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Aminobutyric Acid Type A Receptor $\beta 3$ Subunit Forebrain-Specific Knockout Mice Are Resistant to the Amnestic Effect of Isoflurane

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DISCLOSURES

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Abstract

Background— $\beta 3$ containing γ -aminobutyric acid type A receptors (GABA_A-Rs) mediate behavioral end points of IV anesthetics such as immobility and hypnosis. A knockout mouse with targeted forebrain deletion of the $\beta 3$ subunit of the GABA_A-R shows reduced sensitivity to the hypnotic effect of etomidate, as measured by the loss of righting reflex. The end points of amnesia and immobility produced by an inhaled anesthetic have yet to be evaluated in this conditional knockout.

Methods—We assessed forebrain selective $\beta 3$ conditional knockout mice and their littermate controls for conditional fear to evaluate amnesia and MAC, the minimum alveolar concentration of inhaled anesthetic necessary to produce immobility in response to noxious stimulation, to assess immobility. Suppression of conditional fear was assessed for etomidate and isoflurane, and MAC was assessed for isoflurane.

Results—Etomidate equally suppressed conditional fear for both genotypes. The knockout showed resistance to the suppression of conditional fear produced by isoflurane compared to control littermates. Controls and knockouts did not differ in isoflurane MAC values.

Conclusions—These results suggest that $\beta 3$ containing GABA_A-Rs in the forebrain contribute to hippocampal dependent memory suppressed by isoflurane, but not etomidate.

INTRODUCTION

γ -aminobutyric acid (GABA) type A receptors (GABA_A-Rs) are plausible targets for anesthetic actions.^{1–3} GABA_A-Rs are ligand-gated pentamers composed of several different subunits that include $\alpha 1$ -6, $\beta 1$ -3, $\gamma 1$ -3, δ , ϵ , π , θ and $\rho 1$ -3. In this study, the role of the $\beta 3$ subunit in mediating anesthetic actions was examined.

The $\beta 3$ subunit of the GABA_A-R is highly expressed in the rodent corpus striatum, olfactory bulb and hippocampus.⁴ $\beta 3$ containing GABA_A-Rs mediate immobility and hypnosis produced by IV anesthetics.^{5–6} Global knockout (KO) of $\beta 3$ reduced sensitivity to the immobilizing effects of the inhaled anesthetics halothane and enflurane⁷ but interpretation of these results was confounded by compensation.⁸ To overcome the limitations of the global KO, mice with a conditional $\beta 3$ allele were created.⁶ Forebrain selective KO (including cortex, hippocampus, and amygdala) of $\beta 3$ resulted in reduced sensitivity to the hypnotic effect of etomidate, a 1.5-fold difference, as measured by the loss of righting reflex compared to control littermates.⁶ The anesthetic end points of amnesia produced by etomidate, amnesia and immobility produced by an inhaled anesthetic have yet to be evaluated in this mutant.

In the following set of experiments, the forebrain selective GABA_A $\beta 3$ KO mouse line was tested for resistance to the amnestic effect of etomidate and the amnestic and immobilizing effects of the inhaled anesthetic isoflurane. Etomidate and isoflurane-induced amnesia were

assessed using the behavioral assay of fear conditioning, and isoflurane-induced immobility was assessed by determining the minimum alveolar concentration needed to prevent movement in response to a painful stimulus (MAC).

METHODS

Mice

Mice were generated, bred and genotyped as previously described.⁶ Forebrain selective KO mice were homozygous for the floxed $\beta 3$ allele and positive for an α CamKII-cre transgene. Littermate controls were also homozygous for the floxed $\beta 3$ allele but did not harbor the cre transgene. Mice were group housed in a colony room with a 12-hour alternating light/dark cycle and were given *ad libitum* access to food and water. Adult male mice (ages 10-12 weeks) were used in fear conditioning studies, and both male and female adult mice were used for MAC studies. These protocols were approved by the University of California San Francisco's Institutional Animal Care and Use Committee.

Fear Conditioning

Etomidate—Seven to 11 mice of each genotype were assessed at 0, 11 and 15 mg/kg etomidate (Hospira, Inc; Lake Forest, IL). These doses were chosen based on previous findings indicating that the ED₅₀ for conditional freezing for a similar fear conditioning protocol in wild type mice was 11 mg/kg.⁹ The etomidate was purchased pre-dissolved in a 35% propylene glycol solution at a concentration of 2 mg etomidate/ml solution. A 35% propylene glycol solution (Sigma; St. Louis, MO) was used for all vehicle injections. The intraperitoneal injection of etomidate was given 30 minutes before training, and all mice were injected at 7.7 ml/kg body weight.

Thirty minutes after the injection groups of four mice at a time were transferred to fear conditioning chambers (27 cm L×24.5 cm W×20 cm H) constructed of clear acrylic. The chamber floor was made of 31 stainless steel bars (3 mm in diameter, spaced 7 mm center to center) and was connected to a shock delivery system (San Diego Instruments; San Diego, CA). Before and after each session, the chambers walls were cleaned with a pine solution (5% Pine Scented Disinfectant, Midland, Inc.; Sweetwater, TN). Room lights were left on and background noise (65 db) was played during training.

After a 3-min baseline period, mice received 3 tone (2000 Hz, 90 db)-shock (2 mA, 2 s) pairings, separated by 1 min. Mice were tested for fear to the training context and fear to tone in a counterbalanced order the following day. Context and tone tests were spaced at least an hour apart. To test for fear to the training context, groups of four mice were placed back in their training chamber in the absence of shock for a period of 8 min. To test for fear to tone, groups of four mice were transported in plastic pots (14 cm high×15.5 cm diameter) to a different context in a different room. These test chambers were essentially acrylic triangles (floor dimensions: 28 cm L×25 cm W; sidewall dimensions: 28 cm L×22 cm W, at a 45° angle), fitted with a speaker. The chambers were cleaned with acetic acid (1%, Fisher Scientific; Pittsburgh, PA) before and after each session. The experimental room appeared dark to the mice, being lit by a single red 30 W bulb. There was no background noise during

this test. 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.

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.^{10–11} Each animal's behavior was scored every 8 s during the test. If the animal showed freezing, it was given a score of "1" for that observation; if the animal showed movement, it was given a score of "0" for that observation. A percentage was calculated by dividing the number of freezing observations a mouse had by the total number possible during the observation period. This number represented the animal's freezing score. The observer scoring the behavior was blinded to the genotypes of the mice.

Isoflurane—Eight to 12 mice of each genotype were assessed at the following isoflurane (Baxter Pharmaceutical Products, Inc., Liberty Corner, NJ) concentrations: 0.0%, 0.1%, 0.2%, 0.4%, 0.6%, and 0.8%. Before the training procedure described for etomidate, groups of four mice were placed in a chamber (28 cm L×12.5 cm W×17.5 cm H) equilibrated with the desired isoflurane concentration. A Gow-Mac gas chromatograph (Gow-Mac Instrument Corp.; Bridgewater, NJ) equipped with a flame ionization detector and an infrared analyzer (Daytex, Instrumentarium Corp.; Helsinki, Finland) were used to measure and monitor the concentration 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. Training and testing were conducted as described for etomidate.

MAC

MAC for isoflurane was determined for 9–10 mice of each genotype. Each mouse was placed in a gas-tight plastic chamber (3 cm diameter×13 cm length), which was part of a closed system containing a carbon dioxide absorber, fan, and oxygen source. A vaporizer (as described in the previous section) was used to deliver isoflurane. Groups of 10 mice, approximately half control and half KO, were run at a time. The experimenter was blinded to the animals' genotypes. To prevent hypothermia during the experiment, body temperature was monitored rectally and maintained between 36°C and 38°C by applying heating pads or ice bags to plastic chambers when necessary. Isoflurane concentrations were measured and monitored as described in the previous section.

The initial isoflurane concentration was adjusted to one at which all mice showed movement in response to 1 min of tail stimulation. The isoflurane concentration was then increased by 20% increments until there was no observable movement in response to the stimulus. A 30-min equilibration period was given after each incremental change, before the tail stimulation. MAC for each mouse was calculated as the average between the concentration at which the mouse showed no movement and the previous concentration at which movement was observed.

Statistical Analysis

For fear conditioning with etomidate, analysis of variance (ANOVA) was used to detect significant differences between the means of control and KO mice for the three drug doses tested. For fear conditioning with isoflurane, nonlinear regression was used to calculate EC₅₀ 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:

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

MAC values were averaged for control and KO mice, and a t-test was used to test for statistical significance. A P value = 0.05 was used as criteria for a statistically significant difference between genotypes.

RESULTS

Fear Conditioning

Etomidate—Results are expressed as mean ± standard error of the mean. The genotypes did not differ statistically at 0 mg/kg etomidate for fear to context (control: 65.2% ± 6.4%; KO: 56.25% ± 14.87%) or fear to tone (control: 38.84% ± 5.94%; KO: 39.88% ± 7.38%). ANOVA conducted on fear to context and fear to tone observations revealed a main effect of suppression of fear for both, $F(2,48) = 6.91$, $P < 0.005$; $F(2,48) = 4.07$, $P < 0.05$, for context and tone respectively, but no statistical differences between genotypes. These data are shown in Figure 1.

Isoflurane—The genotypes did not differ statistically at 0% isoflurane for fear to context (control: 50.5% ± 6.6%; KO: 34.8% ± 6.8%) or fear to tone (control: 38.4% ± 4.9%; KO: 51.7% ± 9.0%). For fear to context, the EC₅₀ for control mice was 0.098% ± 0.030% isoflurane, and the EC₅₀ for KO mice was 0.305 ± 0.089% isoflurane (Figure 2a). A t-test revealed a significant difference between these EC₅₀ values, $t(108) = 2.40$; $P < 0.05$. For fear to tone, the EC₅₀ for control mice was 0.709% ± 0.049% isoflurane, and the EC₅₀ for KO mice was 0.728% ± 0.058% isoflurane (Figure 2b). There was no significant difference between these EC₅₀ values.

MAC

MAC for each mouse was calculated as the average between the concentration at which the mouse showed no movement and the previous concentration at which movement was observed. A t-test conducted on isoflurane MAC values revealed no effect of genotype, (control mean = 1.57, SD = 0.03; KO mean = 1.63, SD = 0.03).

DISCUSSION

In these experiments, the contribution of $\beta 3$ subunit containing GABA_A-Rs in the forebrain were assessed for their role in anesthesia-induced amnesia and immobility. Fear

conditioning was used to evaluate etomidate and isoflurane-induced amnesia, and MAC was used to evaluate isoflurane-induced immobility.

We expected that the forebrain selective $\beta 3$ conditional KO mouse line would be resistant to the amnesic effects of both etomidate and isoflurane compared to their control littermates. However, results from the fear conditioning studies showed that the KO mouse line was resistant to the amnesic effect of isoflurane, but not etomidate.

While doses of 11 and 15 mg/kg progressively suppressed conditional freezing, these doses did not differentially affect conditional freezing between the KO and control. In our study, the highest dose of etomidate did not produce total suppression of fear to context or tone. We could not give the mice a dose higher than 15 mg/kg, because our pilot studies showed that the vehicle alone when given at the volume needed to dissolve etomidate concentrations higher than 15 mg/kg produced some suppression of learning and memory. We were unable to find a suitable vehicle that would dissolve concentrations higher than 15 mg/kg etomidate without producing undesired behavioral anxiolytic effects. However, the data collected from the doses given in the present study clearly indicate no difference between genotypes.

There are a number of reasons why we may have not seen a difference in etomidate sensitivity between genotypes: 1) deletion of forebrain containing GABA_A-R subunits $\beta 3$ is not critical for etomidate-induced amnesia; 2) KO of the $\beta 3$ subunit can decrease the expression of other GABA_A-R subunits,¹² and possibly these compensatory changes mask the KO's resistance to etomidate-induced amnesia, and 3) etomidate is acting on subunits other than $\beta 3$, such as $\beta 1$ and $\beta 2$ ¹³ or $\alpha 5$ ¹⁴ For example, Cheng et al.¹⁵ showed that etomidate decreased memory acquisition in $\alpha 5$ wild type controls compared to null mutant mice; the wild type mice showed decreased contextual freezing and decreased time spent in the platform area in a Morris water maze probe trial.¹⁵ Our fear-conditioning data show a similar, but nonsignificant, trend. It is a possibility that $\alpha 5$ is pairing with $\beta 3$ or $\beta 3$ to bring about this effect, or it paired with $\beta 3$ but in cells that did not undergo deletion in our forebrain selective KO mice.

Etomidate acts on GABA_A-Rs containing the $\beta 3$ subunit to produce some hypnotic effects, as the $\beta 3$ conditional KO mice had a reduced duration of the loss of righting reflex compared to control mice.⁶ This is not the case for amnesia, suggesting that different receptor subunits mediate the behavioral end points of hypnosis and amnesia produced by etomidate.

The KO mice showed resistance to the suppression of learning and memory produced by isoflurane compared to control littermates. This is in conflict with a previous study showing that the $\beta 3$ knockin (KI) mouse did not show resistance to the amnesic effect of isoflurane compared to littermate controls.¹⁶ This difference might be due to baseline differences between the KIs and controls. At the baseline of 0% isoflurane, the $\beta 3$ KI mice showed significantly lower freezing values than their littermates. This baseline disparity may mask an actual difference between the genotypes. We have seen this recently with the GABA_A-R $\alpha 4$ KO mouse.¹⁷ The $\alpha 4$ KO and control mice originally showed a statistically significant difference in their freezing scores at 0% isoflurane. There was no difference in the

calculated EC₅₀ values between KOs and controls. However, when freezing levels were equated at 0% isoflurane by adjusting the training protocol (6 tone-shock pairings versus 3 tone-shock pairings in the original study), a significant difference between the genotypes was revealed. We suspect that a similar baseline freezing difference may have masked the finding of an effect of isoflurane to differentially affect the $\beta 3$ KIs and control. In the present study, control and KO mice were not statistically different at 0% isoflurane, ensuring an unconfounded interpretation of the concentration response curves.

In the present study the right shift in EC₅₀ values was observed only for fear to context, and not for fear to tone. This suggests that GABA_A-Rs containing $\beta 3$ subunits in the forebrain are involved in mediating hippocampal-dependent learning, because fear to context is hippocampal-dependent while fear to tone is not.¹⁸

As for the MAC data, our results showed that $\beta 3$ containing GABA_A-Rs in the forebrain are not involved in mediating the immobilizing actions of isoflurane. These results are consistent with previous studies that have demonstrated that the spinal cord, not forebrain structures, are the site of anesthetic-induced immobility.¹⁹ This result is also consistent with the finding that the $\beta 3$ KI mouse shows no difference from its control littermates in terms of MAC values.¹⁶

Together, these results support the hypothesis that different sites of action mediate different anesthetic end points. GABA_A-Rs containing the $\beta 3$ subunit play little if any role in inhaled anesthetic-induced immobility. However, these receptors are key mediators/modulators of inhaled anesthetic-induced hypnosis⁶ and amnesia. Our results also suggest that GABA_A-Rs containing the $\beta 3$ subunit are important for hippocampal-dependent types of memory like declarative memory.

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Figure 1.

Effect of etomidate on conditional fear. (A) Etomidate significantly suppresses fear to context and (B) fear to tone, but there was no statistical difference in this suppression between control and knockout mice.

Figure 2.

Effect of isoflurane on conditional fear. (A) Isoflurane significantly suppresses fear to context, and knockout (KO) mice are resistant to isoflurane's amnesic effect compared to control mice. This is shown by a significant right shift in the EC₅₀ values for KOs compared to controls. (B) Isoflurane significantly suppresses fear to tone similarly for control and KO mice.