements (using 30-IR heat intensity) were taken. After the final concentration of isoflurane was administered, delivery of the anesthetic was terminated, and 30 min elapsed before the HPWL time was remeasured. Throughout the experiments, a heating pad warmed the glass floor. Maintaining the footpads at a constant temperature ensured reproducible HPWL times. In addition, warming the glass floor prevented hypothermia in the mice during anesthesia, although the mice were inaccessible for temperature monitoring during these experiments. Similar experiments using volatile anesthetics indicated that rectal temperatures were maintained between 36 and 39°C and did not differ between the (−/−) and (+/+) mice.

An investigator blinded to the genotype of the mice undertook the behavioral studies.

Electrophysiologic Study

Postnatal Hippocampal Cell Culture. Each postnatal hippocampal cell culture was prepared from single pups born of heterozygous GluR2 (+/−) mice. The 1- to 2-day-old murine pups were killed by decapitation, and their heads were sterilized by a brief submersion in 70% ethanol. The hippocampi of each pup were microdissected, and neurons were dissociated by mechanical trituration using a Pasteur pipette. The cells were plated on a poly-D-lysine-coated glass coverslip situated in a culture dish. Cultures were incubated for the first week in minimal essential medium (Gibco BRL, Grand Island, NY), supplemented with fetal bovine serum (100 μg/ml, Gibco BRL), inactivated horse serum (10 μg/ml, Gibco BRL), and insulin (8 μg/ml crystalline bovine zinc insulin, Gibco BRL) at 37°C in 7% carbon dioxide. On postdissection day 5, 4 mg/ml 5-fluorodeoxyuridine (Sigma Chemical Co., St. Louis, MO) and 10 mg/ml uridine (Sigma Chemical Co.) were added to inhibit glial cell proliferation. After the first week of incubation, the cultures were further maintained in only minimal essential medium and 100 μg/ml horse serum until the time of recording. Cultured mouse hippocampal neurons were used for recording 10–14 days after plating. Tails of the pups were retained for genotyping using polymerase chain reaction.

Acutely Isolated Hippocampal Neurons. In addition to the dissociated culture preparation, pyramidal neurons were acutely isolated from brain slices obtained from newborn pups.¹² These neurons were used to investigate the effects of volatile anesthetics on glutamate-activated AMPA receptors. Unlike cultured neurons, the acutely dissociated cells could be lifted off the bottom of the dish and positioned into the outflow stream of the perfusion barrel. This technique produced a rapid and uniform exchange of the perfusate around the cell and consequently allowed the fast activation and deactivation of AMPA receptors. Briefly, mice older than 6 weeks were anesthetized with halothane and then decapitated by guillotine. The brain was then removed and rinsed in cold (4°C) extracellular solution (ECF) consisting of 140 mM NaCl, 1.3 mM CaCl₂, 5.4 mM KCl, 25 mM HEPES, 33 mM glucose, and 0.0003 mM tetrodotoxin (pH 7.4, adjusted with NaOH; osmolality, 320–325 mOsm). The hippocampi were dissected and manually sectioned by a razor into approximately 500-μm-thick slices. The slices were incubated in 0.4 mg/ml papain latex (Sigma Chemical Co.) dissolved in ECF for 15–30 min at room temperature (20–22°C). The slices were then allowed to acclimate while submersed in oxygenated ECF at room temperature for 2 h after several rinses in papain-free solution. On use, the slice was transferred to a plastic 35-mm tissue culture dish (Nalge Nunc Interna-

tional, Roskilde, Denmark), and the CA1 region was isolated. Pyramidal neurons were isolated from the CA1 region by mechanical trituration using three Pasteur pipettes with consecutively smaller tip diameters (700 μm to 150 μm). The acutely dissociated CA1 pyramidal hippocampal neurons were allowed to settle to the bottom of the dish before the recordings were made.

Whole-cell Recordings. Immediately before electrophysiologic recording, the cultured neurons were washed several times with ECF consisting of 140 mM NaCl, 1 mM CaCl_2 , 5.4 mM KCl, 25 mM HEPES, 33 mM glucose, 0.0003 mM tetrodotoxin, and 2 mM MgCl_2 at room temperature (20–22°C). This solution was buffered to a pH of 7.4 with NaOH, and osmolality was adjusted to between 320 and 325 mOsm. Extracellular solutions containing various concentrations of kainate or glutamate were applied using a multibarreled perfusion apparatus as previously described.¹² The NMDA antagonist, L-(+)-2-amino-4-phosphonovaleric acid, at 40 μM , was added to the ECF in all experiments using glutamate as the AMPA receptor agonist.

Patch electrodes were pulled from thin-walled borosilicate glass (1.5-mm OD; World Precision Instruments, Sarasota, FL) using a two-stage vertical puller (Narishige PP-83, Tokyo, Japan) to a series resistance of 3–10 M Ω . The electrodes were used for whole-cell patch-clamp recordings and contained intracellular solution consisting of 140 mM CsF, 35 mM CsOH, 10 mM HEPES, 2 mM MgCl_2 , 11 mM EGTA, 2 mM tetraethylammonium chloride, 1 mM CaCl_2 , and 4 mM magnesium adenosine 5'-triphosphate. This solution was buffered to a pH 7.4 using CsOH, and osmolality was adjusted to 300–310 mOsm.

Both acutely dissociated and cultured pyramidal hippocampal neurons were voltage clamped at a holding potential of –60 mV. Whole-cell currents evoked by the different concentrations of agonist, kainate (applied to cultured neurons), or glutamate (applied to acutely dissociated neurons) were recorded with an Axopatch amplifier (Axon Instruments Inc., Foster City, CA). Data were filtered (2 kHz), digitized, and acquired on-line using the pCLAMP programs (Axon Instruments). Data were plotted using GraphPad Prism (Graph Pad, San Diego, CA) and SigmaPlot (SPSS Inc., San Rafael, CA).

The concentration–response relations for kainate- and glutamate-evoked currents were fit by the least squares method as previously described.¹² The effective concentration of agonist that produced 50% of the maximal response (EC_{50}) and the Hill coefficient (n_H) in (–/–) and (+/+) neurons were determined according to the following:

$$I = I_{\max} \times 1/(1 + (\text{EC}_{50}/[\text{agonist}])^{n_H}), \quad (1)$$

where I_{\max} is the response at a saturating concentration of agonist.

Extracellular solutions saturated with volatile anesthetic were obtained by storing approximately 100 ml

isoflurane (Abbott Laboratories, Montreal, Canada) and 100 ml of halothane (Halocarbon Laboratories, River Edge, NJ) in separate 500-ml gas-tight glass bottles with approximately 200 ml ECF in each. The halothane and isoflurane solutions at saturated concentrations and at 1:1000, 1:100, and 1:10 dilutions were then coapplied midway through an application of kainate (at the EC_{50} concentration). In other experiments, the solution containing the volatile anesthetic was coapplied with a saturating concentration of glutamate (3 mM).

The concentrations of volatile anesthetic in these solutions were determined using gas chromatography.² Concentrations of halothane and isoflurane that produced 50% of the maximal inhibition (IC_{50}) were determined from the concentration–inhibition curves. Residual current in the presence of anesthetic were normalized to the kainate- or glutamate-evoked current and fit according to the following:

$$I = I_{\max} \times 1/(1 + (\text{IC}_{50}/[\text{antagonist}])^{n_H}), \quad (2)$$

where I_{\max} is the maximal kainate- or glutamate-evoked current.

Only recordings that demonstrated a stable level of inhibition and recovery of the response toward baseline values after drug washout were used for the data analysis.

Statistical Analyses

Statistical analyses of the electrophysiologic and behavioral data were undertaken using SigmaStat (Jandel Scientific Inc.). All data were reported as mean \pm SD. The mean concentration of volatile anesthetics at which LORR and movement to tail clamp occurred were compared between (–/–) and (+/+) mice using the two-tailed Student *t* test with significance at $P < 0.05$. Similarly, the EC_{50} values and Hill coefficients for kainate–glutamate concentration–response relations in (–/–) and (+/+) cultured-acutely dissociated pyramidal hippocampal neurons were compared using the Student *t* test. Compared data for which SD values were determined to be significantly different (using an *F* test) were subsequently analyzed using the alternate *t* test with the Welch approximate.

Results

Increased Sensitivity to Volatile Anesthetics in GluR2 Null Mutant Mice for the Loss of Righting Reflex

The hypnotic effects of isoflurane, halothane, and sevoflurane were quantified by measuring the average concentration at which (–/–) and (+/+) mice demonstrated LORR. Righting reflexes were normal and brisk in both strains of mice in the absence of anesthetic. However, (–/–) mice showed an increased sensitivity to all

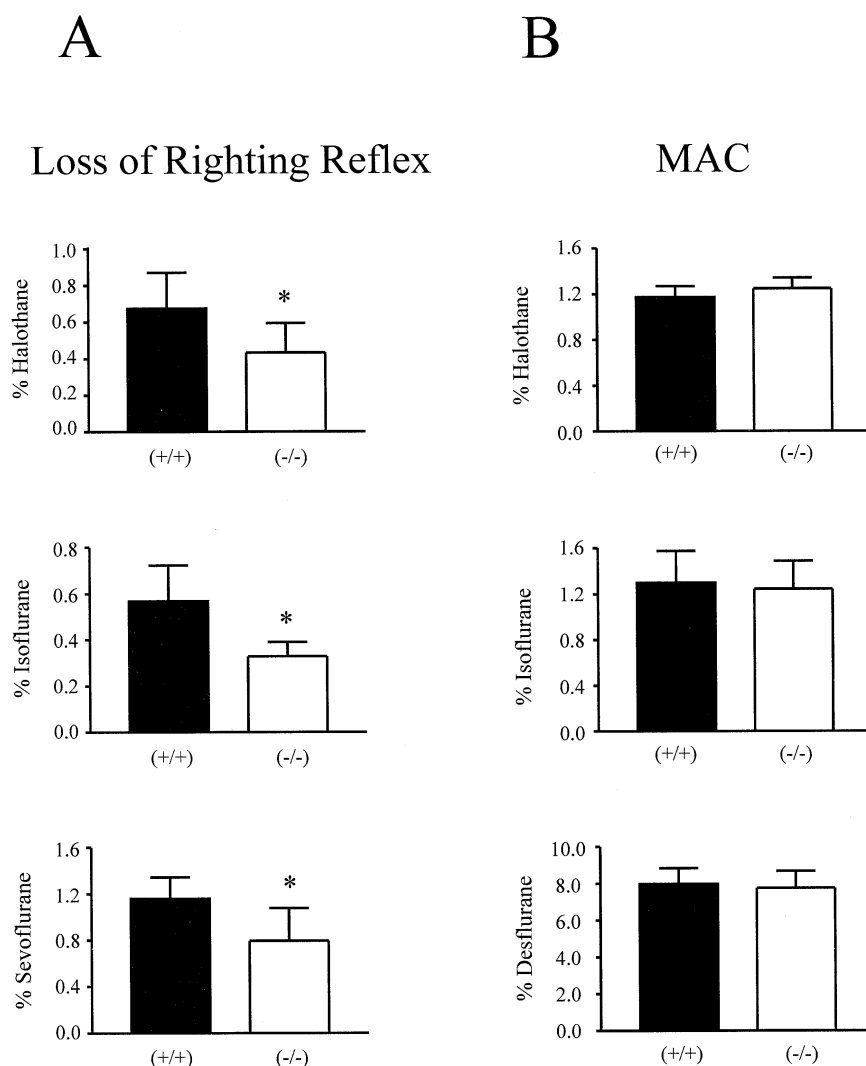


Fig. 1. Average concentrations of volatile anesthetics for the recovery from loss of righting reflex (LORR) and for loss of movement to tail-clamp reflex. (A) The concentrations of isoflurane, halothane, and sevoflurane at which (-/-) mice (unfilled squares) recovered from LORR were reduced compared with (+/+) mice (filled squares). (B) In contrast, concentrations of isoflurane, halothane, and desflurane at which (-/-) mice (open squares) demonstrated a loss of movement to tail clamp were no different from that in (+/+) mice (filled squares). *Significant differences $P < 0.05$.

three volatile anesthetics as evidenced by the lower concentrations required for LORR (fig. 1A). The LORR in (-/-) and (+/+) mice occurred at isoflurane concentrations of $0.33 \pm 0.06\%$ atm ($n = 16$) versus $0.57 \pm 0.16\%$ atm ($n = 16$; $P < 0.05$), at halothane concentrations of $0.43 \pm 0.16\%$ atm ($n = 16$) versus $0.67 \pm 0.20\%$ atm ($n = 16$; $P < 0.05$), and at sevoflurane concentrations of $0.80 \pm 0.28\%$ atm ($n = 16$) versus $1.16 \pm 0.19\%$ atm ($n = 16$; $P < 0.05$).

Unchanged Sensitivity of GluR2 Null Mutant Mice for Anesthetic-induced Immobilization

The classic tail-clamp method was used to investigate the immobilizing effects of isoflurane, halothane, and desflurane. The MAC values of volatile anesthetic that prevented movement in response to a painful stimulus were determined in (-/-) and (+/+) mice. Surprisingly, no differences were detected for the concentrations of isoflurane, halothane, or desflurane required to prevent movement in response to the tail clamp: isoflurane, $1.30 \pm 0.27\%$ atm ($n = 11$) versus $1.25 \pm 0.24\%$

atm ($n = 11$), respectively ($P = 0.62$); halothane, $1.17 \pm 0.09\%$ atm ($n = 10$) versus $1.25 \pm 0.09\%$ atm ($n = 10$) respectively ($P = 0.09$); and desflurane, $8.00 \pm 0.84\%$ atm ($n = 11$) versus $7.76 \pm 0.92\%$ atm ($n = 11$) respectively ($P = 0.54$; fig. 1B). Similar to previous reports for barbiturates, the concentrations of isoflurane and halothane that prevented movement after tail clamp were higher than concentrations causing LORR.¹⁶

Increased Sensitivity of GluR2 Null Mutant Mice to the Antinociceptive Properties of Volatile Anesthetics

The HPWL of (-/-) and (+/+) mice was first determined in the absence of the volatile anesthetics (fig. 2). No differences in HPWL times were detected between the (-/-) and (+/+) mice at IR heat intensities of 25 (11.6 ± 3.6 s, $n = 10$, vs. 9.1 ± 2.9 s, $n = 10$; $P = 0.10$), 30 (7.0 ± 1.5 s, $n = 14$, vs. 7.5 ± 1.9 s, $n = 16$; $P = 0.44$), or 40 (5.0 ± 1.8 s, $n = 10$, vs. 5.6 ± 0.9 s, $n = 10$; $P = 0.36$), respectively.

The (-/-) mice demonstrated an enhanced sensitivity

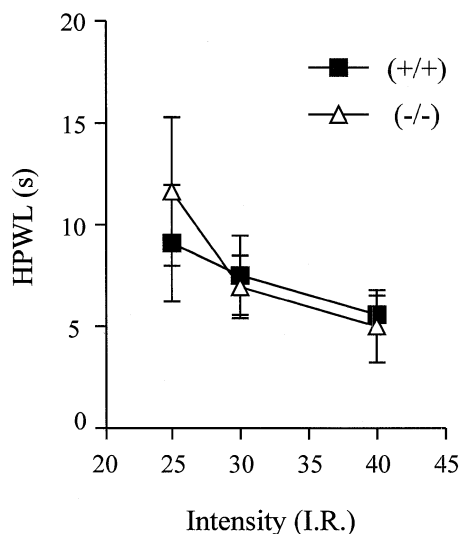


Fig. 2. Normal baseline thermal nociception in the GluR2 null mutant (-/-) mice measured by hind-paw withdrawal latency (HPWL). Heat infrared (I.R.) intensity versus HPWL time relations for HPWL times (in seconds) for wild-type (+/+) ($n = 10$ – 16 mice) and (-/-) ($n = 10$ – 14 mice). The baseline values acquired in oxygen were not significantly different between these two groups of mice at any of the intensities tested.

to isoflurane-induced antinociception at 0.2, 0.4, and 0.6 MAC compared with (+/+) mice (fig. 3). For this analysis, all HPWL times were normalized to baseline values obtained during control conditions. The HPWL time in the (-/-) mice increased in a dose-dependent manner from the baseline value of 7.0 ± 1.5 s to 8.9 ± 2.1 s at 0.1 MAC ($P = 0.02$), 9.4 ± 1.9 s at 0.2 MAC ($P < 0.01$), 11.1 ± 2.6 s at 0.4 MAC ($P < 0.01$), and 16.1 ± 5.0 s at 0.6 MAC ($P < 0.01$). In contrast, the HPWL times for the (+/+) mice increased significantly from the baseline value (7.5 ± 1.9 s) only at the highest concentration of isoflurane: 0.1 MAC, 7.7 ± 1.4 s ($P = 0.77$); 0.2 MAC, 7.4 ± 2.1 s ($P = 0.88$); 0.4 MAC, 8.1 ± 1.7 s ($P = 0.38$); and 0.6 MAC, 11.4 ± 1.4 s ($P < 0.01$). After the washout of isoflurane, the HPWL time returned to baseline values for both the (-/-) (8.0 ± 2.0 s; $P = 0.52$) and (+/+) mice (8.8 ± 2.9 s; $P = 0.02$).

In Vitro Inhibition of AMPA Receptors by Volatile Anesthetics: Higher Potency for Kainate but Not Glutamate in GluR2-deficient AMPA Receptors

To ensure that equi-effective concentrations of agonist were used to elicit control currents from (-/-) and (+/+) neurons in dissociated culture, the concentration-response relations for kainate- and glutamate-evoked currents were examined as previously described.¹² Applications of kainate ($> 10 \mu\text{M}$) activated currents in all cultured hippocampal neurons tested. The concentration-response relation indicated that the EC_{50} value for kainate-evoked current was significantly lower for (-/-) neurons compared with (+/+) neurons ($90 \pm 35 \mu\text{M}$ [$n = 7$] vs. $219 \pm 82 \mu\text{M}$ [$n = 6$]; $P < 0.05$),

respectively (figs. 4A and B). These values for receptors present in dissociated cultured neurons were lower than the EC_{50} value for kainate-evoked current in acutely isolated hippocampal pyramidal (-/-) neurons but not (+/+) neurons ($136 \pm 29 \mu\text{M}$ [$n = 17$] vs. $226 \pm 89 \mu\text{M}$ [$n = 15$]; $P < 0.05$), respectively.¹² Furthermore, the potency of kainate in both cultured and acutely dissociated hippocampal neurons was approximately twofold greater for the (-/-) neurons compared with (+/+) neurons, consistent with previous reports of the higher potency for kainate in recombinant AMPA receptors lacking the GluR2 subunit.¹⁷ In addition, the Hill coefficient was lower for (-/-) neurons compared with (+/+) neurons ($1.13 \pm 0.09 \mu\text{M}$ vs. $1.35 \pm 0.12 \mu\text{M}$; $P < 0.05$; fig. 5), suggesting a decreased cooperativity for activation of GluR2-deficient AMPA receptors by kainate.

Interestingly, the dose-response relations for glutamate-evoked peak and steady state currents did not differ between acutely isolated (-/-) and (+/+) hippocampal neurons (fig. 4). The EC_{50} values for peak responses were $267 \pm 38 \mu\text{M}$ for (-/-) mice ($n = 7$) and $246 \pm 43 \mu\text{M}$ for (+/+) mice ($n = 7$; $P = 0.72$) with Hill coefficients of 1.62 ± 0.17 and 1.49 ± 0.09 ($P = 0.48$), respectively, whereas the EC_{50} values for

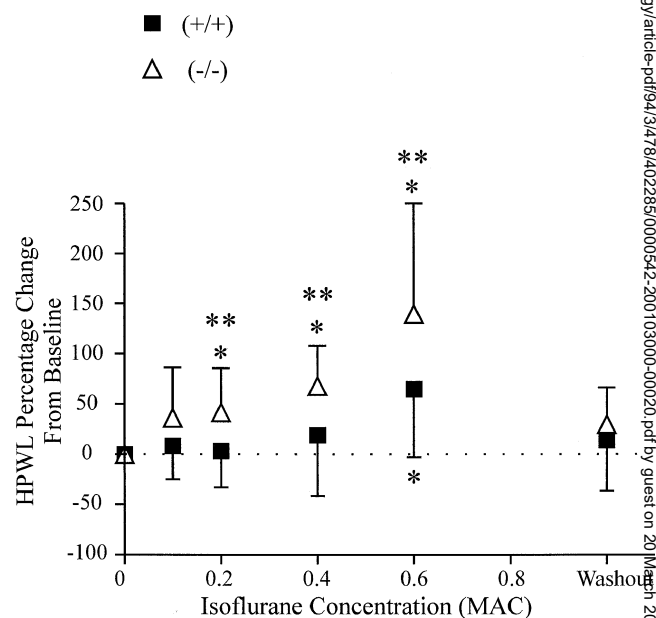


Fig. 3. Increased sensitivity to isoflurane-induced antinociception in the GluR2 null mutant (-/-) mice. Hind-paw withdrawal latency (HPWL) times measured in concentrations of isoflurane (0.1, 0.2, 0.4, and 0.6 MAC) were normalized to baseline values and averaged (mean \pm SD) for wild-type (+/+) mice (closed squares) and (-/-) mice (open triangles). In (-/-) mice, HPWL increased dose-dependently with higher concentrations of isoflurane administered. HPWL increased 36, 42, 68, and 140% above baseline with 0.1, 0.2, 0.4, and 0.6 MAC of isoflurane, respectively ($*P < 0.01$). In contrast, (+/+) mice showed lesser increases in HPWL with 0.1, 0.2, 0.4, and 0.6 MAC of isoflurane (8, 3, 19, and 65%, respectively). The HPWL at 0.2, 0.4, and 0.6 MAC of isoflurane in the (-/-) mice were significantly prolonged compared with values obtained for (+/+) mice ($**P < 0.05$).

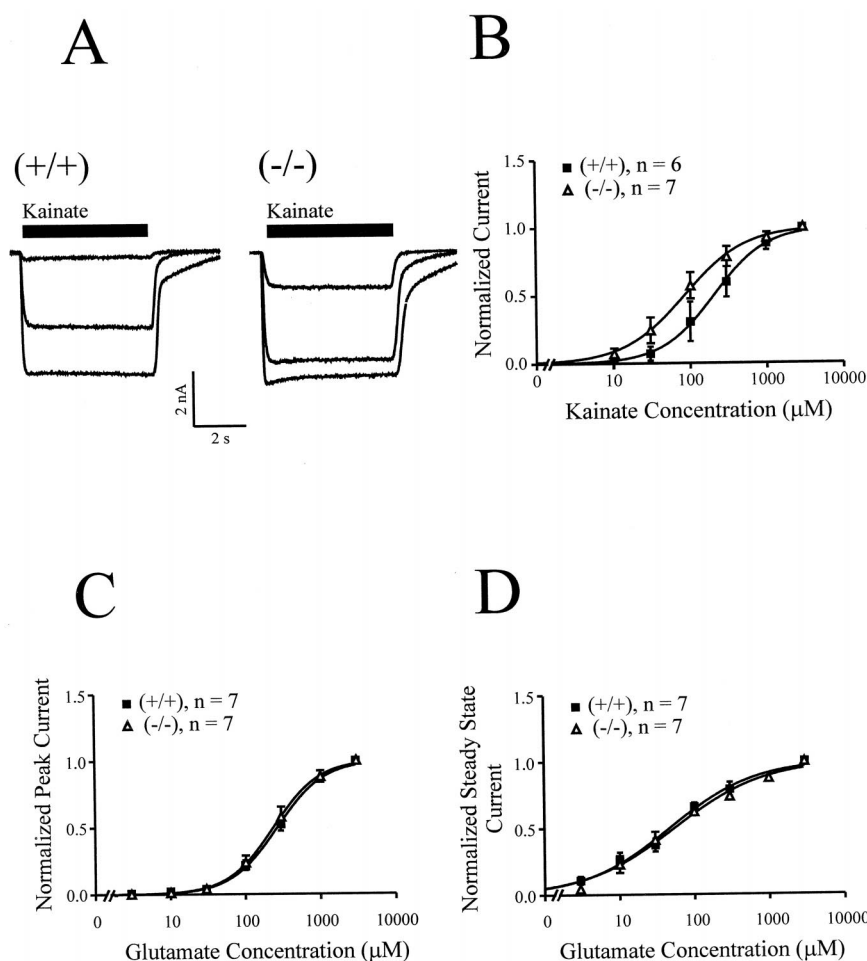


Fig. 4. Kainate- and glutamate-activated currents in acutely isolated hippocampal pyramidal neurons from wild-type (+/+) and mutant (-/-) mice. (A) Representative kainate-evoked currents at subsaturating (30 and 300 μM) and saturating (3,000 μM) concentrations are shown. (B) Concentration-response curves for kainate-evoked currents in (+/+) neurons (squares) and (-/-) neurons (triangles) were constructed by normalizing to the maximal response. (C) Concentration-response curves for glutamate-evoked currents in (+/+) neurons (triangles) and (-/-) neurons (squares).

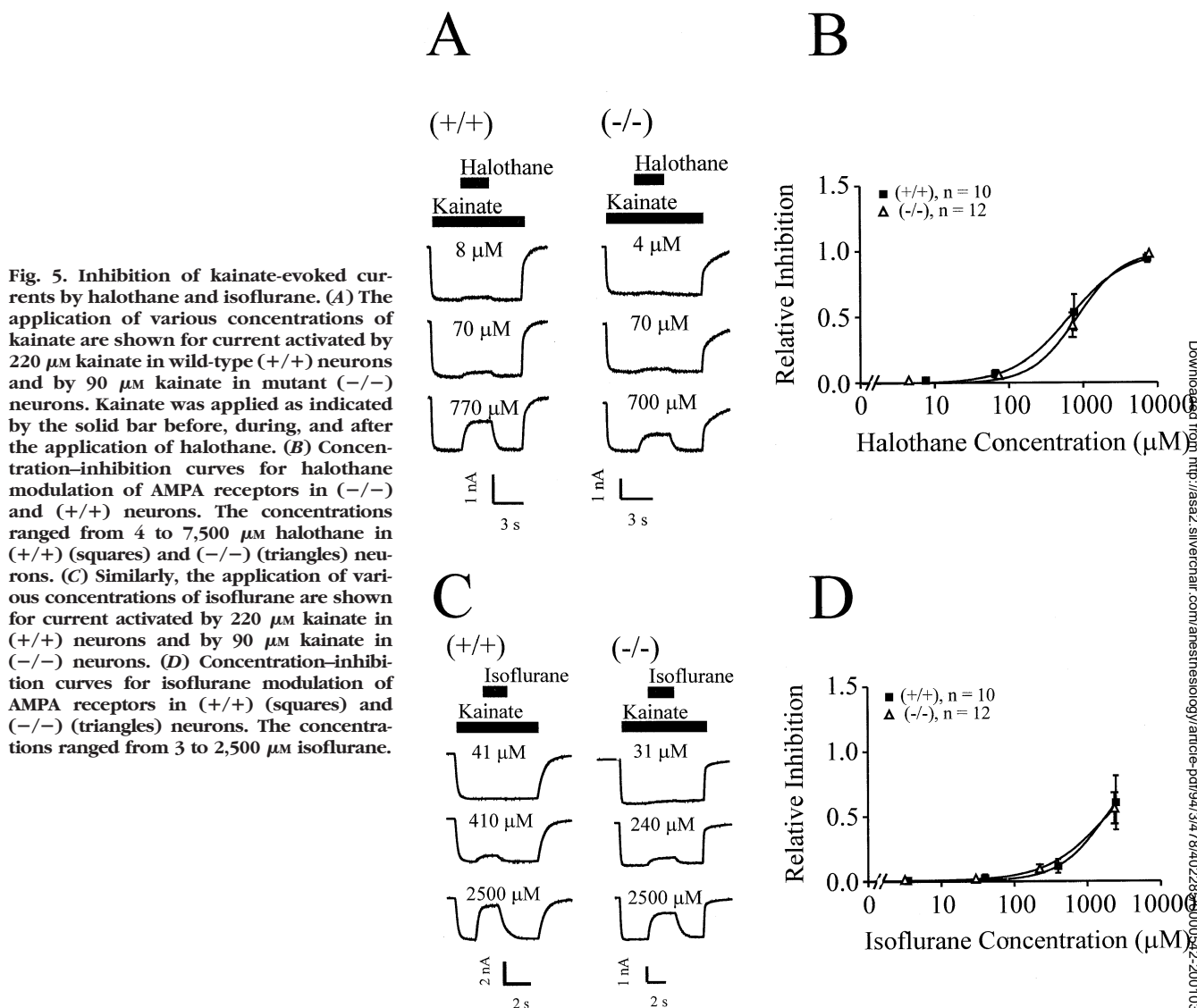
steady state responses were $58 \pm 10 \mu\text{M}$ for (-/-) mice ($n = 7$) and $44 \pm 7 \mu\text{M}$ for (+/+) mice ($n = 7$; $P = 0.27$), with Hill coefficients of 0.78 ± 0.11 and 0.98 ± 0.16 ($P = 0.32$), respectively. The peak amplitude of the responses did not differ between the groups ($3,669 \pm 2,218 \text{ pA}$ vs. $3,206 \pm 1,391 \text{ pA}$; $P = 0.42$); however, the steady state response was dramatically reduced for the (-/-) neurons compared with (+/+) neurons: $181 \pm 133 \text{ pA}$ versus $312 \pm 233 \text{ pA}$ ($P < 0.05$), respectively, consistent with an increase in desensitization in GluR2-deficient receptors.

Glutamate-evoked currents rapidly desensitized, thereby preventing analysis of the drug inhibition under steady state conditions. Consequently, kainate was used as the agonist for most of the experiments designed to investigate the inhibition of AMPA receptors by volatile anesthetics. To investigate the effects of volatile anesthetics on AMPA receptors, equi-effective concentrations of kainate were applied to (-/-) neurons ($\text{EC}_{50} = 90 \mu\text{M}$) or (+/+) neurons ($\text{EC}_{50} = 220 \mu\text{M}$). The agonist was applied for 1.5 s before the application of various concentrations of isoflurane or halothane.

The concentration-inhibition relation for isoflurane and halothane blockade of kainate-evoked currents is illustrated in figures 5A, B, C, and D, respectively. The

IC_{50} value for isoflurane inhibition was similar for (-/-) neurons and (+/+) neurons ($2,292 \pm 1,227 \mu\text{M}$ [$n = 12$] vs. $2,660 \pm 1,972 \mu\text{M}$ [$n = 10$]; $P = 0.96$). Similarly, the IC_{50} values for halothane ($818 \pm 219 \mu\text{M}$ [$n = 12$] vs. $707 \pm 299 \mu\text{M}$ [$n = 10$]; $P = 0.33$) were not different in (-/-) and (+/+) neurons. The Hill coefficients (n_H) for the dose-response plots for isoflurane inhibition were 1.09 ± 0.16 for (-/-) mice and 1.38 ± 0.31 for (+/+) mice, and for halothane were 1.46 ± 0.24 for (-/-) mice and 1.27 ± 0.19 for (+/+) mice.

In other experiments, the effect of a preapplication of near-saturating concentrations of isoflurane (2.5 mM) and halothane (7 mM) on the amplitude of peak current evoked by glutamate was investigated. Consistent with our previous findings, neither isoflurane nor halothane significantly reduced the amplitude of current activated by glutamate (3 mM) recorded from (-/-) and (+/+) neurons. The mean current amplitudes relative to control values observed in the presence of isoflurane or halothane were 0.84 ± 0.2 ($n = 4$) and 1.0 ± 0.23 ($n = 7$), respectively. Similarly, halothane (2.5 mM) produced only a modest inhibition of glutamate currents from (+/+) neurons (0.87 ± 0.07 ; $n = 2$) and mutant neurons (0.89 ± 0.03 ; $n = 2$).



Comparing the In Vitro and In Vivo Concentrations of Volatile Anesthetic

The temperature dependence of the concentrations of volatile anesthetics must be considered when comparing the *in vitro* and *in vivo* experimental results. The MAC equivalent aqueous EC_{50} concentrations at room temperature (T_c) were calculated according to the equation:

$$\text{EC}_{50}(T_c)/\text{EC}_{50}(37^\circ\text{C}) = \exp[-4.08 (37 - T_c)/(273.15 + T_c)]. \quad (3)$$

The EC_{50} (37°C) value is the aqueous concentration of volatile anesthetic that corresponds to MAC, as measured in animals and humans at normal body temperature.¹⁹ Using the predetermined EC_{50} ($T_c = 37^\circ\text{C}$) value for volatile anesthetics in mouse, the EC_{50} values of isoflurane and halothane for *in vitro* experiments performed at room temperature were estimated at 253 and 213 μM , respectively.¹⁸ Therefore, the concentrations of isoflurane and halothane that inhibited 50% of the kainate-evoked current in (+/+) and (-/-) neurons correspond to 10.5 and 4 MAC of the volatile anesthetics, respectively. These results revealed the relative insensitivity of AMPA receptors to inhibition by volatile anesthetics examined at clinically relevant concentrations.

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Discussion

Genetically modified mice were used to investigate the role of AMPA receptors as targets for volatile anesthetics. Anesthetic requirements for the behavioral end points of LORR and HPWL, but not tail clamp-withdrawal (MAC), were reduced in GluR2 (-/-) mice. These findings indicate that neuronal circuits that determine MAC do not require GluR2-containing AMPA receptors in this mouse model. In contrast, the circuitry that mediates HPWL and antinociception was influenced by the absence of the GluR2 subunit. The *in vitro* electrophysiologic experiments confirmed previous reports that

demonstrated clinically relevant concentrations of volatile anesthetics did not appreciably directly inhibit AMPA receptors in hippocampal neurons. Taken together, the behavioral and electrophysiologic results indicate that mechanisms other than direct blockade of postsynaptic AMPA receptors underlie the enhanced behavioral sensitivity of GluR2 ($-/-$) mice to volatile anesthetics. These data support the notion that "separate mechanisms" or receptor populations underlie the different behavioral end points produced by anesthetic drugs.^{19,20}

If we adopt the simplistic notion that anesthetics disrupt the balance between excitatory and inhibitory neurotransmission, what mechanisms might account for the experimental findings? It was postulated that excitatory neurotransmission was reduced in GluR2 ($-/-$) mice, and this alteration resulted in an increased sensitivity to anesthetics that act at other target receptors. In support of this notion, the GluR2 subunit plays a pivotal role in regulating the physiologic properties of AMPA receptors. For example, the GluR2 subunit modifies Ca^{2+} flux through AMPA receptors and thereby influences synaptic plasticity, neuronal development, and maintenance and remodeling of synaptic connections.²¹ A reduction in the amplitude of postsynaptic AMPA currents and the ratio of AMPA-NMDA receptor-mediated currents in hippocampal brain slices from GluR2 mutant mice is consistent with a reduction in excitatory transmission.¹³ The C-terminus cytoplasmic tail of the GluR2 subunit interacts with several anchoring proteins in the postsynaptic density and thereby promotes or stabilizes the surface expression of the GluR2-containing AMPA receptors at the synapse.²² A reduced number of AMPA receptors at dendritic synapses could account for reduced excitatory transmission in these mutant neurons.¹³ It is plausible that volatile anesthetics increase inhibitory neurotransmission, and this action unmasks underlying differences in excitatory transmission in the mutant mice.

It might be argued that impaired motor activity reported for GluR2 ($-/-$) mice contributed to the increased sensitivity to anesthetics. However, both the end points of MAC and HPWL required minimum motor activity, yet an increase anesthetic sensitivity was evident for HPWL but not MAC. Furthermore, heteromeric GluR2 mice ($+/-$) demonstrate a normal phenotype but contain mixed populations of GluR2-containing and -deficient AMPA receptors. In a previous study, the authors demonstrated an intermediate sensitivity of heteromeric ($+/-$) mice to pentobarbital compared with homomeric and wild-type littermates for the end point of LORR. Therefore, drug sensitivity correlates with the complement of GluR2-containing receptors rather than an impairment motor function.²³

Immobility and GluR2 Subunit

The spinal cord is generally believed to be the anatomic site that mediates the immobilizing properties of

volatile anesthetics.²⁴ In particular, neurons in the ventral horn activate nocifensive motor responses after noxious stimuli. AMPA receptors are found ubiquitously throughout the laminae of the dorsal horn, at both presynaptic and postsynaptic sites, and AMPA agonists enhance the response to noxious and non-noxious stimuli. Our results demonstrated that null mutant GluR2-deficient mice were no different in their MAC requirements. This observation suggests that the circuitry mediating the tail-clamp withdrawal motor response is independent of GluR2-containing AMPA receptors. Consistent with this postulate, immunocytochemical analyses of rodent spinal cord revealed that GluR2-containing AMPA receptors are almost absent in motor neurons, whereas GluR1 and GluR3 are prevalent.²⁵ The implication that AMPA receptors lacking the GluR2 subunit mediate the motor response is further supported by the finding that an antagonist selective for AMPA receptors that lack the GluR2 subunit (argiotoxin AR636) caused a greater inhibition of motor responses ($\text{EC}_{50} = 2 \mu\text{g}$) compared with sensory responses ($\text{EC}_{50} = 7 \mu\text{g}$).²⁶

Although the GluR2-containing AMPA receptors may not play a specific role in the immobilizing properties of volatile anesthetics, AMPA receptor function nevertheless influences MAC. Pharmacologic blockade of AMPA receptors by the intrathecal injection of a noncompetitive AMPA receptor antagonist (6-nitro-7-sulfamoylbenzo[f]quinoxaline-2,3-dione) produced a dose-dependent and reversible analgesic effect on tail-flick tests in mice.²⁷ Patch-clamp studies of adult mouse hippocampal slices indicate that halothane inhibits glutamate-mediated excitatory postsynaptic currents.⁵ However, halothane did not inhibit responses evoked by exogenous glutamate,²⁸ suggesting a presynaptic action rather than blockade of postsynaptic AMPA receptors accounted for the reduction in excitatory postsynaptic currents.

The immobilizing properties of volatile anesthetics have also been studied in ($-/-$) mice deficient of GABA_A receptor subunits.^{19,29} Converse to our findings, mice lacking the β_3 subunit of the GABA_A receptor were resistant to enflurane and halothane for loss of the tail-clamp reflex compared with wild-type controls but showed no difference in LORR.^{30,31} Although the effect of the β_3 null mutation on MAC values was small, these results were interpreted as indicating that enhanced inhibitory synaptic transmission mediated by GABA_A receptors may contribute to immobility. Taken together, the behavioral studies suggest that immobilization and hypnosis produced by volatile anesthetics are complex phenomenon mediated by multiple receptor populations.

Inhibition of AMPA Receptors by Volatile Anesthetics

The subunit composition of ligand-gated channels influences the potency of the agonist as well as the potency of antagonists. Consistent with our previous ob-

servations in acutely isolated pyramidal neurons, the potency of kainate for receptor activation in cultured neurons was increased in GluR2-deficient receptors.^{12,28,32} In addition, the *in vitro* studies demonstrated the insensitivity of hippocampal AMPA receptors to clinically relevant concentrations of volatile anesthetics. The IC₅₀ values of halothane and isoflurane were fourfold to 11-fold greater than MAC equivalent concentrations, respectively. Furthermore, isoflurane is a more potent general anesthetic compared with halothane for immobilization (halothane > isoflurane > enflurane), whereas the concentrations of halothane and isoflurane required to reduce kainate-evoked current by 50% determined in this study were 1.27 and 1.38%, respectively. These results are consistent with studies of halothane inhibition of excitatory postsynaptic currents recorded in hippocampal pyramidal neurons from 2–3-week-old rats in which the IC₅₀ values were 660 and 590 μ M, respectively.^{28,33} In addition, recombinant AMPA receptors expressed in oocytes were insensitive to clinically relevant concentrations of halothane, isoflurane, and enflurane.³⁴ These data suggest that inhibition of postsynaptic AMPA receptors by volatile anesthetics contributes little to the sensitivity of both the (+/+) and GluR2 (–/–) mice. Inhibition of excitatory postsynaptic potentials from rat hippocampal slice were notably more sensitive to halothane and isoflurane inhibition, possibly because of a predominant presynaptic effect of volatile anesthetics on the vesicular release of glutamate.³⁵

From a clinical perspective, the GluR2 subunit may be down-regulated as a result of several disease processes, including stroke, cerebral palsy, schizophrenia, Alzheimer disease, and amyotrophic lateral sclerosis. Interestingly, despite the likely reductions in GluR2 subunit expression after forebrain ischemia, MAC values for halothane were unchanged in rats subjected to ischemia and reperfusion.^{36,37} These observations are consistent with our results that indicate MAC was unchanged in GluR2 (–/–) mice, although forebrain ischemia may not induce changes in receptor complement at the level of the spinal cord. Our results predict that the hypnotic and analgesic properties of anesthetics would be enhanced in patients suffering from disorders associated with a reduction in GluR2 receptor complement.

In summary, our findings support the concept that multiple populations of receptor mediate the various behavioral end points associated with the anesthetic state. Genetically modified mice provide a tool to examine the molecular basis for changes in anesthetic sensitivity. However, our findings illustrate the challenge of interpreting the basis for altered anesthetic sensitivity in mutant mouse models. To understand drug actions at the behavioral level, investigation at the single receptor must expand to incorporate dynamic neuronal networks.

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