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GABA_A Receptor Alpha 4 Subunit Knockout Mice Are Resistant to the Amnestic Effect of Isoflurane

Vinuta Rau^{1,*}, Sangeetha V. Iyer^{2,*}, Irene Oh¹, Dev Chandra², Neil Harrison⁴, Edmond I Eger¹, Michael S. Fanselow⁵, Gregg E. Homanics^{2,3}, and James M. Sonner¹

¹ Department of Anesthesia and Perioperative Care, University of California San Francisco, San Francisco, CA 94143

² Department of Pharmacology & Chemical Biology, University of Pittsburgh, Pittsburgh, PA 15261

³ Department of Anesthesiology, University of Pittsburgh, Pittsburgh, PA 15261

⁴ Department of Anesthesiology and Pharmacology, Columbia University, New York, NY 10032

⁵ Department of Psychology, University of California Los Angeles, Los Angeles, CA

Abstract

Background—General anesthesia produces multiple endpoints including immobility, hypnosis, sedation and amnesia. Tonic inhibition via γ -aminobutyric acid type A receptors (GABA_A-Rs) may play a role in mediating behavioral endpoints that are suppressed by low concentrations of anesthetics (e.g., hypnosis and amnesia). GABA_A-Rs containing the α 4 subunit are highly concentrated in the hippocampus and thalamus, and when combined with δ subunits they mediate tonic inhibition, which is sensitive to low concentrations of isoflurane.

Methods—The present study used a GABA_A α 4 receptor knockout mouse line to evaluate the contribution of α 4-containing GABA_A receptors to the effects of immobility, hypnosis and amnesia produced by isoflurane. Knockout mice and their wild-type counterparts were assessed on three behavioral tests: conditional fear (to assess amnesia), loss of righting reflex (to assess hypnosis), and MAC, the minimum alveolar concentration of inhaled anesthetic necessary to produce immobility in response to noxious stimulation in 50% of subjects (to assess immobility).

Results—Genetic inactivation of the α 4 subunit reduced the amnestic effect of isoflurane, minimally affected loss of righting reflex, and had no effect on immobility.

Conclusions—These results lend support to the hypothesis that different sites of action mediate different anesthetic endpoints and suggest that α 4-containing GABA_A-R are important mediators of the amnestic effect of isoflurane on hippocampal-dependent declarative memory.

INTRODUCTION

General anesthesia produces multiple endpoints including immobility, hypnosis, sedation and amnesia.¹ Distinct neuronal pathways likely mediate these endpoints.^{2,3} The neurotransmitter γ -aminobutyric acid (GABA), through its action on GABA type A receptors (GABA_A-Rs), plays a role in mediating these behavioral endpoints of anesthesia.⁴

Address correspondence to Dr. Rau, Department of Anesthesia, S-455, University of California, San Francisco, CA 94143-0464. Telephone 415-514-0410; FAX 415-502-5375.

*Indicates equal contributions

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GABA_A-Rs are ligand-gated pentamers composed of several different subunits including α 1–6, β 1–3, γ 1–3, δ , ϵ , π , θ and ρ 1–3. Specific subunit combinations have unique anatomical distribution, pharmacological properties and functional significance.⁵ GABA acts on GABA_A-Rs to produce two types of inhibitory actions, phasic inhibition and tonic inhibition.^{6,7} Phasic inhibition occurs when a high concentration of GABA is released from presynaptic vesicles, and postsynaptic GABA_A-Rs are transiently activated resulting in an inhibitory postsynaptic potential. Tonic inhibition occurs when low concentrations of GABA in the extracellular space persistently activate extrasynaptic GABA_A-Rs.

GABA_A-Rs in the hippocampus contribute to the amnestic effect of anesthetics on learning and memory^{8,9}, and GABA_A-Rs in the thalamus are one site of action for the hypnotic effects of anesthetics.¹⁰ GABA_A-Rs containing the α 4 subunit are concentrated in the dentate gyrus of the hippocampus,^{11,12} and the dorsal thalamus^{12,13} and they mediate extrasynaptic inhibition.¹⁴ We previously demonstrated that α 4-containing extrasynaptic receptors are essential for the cellular and behavioral responses of the sedative/hypnotic gaboxadol.¹⁴ Similarly, we demonstrated that α 4-mediated extrasynaptic inhibition in the thalamus is markedly potentiated by isoflurane and this potentiation is absent in α 4 knockout (KO) mice.¹⁵ Based on these studies, we hypothesized that as with gaboxadol, α 4-containing receptors may mediate some of the behavioral effects of isoflurane.

The present study used a GABA_A α 4 receptor KO mouse line¹⁴ to evaluate the contribution of α 4-containing GABA_A receptors to the effects of amnesia, hypnosis, and immobility produced by isoflurane. We assessed KO mice and their wild-type (WT) counterparts on three behavioral tests: conditional fear (to assess amnesia), loss of righting reflex (to assess hypnosis), and MAC, the minimum alveolar concentration of inhaled anesthetic necessary to produce immobility in response to noxious stimulation in 50% of subjects (to assess immobility).

METHODS

Mice

Homozygous α 4 KO and WT littermate controls were created by breeding mice heterozygous for the GABA_A-R α 4 subunit KO allele.¹⁴ Mice were of the F_{3–4} generations and their genetic background was a mixture of C57BL/6J and Strain 129S1/X1. At weaning, mice were genotyped using Southern Blot analysis of tail DNA as previously described.¹⁴ Mice were group housed, were kept on a 12 hour alternating light/dark cycle, and were allowed access to food and water *ad libitum*. All protocols were approved by the committees on animal care and use at the University of California, San Francisco and at the University of Pittsburgh.

Fear Conditioning

Mice were assessed for isoflurane-induced amnesia by fear conditioning. Eight to 12 mice of each genotype and sex were assessed at each concentration. Prior to fear conditioning, groups of mice were placed in a chamber (28 cm L $\times$ 12.5 cm W $\times$ 17.5 cm H) equilibrated with the desired isoflurane concentration. Isoflurane concentrations tested included: 0.0%, 0.1%, 0.2%, 0.3%, 0.4%, 0.6%, and 0.8% atm. A Gow-Mac gas chromatograph (Gow-Mac Instrument Corp., Bridgewater, NJ) equipped with a flame ionization detector was used to measure concentrations of isoflurane.

After 30 minutes of equilibration, the mice were quickly transferred to the training chambers, which contained the same isoflurane concentration as in the equilibration chamber. The chambers (27 cm L $\times$ 24.5 cm W $\times$ 20 cm H) were constructed of clear acrylic. The grid floor used to deliver shock was composed of 31 stainless steel bars, each 3 mm in diameter, spaced

7 mm center to center. These floors were connected to a shock delivery system (San Diego Instruments, San Diego, CA). The chambers were wiped down with a pine-scented cleaner (5% Pine Scented Disinfectant, Midland, Inc.; Sweetwater, TN) before and after each session. In the room in which training took place, the overhead fluorescent bulbs were left on and a ventilation fan provided background noise (65 db). The lighting, background noise, appearance, odor, and texture of the chambers and room comprised the training context.

After a 3 min period in the chambers, the mice received 3 tone (2000 Hz, 90 db)-shock (1 mA, 2 s) pairings, separated by 1 min. During training, freezing was scored only in animals receiving 0% isoflurane. This is because freezing may be visually difficult to distinguish from sedation in mice treated with isoflurane, thus confounding scoring procedures. Freezing, the absence of all movement except that necessary for respiration, is an innate defensive response in rodents and is a reliable measure of learned fear.^{16,17} Each animal's behavior was scored every 8 s during the observation period and a percentage was calculated by dividing the number of freezing observations a mouse had by the total number possible during the observation period.

The following day, mice were tested for fear to the training context and fear to tone. For the context test, each mouse was placed back in the chamber in which it was trained for a period of 8 min (in the absence of shock). For the tone test, groups of mice were transported in separate plastic pots (14 cm high $\times$ 15.5 cm diameter) to a different context in a different room. The test chambers were triangular in shape with an acrylic floor (28 cm L $\times$ 25 cm W) and two acrylic sidewalls (28 cm L $\times$ 22 cm W) at a 45° angle. The chambers were equipped with a speaker and were wiped down with acetic acid (1%, Fisher Scientific) before and after each session. The room appeared dark to the mice, being lit by a single red 30 W bulb. White noise (65 dB) was used for background noise. Mice were given a 3 min exploratory period, then 6 30 s tones (2000 Hz, 90 db) were presented, separated by 60 s. As with the context test, no shocks were administered during the tone test. Animals were removed from the chamber after an additional 30 s. The order of the context and tone tests was counterbalanced, such that half of each treatment group was tested to context first and tone second and vice versa. Context and tone tests were spaced at least an hour apart. Freezing was scored during training and both tests.

Loss of Righting Reflex

Mice (10–20 weeks of age) were tested for halothane (n=7 males (M), 9 females (F)/genotype) and isoflurane (WT: n=2M, 9F; KO: n=4M, 9F) -induced loss of righting reflex as previously described.¹⁸ Controlled concentrations of halothane (Halocarbon Laboratories, River Edge, NJ) or isoflurane (Abbot Laboratories, North Chicago, IL) were delivered to the testing chambers by means of agent-specific vaporizers. A Datomex Capnomac Ultima device continuously monitored the concentration of anesthetic gas within the chamber. The mice were equilibrated with anesthetic for 15 minutes and then scored for the righting reflex while the carousel rotated at 4 rpm. After testing at one concentration of anesthetic, the mice were allowed to recover in anesthetic-free air for 20 min. Anesthetic gas concentrations tested ranged from 0.6%–1.1% atm with an increment of 0.1% in each trial.

MAC

Mice (10–20 weeks of age) were monitored for response to noxious stimuli in the presence of the halothane (n=7M, 9F/genotype) and isoflurane (WT: n=12M, 14F; KO: n=13M, 14F) using the tail clamp-withdrawal assay as described.¹⁸ Following equilibration with anesthetic, a hemostat was applied to the tail and the mouse was scored for purposeful, gross movement in response to the tail clamp. Mice were allowed to recover for 15 minutes in air before testing at the next concentration. The range of concentrations tested was 1.0%–1.8% with 0.2% increments.

Statistical Analysis

For loss of righting reflex and MAC assays, half maximal effective concentration values (EC_{50}) were obtained from concentration response data using the iterative nonlinear least-squares method as previously described¹⁹ for quantal responses.¹⁸ The Z statistic was used to compare genotypes on both assays.²⁰ Because of the limited number of genetically engineered mice available for these studies, males and females were combined for analysis. Previous studies demonstrated that males and females respond similarly on the behavioral assays of loss of righting reflex and MAC in response to anesthetics.^{21,22} Data are presented as $EC_{50} \pm SEM$.

For fear conditioning, analysis of variance (ANOVA) was used to analyze freezing scores during training, and nonlinear regression was used to calculate EC_{50} values and the maximum value of the dose response curve for context and tone freezing scores. The following equation was used in the regression, with n =the Hill coefficient, and A =the maximal value

$$\text{Freezing} = A \times \left(\frac{\text{isoflurane}^n}{\text{isoflurane}^n + EC_{50}^n} \right)$$

RESULTS

Fear Conditioning

Pilot data suggested that male and female WT mice showed differences in freezing scores at 0% isoflurane. Given these results, we analyzed data from male and female mice separately.

Training—Freezing during training was only scored in mice receiving 0% isoflurane. This is because freezing may be visually difficult to distinguish from sedation in mice treated with isoflurane, thus confounding scoring procedures. All mice regardless of genotype or sex showed increased freezing over the duration of training, with all groups arriving at the same level of freezing by the third tone-shock presentation (Figure 1). This observation was confirmed with a repeated measures ANOVA with genotype and sex as factors. Freezing significantly increased with each tone-shock presentation during training, $F(4, 168) = 48.50$, $P < 0.0001$, but there was no reliable effect of genotype or sex and no interactions.

Context Test—The isoflurane EC_{50} s for female WT and KO mice were $0.08 \pm 0.03\%$ and $0.28 \pm 0.03\%$, respectively (Figure 2). A t-test revealed a statistically significant difference between these EC_{50} measures ($P < 0.005$). Female WT and KOs did not differ significantly at 0% isoflurane.

There was no statistical difference between the isoflurane EC_{50} s for male WT mice ($0.16 \pm 0.05\%$ isoflurane) and male KO mice ($0.14 \pm 0.03\%$ isoflurane), as shown in Figure 3. However, male mice differed ($P < 0.05$) on context freezing scores at 0% isoflurane ($30.41 \pm 8.59\%$ for WT vs. $55.29 \pm 6.78\%$ for KO). Because of this difference in freezing at 0% isoflurane, it was not possible to accurately compare EC_{50} values for WT and KO mice. To address this issue in a manner that would allow for a valid comparison of genotypes, we conducted a follow-up experiment in which freezing levels were made equivalent in male WT and KO mice at 0% isoflurane by training them with 6 tone-shock pairings (versus 3 tone-shock pairings in the original experiment). We subsequently tested both genotypes at the EC_{50} for WT mice for fear to context, 0.16% isoflurane, and the EC_{50} for WT mice for fear to tone, 0.52% isoflurane. When freezing was equated between male WT and KO at 0% isoflurane, an effect of genotype on freezing scores was observed, $F(1, 34) = 4.19$, $P < 0.05$; Figure 4). There was also a trend toward an interaction between genotype and isoflurane, F

(1,34) = 3.35, $P=0.076$. Preplanned comparisons show that KO were reliably higher than WT at both 0.16% and 0.52% isoflurane ($P<.05$). Thus, while 0.16% isoflurane had no effect on knockout mice trained with the 6 shock paradigm, it had a significant effect on the wildtypes littermates.

Tone Test—The EC_{50} for female WT mice was $0.43 \pm 0.06\%$ isoflurane, and the EC_{50} for female KO mice was $0.43 \pm 0.06\%$ isoflurane (Figure 5). A t-test revealed no significant difference between these EC_{50} measures. There was no difference at 0% isoflurane between female WT and KO mice.

The EC_{50} for male WT mice was $0.52 \pm 0.08\%$ isoflurane, and the EC_{50} for male KO mice was $0.42 \pm 0.06\%$ isoflurane (Figure 6). A t-test revealed no significant difference between these EC_{50} measures. Fear to tone was also measured in the follow-up experiment with 6 shocks described in the previous section. There was no reliable difference in fear to tone between WT and KO mice in this follow-up study as determined by ANOVA.

Loss of Righting Reflex

For isoflurane-induced loss of righting reflex (Figure 7), the EC_{50} for WT (0.73 ± 0.02) and KO (0.74 ± 0.02) mice did not differ. For halothane-induced loss of righting reflex (Figure 8), the EC_{50} for KO mice (0.90 ± 0.01) was increased ($P<0.05$) compared to WT controls (0.84 ± 0.02).

MAC

WT and KO mice did not differ in their isoflurane MAC (also shown in Figure 7) (WT $EC_{50}=1.45 \pm 0.03$ vs. KO $=1.37 \pm 0.03$). Similarly, genotype did not affect response to halothane (also shown in Figure 8) on this assay (WT $EC_{50}=1.61 \pm 0.03$ vs. KO $=1.59 \pm 0.03$).

DISCUSSION

In this study, we tested the role of $\alpha 4$ -containing GABA_A-Rs in behavioral responses to isoflurane and halothane. We found that GABA_A-R $\alpha 4$ KO mice were resistant to the effects of isoflurane on learning and memory. In other words, genetic inactivation of the $\alpha 4$ subunit reduced the amnesic effect of isoflurane. In contrast, KO of $\alpha 4$ slightly but significantly affected loss of righting reflex for halothane but not for isoflurane, and had no effect on immobility as determined by MAC for either isoflurane or halothane.

KO mice were resistant to isoflurane's effect on context conditioning, but not conditioning to tone. This finding is consistent with the observations that GABA_A $\alpha 4$ -containing receptors are highly expressed in the hippocampus^{11,12}, and the hippocampus is selectively involved in fear conditioned to context, but not tone. The basolateral amygdala is the site at which convergence of information about the shock and its predictive cues occurs.²³ The roles of the amygdala and hippocampus in fear conditioning have been well characterized. Lesions of the amygdala block fear to tone and context, whereas lesions of the hippocampus block only fear to context and leave fear to tone intact.^{24,25} Our findings and the selective role of the hippocampus in fear conditioned to context suggest a specific role for the GABA_A $\alpha 4$ receptor subtype in inhaled anesthetic-induced impairment of hippocampal-dependent learning.

While the majority of hippocampal GABA_A $\alpha 4$ receptors co-localize with the δ subunit extrasynaptically, there is a substantial portion of GABA_A $\alpha 4$ containing receptors that co-localize synaptically with the GABA_A $\gamma 2$ subunit.¹¹ Therefore, it is possible that isoflurane produces its suppression of learning and memory by extrasynaptic as well as synaptic inhibition. However, this is likely related to isoflurane concentration. We have shown

previously that knockout of the GABA_A α 1 receptor subunit, which is thought to primarily mediate phasic inhibition^{26,27} also produces a resistance to the amnestic effects of isoflurane.⁸ The EC₅₀ for GABA_A α 1 WT mice (males and females combined) was approximately 0.15% isoflurane, and the EC₅₀ for GABA_A α 1 KO mice was approximately 0.37% isoflurane.⁸ This is much higher for the KO in comparison to the results in this study: EC₅₀ for GABA_A α 4 WT mice (averaged between males and females) was 0.12% isoflurane while the EC₅₀ for GABA_A α 4 KO mice 0.21% isoflurane. While the EC₅₀ values for both GABA_A α 1 and α 4 WTs are similar, more isoflurane was necessary to suppress freezing by 50% in the GABA_A α 1 KO than the α 4 KO. This is consistent with the finding that isoflurane at a very low concentration (25 μ M) directly activates GABA_A-R containing the α 4 subunit, but only higher concentrations (higher than 200 μ M) are able to directly activate GABA_A-R containing the α 1 subunit.²⁸

There was no significant difference between WT and KO mice on isoflurane-induced loss of righting reflex. However, a modest, but statistically significant difference on halothane-induced loss of righting reflex was observed between genotypes. The halothane EC₅₀ that was required to suppress this response in KO mice was increased by approximately 7% compared to WT mice. We are uncertain as to the biological significance of this modest change in anesthetic response. We also cannot rule out that compensatory responses may lead to over- or underestimation of the true contribution of α 4-containing receptors to anesthetic-induced loss of righting. Nonetheless, we conclude that α 4-containing GABA_A-Rs are not required for mediating the hypnotic effect of inhaled anesthetics.

Lastly, we observed no effect of α 4 gene KO on immobility in response to halothane or isoflurane as measured by MAC. These results are consistent with previous pharmacologic studies that have demonstrated that GABA_A-Rs in the spinal cord are not key mediators of anesthetic-induced immobility.^{29,30}

The present results lend support to the hypothesis that different sites of action mediate different anesthetic endpoints. GABA_A-Rs containing the α 4 subunit play little if any role in inhaled anesthetic-induced hypnosis and immobility. In contrast, these receptors are key mediators/modulators of inhaled anesthetic-induced amnesia. GABA_A-Rs containing the α 4 subunit are important for hippocampal-dependent fear to context but not hippocampal-independent fear to tone. These results support an important role for α 4 containing GABA_A-Rs specifically on hippocampal-dependent types of memory like declarative memory.

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Figure 1.

Conditional freezing during training. Training consisted of three tones (labeled here at “T”) that coterminated each with a shock. Freezing, scored during tones and after (Post-Tones: “PT”) significantly increased over the tone-shock pairings during training, ($P < .0001$), but scores did not differ between genotype and sex.

Fear to Context FEMALES

Figure 2.

The dose response curve and EC₅₀ values for female WT and KO mice for fear to context. There was a statistically significant shift between the EC₅₀ for female WT mice ($0.082 \pm 0.03\%$ isoflurane) and the EC₅₀ for female KO mice ($0.28 \pm 0.03\%$ isoflurane). Female KO mice show resistance to the amnestic effect of isoflurane.

Figure 3.

The dose response curves and EC₅₀ values for context freezing for male WT mice and male KO mice. There was no statistical difference between the mice ($0.16 \pm 0.05\%$ isoflurane) versus male KO mice ($0.14 \pm 0.03\%$ isoflurane). However, male mice differed ($P < 0.05$) on context freezing scores at 0% isoflurane ($30.41 \pm 8.59\%$ for WT vs. $55.29 \pm 6.78\%$ for KO).

Figure 4.

When freezing was equated between male WT and KO by increasing the number of shocks during training from 3 to 6, there was a reliable effect of genotype on context freezing scores at the EC₅₀ value for context conditioning in WT male mice, 0.16% isoflurane, and the EC₅₀ value for tone conditioning in WT male mice, 0.52% isoflurane ($P < .05$).

Figure 5. Concentration response curves and EC_{50} values for female WT and KO mice for fear to tone. The female WT EC_{50} ($0.43 \pm 0.06\%$ isoflurane) did not differ from the female KO EC_{50} ($0.43 \pm 0.06\%$ isoflurane).

Figure 6. Concentration response curves and the EC₅₀ values for male WT and KO mice for fear to tone. Male WT mice did not differ from KO mice on their EC₅₀ values (0.52 $\pm$ 0.08% isoflurane vs. 0.42 $\pm$ 0.06% isoflurane).

Figure 7.

For isoflurane-induced loss of righting reflex (LORR), the EC_{50} for WT (0.73 ± 0.02) and KO (0.74 ± 0.02) mice did not differ. The MAC value also did not differ between WT and KO (WT $EC_{50}=1.45 \pm 0.03$ vs. KO= 1.37 ± 0.03).

Figure 8.

For halothane-induced loss of righting reflex (LORR), the EC_{50} for KO mice (0.90 ± 0.01) was increased ($p < 0.05$) compared to WT controls (0.84 ± 0.02). The MAC value did not differ between WT and KO (WT $EC_{50} = 1.61 \pm 0.03$ vs. KO = 1.59 ± 0.03).