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# $\alpha$1 Subunit-Containing GABA Type A Receptors in Forebrain Contribute to the Effect of Inhaled Anesthetics on Conditioned Fear

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**ABSTRACT**

Inhaled anesthetics are believed to produce anesthesia by their actions on ion channels. Because inhaled anesthetics robustly enhance GABA A receptor ($GABA_A$-R) responses to GABA, these receptors are considered prime targets of anesthetic action. However, the importance of $GABA_A$-Rs and individual $GABA_A$-R subunits to specific anesthetic-induced behavioral effects in the intact animal is unknown. We hypothesized that inhaled anesthetics produce amnesia, as assessed by loss of fear conditioning, by acting on the forebrain $GABA_A$-Rs that harbor the $\alpha$1 subunit. To test this, we used global knockout mice that completely lack the $\alpha$1 subunit and forebrain-specific, conditional knockout mice that lack the $\alpha$1 subunit only in the hippocampus, cortex, and amygdala. Both knockout mice were 75 to 145% less sensitive to the amnestic effects of the inhaled anesthetic isoflurane. These results indicate that $\alpha$1-containing $GABA_A$-Rs in the hippocampus, amygdala, and/or cortex influence the amnestic effects of inhaled anesthetics and may be an important molecular target of the drug isoflurane.

Inhaled anesthetics produce anesthesia by unknown mechanisms. The prevailing theory, initially proposed by Franks and Lieb (1984), posits that specific protein targets in the nervous system are the molecular sites of action of inhaled anesthetics. Numerous putative protein targets have been identified, including a wide variety of ion channels (Campagna et al., 2003). However, the contribution that each of these targets makes to whole-animal behavioral responses to inhaled anesthetics is not clear.

A plausible target that has received considerable attention is the GABA A receptor ($GABA_A$-R). The $GABA_A$-R is a five-subunit chloride channel activated by GABA and muscimol and is blocked competitively by bicuculline and noncompetitively by picrotoxin (Olsen, 1982). Eccles et al. (1963) noted that many general anesthetics prolong the inhibition of spinal motoneurons, an effect mediated by GABA. Nicoll (1972) suggested that GABA-mediated enhancement of synaptic inhibition might underlie anesthetic actions. Consistent with this suggestion, Pearce et al. (1989) reported that anesthetics greatly prolong the time course of recurrent inhibition in the rat hippocampus. However, our knowledge of the importance of $GABA_A$-Rs and individual $GABA_A$-R subunits to anesthetic-induced behavioral effects remains incomplete.

Inhaled anesthetics produce two universal clinical effects: amnesia for events during surgery, and immobility in response to noxious stimulation (e.g., surgical incision). Although the primary neuroanatomic site at which inhaled anesthetics act to produce immobility is the spinal cord (Antognini and Schwartz, 1993; Rampil et al., 1993), supraspinal structures probably mediate amnestic effects. A plausible site of action by which inhaled anesthetics interfere with memory is the hippocampus, where $GABA_A$-Rs participate in memory formation (Bailey et al., 2002; Collinson et al., 2002).

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**ABBREVIATIONS:** $GABA_A$-R, GABA type A receptor; MAC, minimum alveolar concentration; LORR, loss of righting reflex; Ro 15-4513, ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*]benzodiazepine-3-carboxylate; TBPS, *t*-butylbicyclophosphorothionate.

The $\alpha$1 subunit seems to be of particular importance in this process. The $\alpha$1 subunit of the $GABA_A$-R is the most abundant $\alpha$ subunit in the adult brain (McKernan and Whiting, 1996) and is expressed at high levels in many brain regions, including the hippocampus (Sperk et al., 1997). Benzodiazepines, which only act by allosterically enhancing the action of GABA, specifically cause amnesia by an action on the $\alpha$1 $GABA_A$-R (Rudolph et al., 1999).

This study investigated the hypothesis that $\alpha$1 subunit-containing hippocampal $GABA_A$-Rs partly mediate the amnesia caused by inhaled anesthetics. We used genetically engineered mice that completely lack the $\alpha$1 subunit (Vicini et al., 2001) in all cells of the body (i.e., a global knockout) and mice that conditionally lack the $\alpha$1 subunit in restricted neuronal populations (i.e., forebrain-specific knockout) to address this hypothesis. We assessed amnesia by Pavlovian fear conditioning. For comparison, we tested whether either knockout would influence the capacity of an inhaled anesthetic to produce loss of the righting reflex or suppression of nociceptive reflexes. We predicted that these effects would not be influenced by the $\alpha$1 subunit.

## Materials and Methods

**Mouse Production.** Global $\alpha$1 knockout mice were produced as described previously (Vicini et al., 2001). Mice heterozygous for a floxed $\alpha$1 allele (exon 8 flanked by loxP sites) and a cre-recombined, inactive $\alpha$1 allele that lacks exon 8 were interbred to produce homozygous floxed control mice and heterozygous and homozygous global knockout mice. Expression of the unrecombined floxed allele does not differ from wild-type $\alpha$1 expression; the recombined $\alpha$1 allele is a true null allele (Vicini et al., 2001; Kralic et al., 2002). Global knockout mice and control mice were of a mixed C57BL/6J X strain 129S1/X1 $\times$ FVB/N hybrid background (Vicini et al., 2001) of the $F_{6+}$ generation.

$\alpha$CamKII-cre transgenic mice, line T29-1 (Tsien et al., 1996), were crossed with B6;129S4-*Gt(ROSA)26Sor*$^{tm1Sor}$*/J* (Soriano, 1999) or B6.Cg-*Tg(xstpx-lacZ)32*$^{And}$/J (Zinyk et al., 1998) reporter mice obtained from The Jackson Laboratory (Bar Harbor, ME). Adult (56 days of age) $F_1$ generation mice from these crosses were analyzed for functional $\beta$-galactosidase activity to reveal tissue-specific patterns of cre-mediated recombination, as described below.

Crossing the $\alpha$CamKII-cre transgene (Tsien et al., 1996) onto the $\alpha$1 floxed background (Vicini et al., 2001) produced forebrain-specific $\alpha$1 knockout mice. Breeding pairs were used in which the male lacked the $\alpha$CamKII-cre transgene (Cre-) but was homozygous floxed $\alpha$1, and the female was hemizygous for the $\alpha$CamKII-cre transgene (Cre+) and homozygous for the floxed $\alpha$1 gene. Conditional knockout mice and control mice were of a mixed C57BL/6J X strain 129Sv/SvJ hybrid background of the $F_{6-9}$ generation.

All mice were maintained under a 12-h light/dark schedule with lights on at 7:00 AM. Mice were group housed, provided ad libitum access to food and water, and genotyped by Southern blot analysis. All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health and were approved by the Institutional Animal Care and Use Committees at the University of California at San Francisco and the University of Pittsburgh.

**Histology and Immunohistochemistry.** Tissue sections derived from mice at 8 weeks of age were analyzed for cre-activated $\beta$-galactosidase staining to determine the extent of recombination throughout the brain using standard techniques (Tsien et al., 1996). Slides were counterstained with eosin and examined by light microscopy.

For $GABA_A$-R $\alpha$1 immunostaining, animals (56 days of age) were deeply anesthetized with pentobarbital (Nembutal 40 mg/kg; Ovation Pharmaceuticals Inc., Deerfield, IL) and perfused transcardially (Fritschy and Mohler, 1995). The $GABA_A$-R $\alpha$1 subunit was visualized in 40-$\mu$m sections processed for immunoperoxidase staining with subunit-specific antisera raised against amino acids 1 to 16 of the $\alpha$1 subunit (Gao et al., 1993). Free-floating sections were washed three times for 10 min each in Tris buffer (Tris saline, pH 7.4, and 0.05% Triton X-100) and incubated at 4°C overnight in primary antibody solution (1:20,000) diluted in Tris buffer containing 2% normal goat serum. Sections were then washed three times for 10 min each in Tris buffer and incubated in biotinylated secondary antibody solution (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) diluted 1:300 in Tris buffer containing 2% normal goat serum for 30 min at room temperature. After additional washing, sections were transferred to the avidin-peroxidase solution (Vectastain Elite Kit; Vector Laboratories, Burlingame, CA) for 20 min, washed, and processed using diaminobenzidine hydrochloride (Sigma-Aldrich, St. Louis MO) as the chromogen. Slides were air-dried, dehydrated with ascending series of ethanol, cleared with xylene, and coverslipped with Eukitt (Electron Microscopy Services, Hatfield, PA). Changes in regional distribution of $GABA_A$-R $\alpha$1 subunits were analyzed by light microscopy (Zeiss Axioplan microscope; Carl Zeiss Inc., Thornwood, NY).

**Ligand Autoradiography.** Whole brains from 5 adult (11.6–12.7 weeks of age) male mice of each genotype were used. The autoradiographic procedures for regional localization of the benzodiazepine (labeled with [$^3$H]Ro 15-4513; PerkinElmer Life and Analytical Sciences, Boston, MA), GABA ([$^3$H]muscimol; PerkinElmer Life and Analytical Sciences) and convulsant ionophoric (*t*-butylbicyclophosphoro[$^{35}$S]thionate, [$^{35}$S]TBPS; PerkinElmer Life and Analytical Sciences) binding sites were as described previously (Mäkelä et al., 1997). Nonspecific binding was determined with 10 $\mu$M flumazenil (donated by F. Hoffmann-La Roche, Basel, Switzerland), 100 $\mu$M picrotoxinin (Sigma-Aldrich), and 100 $\mu$M GABA (Sigma-Aldrich) in [$^3$H]Ro 15-4513, [$^{35}$S]TBPS, and [$^3$H]muscimol assays, respectively.

Autoradiography films were quantified using AIS image analysis system (Imaging Research, St. Catherines, ON, Canada) as described previously (Mäkelä et al., 1997). Binding densities for each brain area were averaged from measurements of one to three sections per brain. The standards exposed simultaneously with brain sections were used as reference with the resulting binding values given as radioactivity levels estimated for gray matter areas (in nanocuries per milligram for $^3$H and nanocuries per gram for $^{14}$C). The significance of the differences between the mouse lines in each brain region was assessed with analysis of variance followed by Tukey-Kramer post-test.

**Fear Conditioning and Minimum Alveolar Concentration Determinations.** At 8 to 12 weeks of age, 99 control, 154 heterozygous, and 100 homozygous $GABA_A$-R $\alpha$1 global knockout mice, and 40 control and 37 conditional $GABA_A$-R $\alpha$1 knockout mice were studied.

Fear conditioning was performed as described previously (Eger et al., 2003). In brief, animals were exposed to target concentrations of isoflurane in oxygen or oxygen alone (control) for 30 min and then were placed in a gas-tight training chamber containing the same concentration of isoflurane. After allowing 3 min for exploration of the training chamber, a 90-dB, 2-kHz tone sounded, coterminating with a 2-s foot shock. This was repeated twice, with 1 min between tones. Foot shock intensity varied between 1 and 3 mA as a function of anesthetic concentration to equalize the response of mice to the foot shock. Animals were observed by closed-circuit television.

The following day, fear to tone was tested by placing animals in a different context. After allowing 3 min for exploration, the animals were exposed to a 90-dB, 2-kHz tone for 8 min and then were immediately returned to the animal's home container. Fear to context was tested later that day by placing the mice in the original training chamber for 8 min with no tone imposed. Animals were observed by closed-circuit television. Fear was assessed by behav-

ioral freezing (i.e., immobility except for respiration) and was measured every 8 s for 8 min per mouse by a blinded observer. The number of freezes of the 60 measurements gave the probability of freezing ("freeze score") for each animal.

The minimum alveolar concentration (MAC) of anesthetic preventing movement in 50% of animals in response to a noxious stimulus (a tail clamp) is a standard $EC_{50}$ measure of anesthetic potency. MAC values for desflurane, halothane, and isoflurane were measured as described previously (Sonner et al., 2000).

For each anesthetic, MAC values and regression parameters estimated in calculating amnestic $EC_{50}$ values for different genotypes were compared using either an analysis of variance with a Student-Newman-Keuls test for post hoc multiple comparison testing or a Student's $t$ test. Nonlinear regression was performed to calculate an $EC_{50}$ value and the maximum value of the dose-response curve (A) for fear conditioning according to the following equation:

$$\text{Freeze Score} = A \times \left(1 - \frac{\text{isoflurane}^n}{\text{isoflurane}^n + \text{ED}_{50}{}^n}\right)$$

Means ± S.E. are reported except where otherwise noted. $P \leq 0.05$ was considered statistically significant.

**Loss of Righting Reflex.** Groups of six to eight mice (8–19 weeks old; 15.9–33.1 g) were tested for loss of righting reflex (LORR) in individual wire-mesh cages in a rotating carrousel enclosed in a sealed acrylic chamber as described previously (Homanics et al., 1997; Quinlan et al., 1998). Halothane and isoflurane (both from Halocarbon Laboratories, River Edge, NJ) mixed with oxygen were monitored with an infrared anesthesia analyzer (Datex-Ohmeda Inc., Andover, MA). Constant anesthetic concentrations were supplied for 15 min before testing. A blinded observer scored the mice as positive for LORR if they passively rolled twice in a 75-s time period while the carousel rotated at 4 rpm. Mice tested with both volatile drugs were given at least 7 days to recover between anesthetics.

**Sleep Time.** The sleep time (duration of the loss of the righting reflex) was used to assess the sedative/hypnotic effects of pentobarbital (45 mg/kg, i.p.) and zolpidem (60 mg/kg, i.p.). Normothermia was maintained with a heat lamp.

## Results

**Production and Characterization of Genetically Engineered Mice.** To test the role of $\alpha$1 GABA$_A$-Rs in specific brain regions in anesthetic mechanisms, we set out to create conditional $\alpha$1 knockout mice in which the $\alpha$1 gene was inactivated only in hippocampal pyramidal cells of the CA1 region. We obtained $\alpha$CamKII-cre mice purported to induce postnatal hippocampal CA1 pyramidal cell-specific recombination (Tsien et al., 1996). However, when we crossed this mouse line with the *Gt(ROSA)26$^{Sor}$* floxed $\beta$-galactosidase reporter mouse line (Soriano, 1999), we observed high levels of recombination-induced $\beta$-galactosidase expression in many areas of the hippocampus in addition to CA1 and a lower level of staining in cortex and amygdala, but no staining in other areas of the brain (Fig. 1, A–C, F, and I). We also tested the $\alpha$CamKII-cre mice by crossing to the *Tg(xstpx-lacZ)32$^{And}$*/J floxed $\beta$-galactosidase reporter mouse line (Zinyk et al., 1998). This reporter mouse line revealed hippocampal CA1-specific staining (data not shown) as originally reported by Tsien et al. (1996). Thus, the pattern of recombination seems to depend on the reporter mouse line used.

We next created tissue-specific conditional $\alpha$1 knockout mice by crossing $\alpha$CamKII-cre transgenic mice (Tsien et al., 1996) with floxed $\alpha$1 mice (Vicini et al., 2001). Immunohistochemistry with an $\alpha$1-specific antibody showed a pattern of reduction of $\alpha$1 staining in the conditional knockout mice which paralleled that observed in the *Gt(ROSA)26$^{Sor}$* $\beta$-galactosidase reporter mouse study. This indicated successful creation of a forebrain-specific $\alpha$1 knockout mouse line in which $\alpha$1 is selectively inactivated in hippocampus, cortex, and amygdala. The pattern of staining was as follows. In control mice, $\alpha$1 immunoreactivity was found on cell bodies and on densely packed dendritic processes in all layers of the cerebral cortex (Fig. 1D). Staining for $\alpha$1 was decreased in the outer cortical layers (Fig. 1E) of the mutant in a pattern similar to that shown in Fig. 1C. A loss of $\alpha$1 immunoreactivity was observed in the CA1 region and subiculum of the mutant hippocampus and to a lesser extent in the CA3 and dentate gyrus (Fig. 1H) compared with control (Fig. 1G). The staining pattern follows the expected loss of $\alpha$1 on pyramidal cells because $\alpha$1 on this cell type is localized mostly to dendritic processes (Fritschy and Mohler, 1995; Sperk et al., 1997). Note that staining does not completely disappear in

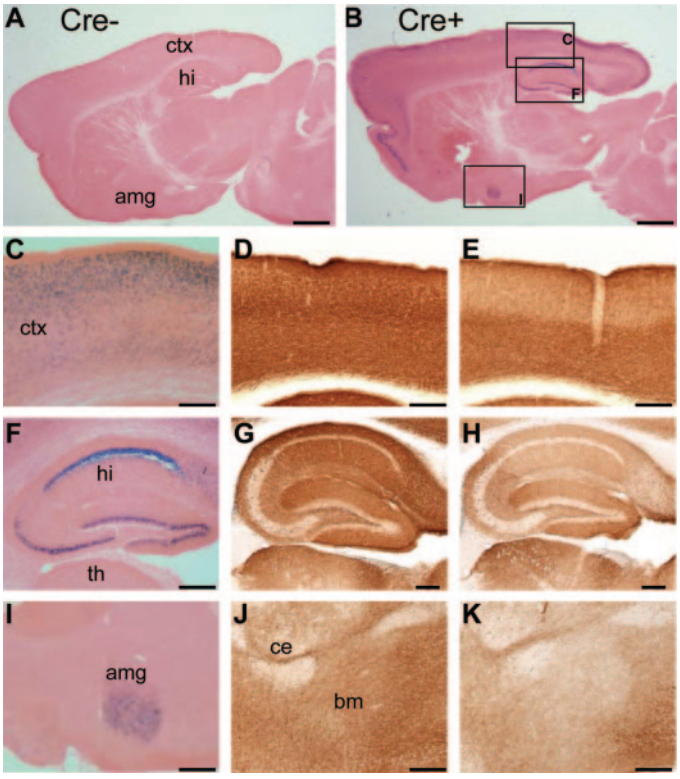


**Fig. 1.** A, in $\beta$-galactosidase staining of a sagittal section from a mouse that harbors only the $\alpha$CamKII-cre transgene, no cells are stained blue. B, $\beta$-galactosidase staining (blue cells) in a sagittal section from a mouse that harbors the $\alpha$CamKII-cre and ROSA26 reporter transgenes indicates regions of the hippocampus, cerebral cortex, and amygdala that have undergone cre-mediated recombination of the reporter. (C, F, and I, higher magnification view of areas boxed in B shows $\beta$-galactosidase staining in cortex, hippocampus, and amygdala, respectively. D and E, immunostaining for $\alpha$1 in cortex of control and conditional $\alpha$1 knockout, respectively, shows decreased $\alpha$1 staining in outer cortical layers of mutant compared with control mice. G and H, immunostaining for $\alpha$1 in hippocampus of control and conditional $\alpha$1 knockout, respectively, shows decreased $\alpha$1 staining in CA1 and subiculum and, to a lesser extent, in CA3 and dentate gyrus but not in the thalamus of the mutant compared with control. J and K, immunostaining for $\alpha$1 in amygdala of control and conditional $\alpha$1 knockout, respectively, shows decreased $\alpha$1 staining in basomedial nucleus of amygdala of mutant compared with control. Scale bars, 1 mm (A and B) and 200 $\mu$m (C–K). amg, amygdala; bm, basomedial nucleus of amygdala; ce, central nucleus of amygdala; ctx, cerebral cortex; hi, hippocampus; th, thalamus.

these areas because the $\alpha$1 gene was not inactivated in hippocampal interneurons as observed, for example, by the presence of $\alpha$1-stained interneurons in the CA3 region of both control and mutant mice. A reduction in $\alpha$1 staining was observed in the basomedial (Fig. 1K) and basolateral amygdala (data not shown) of mutant mice compared with control (Fig. 1J). Staining for $\alpha$1 in other brain regions of mutant mice did not differ from control.

Ligand autoradiography was used to visualize the brain regional distribution of binding sites. We used three different ligands, each known to have heterogenous brain regional distribution (Korpi et al., 2002a). Flumazenil-sensitive benzodiazepine sites labeled by [$^3$H]Ro 15-4513 were rather evenly distributed throughout the brain, with the lowest binding densities being observed in the thalamus (Fig. 2A). The conditional knockout mice had significantly lower binding only in the cerebral cortex of the brain horizontal sections compared with control mice (Fig. 2A and Table 1). This ligand detects primarily the $\gamma$2 subunit-containing $GABA_A$-Rs, irrespective of the $\alpha$ subunits in the receptor subunit complex (Pritchett et al., 1989; Lüddens and Korpi, 1996). GABA-sensitive [$^3$H]muscimol binding resulted in a different pattern, labeling strongly the cerebellar granule cell layer and more faintly the thalamus, cerebral cortex, caudate-putamen, and the olfactory bulbs in both mouse lines (Fig. 2B). This binding is largely caused by the high-affinity binding to $\delta$ subunit-containing receptors, which are assembled in the forebrain mostly with $\alpha$4 subunits and in the cerebellum with the $\alpha$6 subunits, because [$^3$H]muscimol binding signal is abolished in $\alpha$6- and $\delta$-deficient mouse brains (Korpi et al., 2002b). Quantification of this signal in the conditional knockout mice and control mice failed to reveal any differences in the brain regions analyzed (Table 1). Finally, we applied picrotoxin-sensitive [$^{35}$S]TBPS binding to label the $GABA_A$ receptor-associated ionophores. The labeling pattern of [$^{35}$S]TBPS again differed from the previous ligands (Fig. 2C). With this ligand, several brain regions were less labeled in the conditional knockout mice than in the control mice. These regions included the cerebral cortex, caudate-putamen, and hippocampus (Table 1). There were no differences in the thalamus and cerebellum (Table 1) or in the strongly labeled inferior colliculus (Fig. 2C).

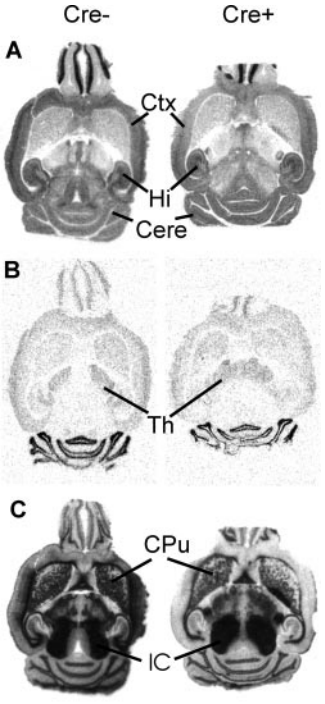


**Fig. 2.** Representative autoradiographic images show the flumazenil-sensitive benzodiazepine site-binding labeled by [$^3$H]Ro 15-4513 (A), the GABA-sensitive agonist site binding labeled by [$^3$H]muscimol (B), and the picrotoxin-sensitive ion channel site binding labeled by [$^{35}$S]TBPS (C) for the Cre-negative (controls) and Cre-positive (conditional knockout) $GABA_A$-R $\alpha$1 mice. The images were scanned with identical brightness and contrast in both genotypes. Ctx, cerebral cortex; Th, thalamus; Hi, hippocampus; CPu, caudate-putamen; Cere, cerebellar cortex; IC, inferior colliculus.

**Global Deletion of $\alpha$1 Subunits Reduced the Amnestic Effects of Isoflurane.** Homozygous global knockout mice were ~2.3-fold more resistant to the amnestic effects of isoflurane, as assessed by fear to context, compared with control littermates ($p < 0.05$; Fig. 3A). Mice heterozygous for the global knockout were also more resistant to the amnestic effects of isoflurane compared with control mice ($p < 0.05$; Fig. 3A). Baseline freeze scores in the absence of anesthetic did not differ by genotype: wild-type mice had a freeze score of 38.8 ± 6.8%; heterozygous mice, 45.3 ± 5.5%; and knockout mice, 40.7 ± 11.9% without anesthetic.

Genotype influenced the baseline responses for tone conditioning in the absence of anesthetic, making the comparison of $EC_{50}$ values problematic. Wild-type animals had a starting freeze score of 49.4 ± 9.6%; heterozygous mice, 62.5 ± 4.8%; and knockout mice, 77.8 ± 9.2%. This problem was surmounted, as noted below, in the conditional knockout of the

TABLE 1

Autoradiographic analysis of $GABA_A$-R binding sites in horizontal sections of control and conditional $GABA_A$-R $\alpha$1 subunit knockout mice

The data are means ± S.D. for five mice in each genotype and are expressed as nanocuries per milligram for $^3$H ligands and as nanocuries per gram for $^{35}$S ligands.

| Ligand | Brain Region | | | | |
|---|---|---|---|---|---|
| | Ctx | Th | Hi | CPu | Cere |
| [$^3$H]Ro 15-4513 binding to benzodiazepine sites | | | | | |
| Cre−, $\alpha$1F/F mice | 121 ± 10 | 50 ± 3 | 115 ± 5 | 51 ± 4 | 97 ± 5 |
| Cre+, $\alpha$1F/F mice | 90 ± 13* | 47 ± 7 | 108 ± 6 | 44 ± 6 | 103 ± 9 |
| [$^3$H]Muscimol binding to GABA sites | | | | | |
| Cre−, $\alpha$1F/F mice | 12.3 ± 1.4 | 17.5 ± 5.4 | 8.2 ± 1.3 | 8.8 ± 2.0 | 35.4 ± 5.6 |
| Cre+, $\alpha$1F/F mice | 11.5 ± 3.1 | 15.0 ± 1.0 | 8.1 ± 1.9 | 8.3 ± 1.2 | 31.8 ± 5.3 |
| [$^{35}$S]TBPS binding to ionophore sites | | | | | |
| Cre−, $\alpha$1F/F mice | 226 ± 54 | 503 ± 94 | 157 ± 26 | 338 ± 32 | 122 ± 26 |
| Cre+, $\alpha$1F/F mice | 70 ± 14** | 472 ± 128 | 121 ± 22* | 193 ± 36** | 115 ± 21 |

Significance of the difference from control (Student's $t$ test): * $P < 0.01$; ** $P < 0.001$.
Ctx, cerebral cortex; Th, thalamus; Hi, hippocampus; CPu, caudate-putamen; Cere, cerebellar cortex.

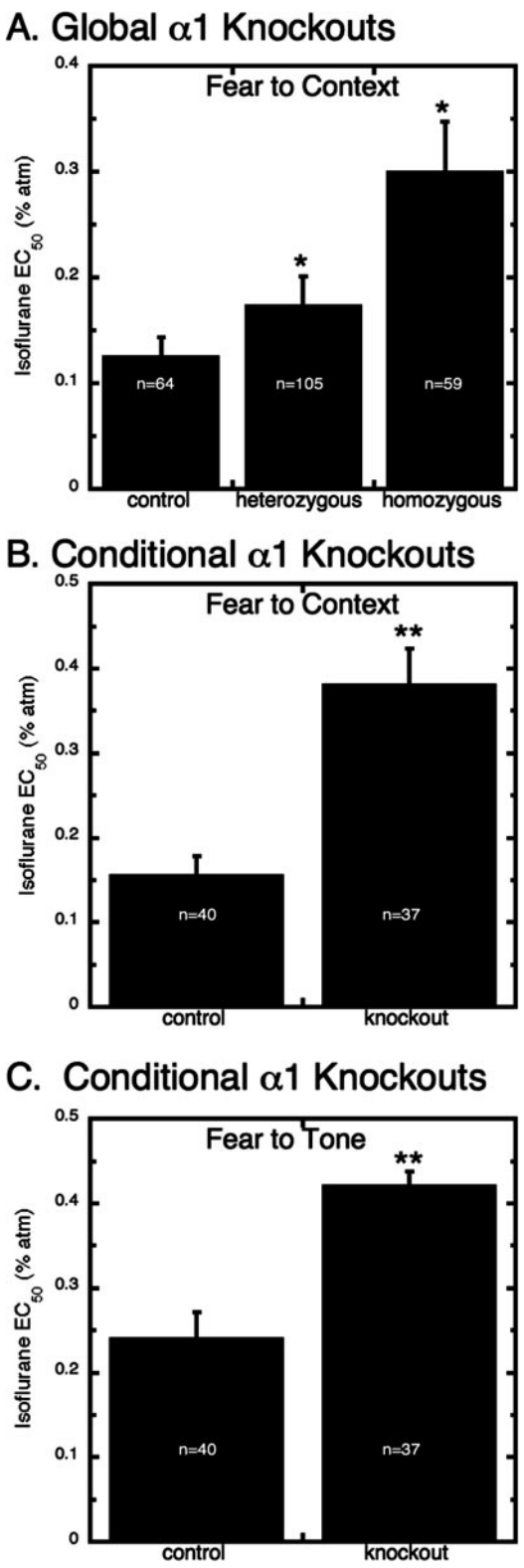


**Fig. 3.** A, global knockout of the GABA$_A$-R $\alpha$1 subunit significantly increases the $EC_{50}$ value at which isoflurane interferes with fear conditioning to context in heterozygous and homozygous knockout mice compared with control mice. B, forebrain-specific knockout of the GABA$_A$-R $\alpha$1 subunit significantly increases the $EC_{50}$ value at which isoflurane interferes with fear conditioning to context compared with control mice. C, forebrain-specific knockout significantly increases the $EC_{50}$ value at which isoflurane interferes with fear conditioning to tone compared with control mice. Values presented are the mean ± S.E.M.; ⋆, $P < 0.05$; ⋆⋆, $P < 0.001$.

$\alpha$1 GABA$_A$-R, in which the different genotypes showed similar baseline responses.

**Forebrain-Specific Deletion of $\alpha$1 also Reduced Amnestic Effects of Isoflurane.** For contextual fear conditioning, conditional deletion of the $\alpha$1 GABA$_A$-R increased the isoflurane amnestic $EC_{50}$ value compared with control mice by 145% (Fig. 3B; $p < 0.001$). The baseline responses in the absence of anesthetic did not differ between genotypes (freeze scores were 58.1 ± 11.0 and 76.8 ± 6.7% for knockout and wild-type mice, respectively; $p = 0.18$).

For tone fear conditioning, the conditional deletion increased the isoflurane amnestic $EC_{50}$ values compared with control mice by 75% (Fig. 3C; $p < 0.001$). The baseline responses in the absence of anesthetic (82.2 ± 5.3 and 82.7 ± 8.7% for knockout and wild-type mice, respectively) did not differ for the two genotypes.

**Deletion of $\alpha$1 Minimally Affected Other Anesthetic-Induced Behaviors.** Isoflurane MAC did not differ between control, heterozygous, and global $\alpha$1 knockout littermates (Table 2). As would be expected from the importance of the spinal cord for the capacity of inhaled anesthetics to produce immobility, MAC did not differ between control and littermate forebrain-specific knockout mice for halothane or desflurane (Table 2). There was a small (11%) increase in MAC for isoflurane ($p < 0.05$; Table 2).

Global knockout mice were less sensitive to the effects of halothane compared with control mice for LORR, but by only 19% (Table 3; $p < 0.0001$). Global knockout mice had comparable $EC_{50}$ values for isoflurane-induced LORR. LORR $EC_{50}$ values were similar for conditional knockout mice and control mice for both isoflurane and halothane.

Sleep time of forebrain-specific $\alpha$1 knockout mice (23.2 ± 2.4 min, mean ± S.E.M.; $n = 19$) in response to zolpidem did not differ from control mice (19.6 ± 3.1; $n = 15$). Furthermore, sleep time induced by pentobarbital in forebrain-specific $\alpha$1 knockout mice (46.8 ± 2.9; $n = 19$) also did not differ from control mice (50.5 ± 3.1; $n = 13$).

## Discussion

These studies reveal two novel findings about the mechanism of action of inhaled anesthetics. First, we demonstrated that $\alpha$1-containing GABA$_A$-Rs contribute to the amnestic effects of isoflurane, as assessed by fear conditioning in two different mouse lines lacking these receptors. The more than 2-fold increase in amnestic $EC_{50}$ values we observed is among the largest relative increase in anesthetic effect ever observed. Second, these studies provide novel insight into the brain regions that are responsible for the amnestic effects of inhaled anesthetics. GABA$_A$-Rs containing the $\alpha$1 subunit in hippocampus, amygdala, and/or cortex seem to be critically important in the amnestic effects of isoflurane. This is the first demonstration of specific brain regions involved in this universal effect of inhaled anesthetics.

We found that mice that completely lacked the $\alpha$1 subunit (i.e., global $\alpha$1 knockout mice), were 139% less sensitive to the amnestic effects of isoflurane as assessed by fear conditioning to context compared with control littermates (Fig. 3A). These results parallel results from other studies which demonstrate that benzodiazepines cause amnesia by interacting primarily with the $\alpha$1 subunit (Rudolph et al., 1999).

We hypothesized that the hippocampus, because of its cen-

tral role in learning and memory, mediates the amnestic effects of inhaled anesthetics. To test this hypothesis, we used conditional $\alpha$1 knockout mice that selectively lacked the $\alpha$1 $GABA_A$-R subunit in forebrain structures including the hippocampus, cortex, and amygdala. Like the global knockout mice, forebrain-specific $\alpha$1 knockout mice also resisted the amnestic effects of isoflurane. The $EC_{50}$ values for isoflurane increased 145 and 75% compared with control values for fear to context and fear to tone, respectively (Fig. 3, B and C). Both tone and context conditioning depend on the amygdala; in addition, context conditioning is hippocampus-dependent (Fendt and Fanselow, 1999). Because this conditional knockout affected $\alpha$1 subunits in the amygdala, it is not surprising that the amnestic effect of isoflurane decreased for both tone and context conditioning. Furthermore, the greater decrease in context conditioning may be attributed to the additional dependence on the hippocampus for this behavior.

In contrast to an important role of $\alpha$1 $GABA_A$-Rs in amnesia, these $GABA_A$-R isoforms seem to contribute little to MAC. Global knockout mice did not differ in response to isoflurane for MAC. Conditional knockout mice were slightly less sensitive to isoflurane but were normally sensitive to halothane and desflurane for MAC. Thus, the $\alpha$1 subunit of the $GABA_A$-R seems to contribute little to the mechanism by which these inhaled drugs produce immobility in response to a noxious stimulus. Studies of other $GABA_A$-R mutant mice have also failed to find an effect of subunit deletion on MAC (Homanics et al., 1997; Mihalek et al., 1999), although $\beta$3 knockout mice and point mutants are an exception because they are modestly less sensitive to inhaled anesthetics (MAC is increased by approximately 20%) (Quinlan et al., 1998; Jurd et al., 2002). These results from mutant mice are in accord with our pharmacological studies demonstrating that GABA plays at most a minor role in the immobilizing effect of anesthetics (Sonner et al., 2003).

$GABA_A$-Rs containing the $\alpha$1 subunit similarly do not seem to be key mediators of inhaled anesthetic action for the righting-reflex behavioral endpoint. Although global knockout mice were slightly less sensitive to halothane for LORR, they did not differ in response to isoflurane. Conditional knockout mice did not differ from control mice in response to either halothane or isoflurane. Studies of other $GABA_A$-R mutant mice have also failed to find a robust effect of $GABA_A$-R manipulation on the righting reflex for inhaled anesthetics (Homanics et al., 1997; Quinlan et al., 1998; Mihalek et al., 1999; Jurd et al., 2002).

To produce the conditional knockout mouse line, we relied on tissue-specific inactivation of the $\alpha$1 gene using cre-lox technology. The mouse line used to direct tissue-specific expression of cre recombinase was a transgenic mouse line that had been reported to induce hippocampal CA1 pyramidal cell-specific recombination (Tsien et al., 1996). We did observe CA1-specific recombination when we tested this transgenic mouse line by crossing with the *Tg(xstpx-lacZ)32*$^{And}$/J reporter mouse line (Zinyk et al., 1998). However, we observed that this $\alpha$CamKII-cre mouse line induced recombination in a much broader tissue-specific pattern when mated to the *Gt(ROSA)26*$^{Sor}$ reporter line (Soriano, 1999) and to our floxed $\alpha$1 mouse line (Fig. 1). We found recombination in CA1, CA3, and dentate gyrus of the hippocampus, the amygdala, and the cortex.

Ligand autoradiographic analyses of brain $GABA_A$-R binding activities also documented forebrain, but not cerebellar, alterations in the conditional knockout animals. First, [$^3$H]muscimol binding, which depends on $\alpha$6 subunits in the cerebellum and on $\delta$ subunits in the forebrain (Korpi et al., 2002b), was not altered. This excludes a general alteration in the transcription of $GABA_A$-R subunit genes located on chromosome 11 close to the $\alpha$1 subunit (i.e., the $\alpha$6 and $\gamma$2 genes) (Russek, 1999). This contrasts with targeting of the $\alpha$6 subunit, which resulted in attenuated transcription of $\alpha$1 and $\beta$2 genes (Uusi-Oukari et al., 2000). Second, [$^3$H]Ro 15-4513 is a benzodiazepine site ligand that has little subtype-selectivity between $\alpha$ subunits, but the $\gamma$2 subunit is obligatory for its high-affinity binding (Lüddens and Korpi, 1996). The small alterations in its binding to the cortical and hippocampal region of the forebrain-specific $\alpha$1 knockout mice may reflect the fact that $\alpha$1 is not inactivated in all cells in these brain regions. Third, [$^{35}$S]TBPS autoradiography reflects most receptor subtypes, but much less is known about its subtype-dependence compared with [$^3$H]muscimol and [$^3$H]Ro 15-4513. In recombinant receptor studies, [$^{35}$S]TBPS binding is

TABLE 3
Loss of righting reflex assay results for global and conditional $\alpha$1 $GABA_A$-R subunit knockout mice
Values in parentheses indicate the number of male and female mice, respectively.

| Genotype | *n* | Anesthetic | $EC_{50}$ |
|---|---|---|---|
| | | | *atm% ± S.E.* |
| Control | 17 (9/7) | Halothane | 0.82 ± 0.02 |
| Global KO | 19 (14/5) | | 0.98 ± 0.02* |
| Control | 18 (9/9) | Isoflurane | 0.80 ± 0.01 |
| Global KO | 18 (14/4) | | 0.80 ± 0.02 |
| Control | 12 (6/6) | Halothane | 0.94 ± 0.03 |
| Conditional KO | 10 (5/5) | | 0.92 ± 0.03 |
| Control | 11 (5/6) | Isoflurane | 0.79 ± 0.02 |
| Conditional KO | 11 (5/6) | | 0.76 ± 0.03 |

* $P < 0.0001$.
KO, knockout.

TABLE 2
MAC values for global and conditional $GABA_A$-R $\alpha$1 subunit knockout mice
Values in parentheses represent *n*. MAC values are reported as mean ± S.D. (*n* = number of animals studied).

| Genotype | MAC | | |
|---|---|---|---|
| | Isoflurane | Halothane | Desflurane |
| Control | 1.54 ± 0.17 (6) | N.D. | N.D. |
| Heterozygous global knockout | 1.59 ± 0.11 (10) | N.D. | N.D. |
| Homozygous global knockout | 1.55 ± 0.11 (9) | N.D. | N.D. |
| Control | 1.37 ± 0.16 (10) | 1.27 ± 0.11 (8) | 7.24 ± 0.36 (9) |
| Conditional knockout | 1.52 ± 0.08 (10)* | 1.25 ± 0.11 (8) | 7.49 ± 0.50 (10) |

* $P < 0.05$.
N.D., not determined.

not always formed by theoretically relevant subunit combinations (Lüddens and Korpi, 1995). However, it is clear that $\alpha$1 subunit-containing receptors do bind [$^{35}$S]TBPS efficiently (Korpi and Lüddens, 1993). This is consistent with the present findings, because we observed the greatest reductions in the cortical and hippocampal regions. We also had global $\alpha$1 knockout brains in the same assay, and they showed widespread reduction in both [$^{35}$S]TBPS and [$^{3}$H]Ro 15-4513 autoradiographic signals (Kralic et al., 2002) but no alteration in [$^{3}$H]muscimol signal (data not shown). The reduction of [$^{35}$S]TBPS binding in the caudate-putamen is difficult to explain, because cre expression is low there, as is the expression of $\alpha$1 subunits (Korpi et al., 2002a). [$^{35}$S]TBPS binding was reduced in this brain region also in the global $\alpha$1 knockout mice (Kralic et al., 2002).

In summary, the immunohistochemical and autoradiographic analyses indicate that the conditional $\alpha$1 knockout mice have regional alterations in GABA$_A$-R subtypes restricted to forebrain. The differences in recombination patterns observed in the present studies and those of Tsien et al. (1996) are most likely caused by differences in accessibility of the floxed loci to the recombinase. This may be influenced by differences in genetic background between mouse lines. The differences are not caused by age-dependent changes in the pattern of cre expression because recombination was studied in mice at 8 weeks of age in the present studies and in those of Tsien et al. (1996).

A caveat to these experiments is that global deficiency of a GABA$_A$-R gene may induce compensatory changes in other genes and/or gene products (Brickley et al., 2001; Korpi et al., 2002b; Peng et al., 2002). We have observed an increase in $\alpha$2 and $\alpha$3 subunits and a decrease in $\beta$2/3 and $\gamma$2 subunits in $\alpha$1 global knockout mice (Kralic et al., 2002). Such changes should be less widespread in the conditional knockout animals. Note that the magnitude of changes in amnestic and other effects did not differ between global and conditional knockout mice. However, it is possible that compensation occurred in the cells of these conditional mice in which the $\alpha$1 gene was inactivated. This caution must be considered when interpreting the results of our studies. Thus, compensatory changes, rather than a direct effect on $\alpha$1-containing receptors, could mediate the phenotypic changes in anesthetic responsiveness.

The gene knock-in approach has recently been used to determine the contribution of GABA$_A$-R subunits to the behavioral effects of benzodiazepines and intravenous anesthetics (Rudolph et al., 1999; Low et al., 2000; Jurd et al., 2002; Reynolds et al., 2003; Cope et al., 2004). These studies created individual mouse lines with point mutations that reduced/eliminated sensitivity to these drugs. The results provide compelling evidence for subunit-specificity in behavioral response to these drugs. Furthermore, these studies demonstrate that different anesthetics have different molecular mechanisms of action, and different mechanisms can mediate different behavioral responses to the same drug. The results presented here build on these studies and suggest that inhaled anesthetics may also have distinct mechanisms of action for specific behavioral endpoints. These studies support the multisite agent-specific mechanism of anesthetic action (Pittson et al., 2004).

In summary, using mice harboring global and conditional knockout mice of the $\alpha$1 subunit of the GABA$_A$-R, we found that $\alpha$1-containing GABA-Rs in the hippocampus, amygdala, and/or cortex are important molecular targets influencing the amnestic effects of the inhaled anesthetic isoflurane. In contrast, these receptors seem to contribute little to the suppression of pain-evoked movement and righting reflexes by this inhaled anesthetic.

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**References**

Antognini JF and Schwartz K (1993) Exaggerated anesthetic requirements in the preferentially anesthetized brain. *Anesthesiology* **79:**1244–1249.

Bailey DJ, Tetzlaff JE, Cook JM, He X, and Helmstetter FJ (2002) Effects of hippocampal injections of a novel ligand selective for the alpha 5 beta 2 gamma 2 subunits of the GABA/benzodiazepine receptor on Pavlovian conditioning. *Neurobiol Learn Mem* **78:**1–10.

Brickley SG, Revilla V, Cull-Candy SG, Wisden W, and Farrant M (2001) Adaptive regulation of neuronal excitability by a voltage- independent potassium conductance. *Nature (Lond)* **409:**88–92.

Campagna JA, Miller KW, and Forman SA (2003) Mechanisms of actions of inhaled anesthetics. *N Engl J Med* **348:**2110–2124.

Collinson N, Kuenzi FM, Jarolimek W, Maubach KA, Cothliff R, Sur C, Smith A, Otu FM, Howell O, Atack JR, et al. (2002) Enhanced learning and memory and altered GABAergic synaptic transmission in mice lacking the alpha 5 subunit of the GABA$_A$ receptor. *J Neurosci* **22:**5572–5580.

Cope DW, Wulff P, Oberto A, Aller MI, Capogna M, Ferraguti F, Halbsguth C, Hoeger H, Jolin HE, Jones A, et al. (2004) Abolition of zolpidem sensitivity in mice with a point mutation in the GABA$_A$ receptor gamma2 subunit. *Neuropharmacology* **47:**17–34.

Eccles J, Schmidt R, and Willis W (1963) Pharmacological studies on presynaptic inhibition. *J Physiol* **168:**500–530.

Eger EI 2nd, Xing Y, Pearce R, Shafer S, Laster MJ, Zhang Y, Fanselow MS, and Sonner JM (2003) Isoflurane antagonizes the capacity of flurothyl or 1,2-dichlorohexafluorocyclobutane to impair fear conditioning to context and tone. *Anesth Analg* **96:**1010–1018.

Fendt M and Fanselow MS (1999) The neuroanatomical and neurochemical basis of conditioned fear. *Neurosci Biobehav Rev* **23:**743–760.

Franks NP and Lieb WR (1984) Do general anaesthetics act by competitive binding to specific receptors? *Nature (Lond)* **310:**599–601.

Fritschy JM and Mohler H (1995) GABA$_A$-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. *J Comp Neurol* **359:**154–194.

Gao B, Fritschy JM, Benke D, and Mohler H (1993) Neuron-specific expression of GABA$_A$-receptor subtypes: differential association of the alpha 1- and alpha 3-subunits with serotonergic and GABAergic neurons. *Neuroscience* **54:**881–892.

Homanics GE, Ferguson C, Quinlan JJ, Daggett J, Snyder K, Lagenaur C, Mi ZP, Wang XH, Grayson DR, and Firestone LL (1997) Gene knockout of the $\alpha$6 subunit of the $\gamma$-aminobutyric acid type A receptor: lack of effect on responses to ethanol, pentobarbital and general anesthetics. *Mol Pharmacol* **51:**588–596.

Jurd R, Arras M, Lambert S, Drexler B, Siegwart R, Crestani F, Zaugg M, Vogt KE, Ledermann B, Antkowiak B, et al. (2002) General anesthetic actions in vivo strongly attenuated by a point mutation in the GABA$_A$ receptor beta3 subunit. *FASEB J* **17:**250–252.

Korpi ER, Grunder G, and Luddens H (2002a) Drug interactions at GABA$_A$ receptors. *Prog Neurobiol* **67:**113–159.

Korpi ER and Lüddens H (1993) Regional $\gamma$-aminobutyric acid sensitivity of *t*-butylbicyclophosphoro[$^{35}$S]thionate binding depends on $\gamma$-aminobutyric acid$_A$ receptor $\alpha$ subunit. *Mol Pharmacol* **44:**87–92.

Korpi ER, Mihalek RM, Sinkkonen ST, Hauer B, Hevers W, Homanics GE, Sieghart W, and Lüddens H (2002b) Altered receptor subtypes in the forebrain of GABA$_A$ receptor $\delta$ subunit-deficient mice: recruitment of $\gamma$2 subunits. *Neuroscience* **109:**733–743.

Kralic JE, Korpi ER, O'Buckley, Homanics GE, and Morrow AL (2002) Molecular and pharmacologic characterization of GABA$_A$ receptor $\alpha$-1 subunit knockout mice. *J Pharmacol Exp Ther* **302:**1037–1045.

Low K, Crestani F, Keist R, Benke D, Brunig I, Benson JA, Fritschy JM, Rulicke T, Bluethmann H, Mohler H, et al. (2000) Molecular and neuronal substrate for the selective attenuation of anxiety. *Science (Wash DC)* **290:**131–134.

Lüddens H and Korpi ER (1995) GABA antagonists differentiate between recombinant GABAA/benzodiazepine receptor subtypes. *J Neurosci* **15:**6957–6962.

Lüddens H and Korpi ER (1996) GABA$_A$ receptors: pharmacology, behavioral roles and motor disorders. *Neuroscientist* **2:**15–23.

Mäkelä R, Uusioukari M, Homanics GE, Quinlan JJ, Firestone LL, Wisden W, and Korpi ER (1997) Cerebellar $\gamma$-aminobutyric acid type A receptors: pharmacological subtypes revealed by mutant mouse lines. *Mol Pharmacol* **52:**380–388.

McKernan RM and Whiting PJ (1996) Which GABA$_A$-receptor subtypes really occur in the brain? *Trends Neurosci* **19:**139–143.

Mihalek RM, Banjeree PK, Korpi E, Quinlan JJ, Firestone LL, Mi Z-P, Lagenaur C, Tretter V, Sieghart W, Anagnostaras S, et al. (1999) Attenuated sensitivity to

neuroactive steroids in GABA type A receptor delta subunit knockout mice. *Proc Natl Acad Sci USA* **96:**12905–12910.
Nicoll R (1972) The effects of anesthetics on synaptic excitation and inhibition in the olfactory bulb. *J Physiol* **223:**803–814.
Olsen RW (1982) Drug interactions at the GABA receptor-ionophore complex. *Annu Rev Pharmacol* **22:**245–277.
Pearce RA, Stringer JL, and Lothman EW (1989) Effect of volatile anesthetics on synaptic transmission in the rat hippocampus. *Anesthesiology* **71:**591–598.
Peng Z, Hauer B, Mihalek RM, Homanics GE, Sieghart W, Olsen RW, and Houser CR (2002) $GABA_A$ receptor changes in delta subunit-deficient mice: altered expression of alpha4 and gamma2 subunits in the forebrain. *J Comp Neurol* **446:** 179–197.
Pittson S, Himmel AM, and MacIver MB (2004) Multiple synaptic and membrane sites of anesthetic action in the CA1 region of rat hippocampal slices. *BMC Neurosci* **5:**52.
Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenmann H, Schofield PR, and Seeburg PH (1989) Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. *Nature (Lond)* **338:**582–585.
Quinlan JJ, Homanics GE, and Firestone LL (1998) Anesthesia sensitivity in mice lacking the $\beta$3 subunit of the $GABA_A$ receptor. *Anesthesiology* **88:**775–780.
Rampil IJ, Mason P, and Singh H (1993) Anesthetic potency (MAC) is independent of forebrain structures in the rat. *Anesthesiology* **78:**707–712.
Reynolds DS, Rosahl TW, Cirone J, O'Meara GF, Haythornthwaite A, Newman RJ, Myers J, Sur C, Howell O, Rutter AR, et al. (2003) Sedation and anesthesia mediated by distinct $GABA_A$ receptor isoforms. *J Neurosci* **23:**8608–8617.
Rudolph U, Crestani F, Benke D, Brunig I, Benson JA, Fritschy JM, Martin JR, Bluethmann H, and Mohler H (1999) Benzodiazepine actions mediated by specific gamma-aminobutyric $acid_A$ receptor subtypes. *Nature (Lond)* **401:**796–800.
Russek SJ (1999) Evolution of $GABA_A$ receptor diversity in the human genome. *Gene* **227:**213–222.
Sonner JM, Gong D, and Eger EI 2nd (2000) Naturally occurring variability in anesthetic potency among inbred mouse strains. *Anesth Analg* **91:**720–726.
Sonner JM, Zhang Y, Stabernack C, Abaigar W, Xing Y, and Laster MJ (2003) $GABA_A$ receptor blockade antagonizes the immobilizing action of propofol but not ketamine or isoflurane in a dose-related manner. *Anesth Analg* **96:**706–712.
Soriano P (1999) Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* **21:**70–71.
Sperk G, Schwarzer C, Tsunashima K, Fuchs K, and Sieghart W (1997) $GABA_A$ receptor subunits in the rat hippocampus I: immunocytochemical distribution of 13 subunits. *Neurosci* **80:**987–1000.
Tsien JZ, Chen DF, Gerber D, Tom C, Mercer EH, Anderson DJ, Mayford M, Kandel ER, and Tonegawa S (1996) Subregion- and cell type-restricted gene knockout in mouse brain. *Cell* **87:**1317–1326.
Uusi-Oukari M, Heikkila J, Sinkkonen ST, Makela R, Hauer B, Homanics GE, Sieghart W, Wisden W, and Korpi ER (2000) Long-range interactions in neuronal gene expression: evidence from neomycin gene insertions in the GABAa receptor $\beta$2-$\alpha$6-$\alpha$1-$\gamma$2 gene cluster. *Mol Cell Neurosci* **16:**34–41.
Vicini S, Ferguson C, Prybylowski K, Kralic J, Morrow AL, and Homanics GE (2001) $GABA_A$ receptor $\alpha$1 subunit deletion prevents developmental changes of inhibitory synaptic currents in cerebellar neurons. *J Neurosci* **21:**3009–3016.
Zinyk DL, Mercer EH, Harris E, Anderson DJ, and Joyner AL (1998) Fate mapping of the mouse midbrain-hindbrain constriction using a site-specific recombination system. *Curr Biol* **8:**665–668.

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