Genetics and Molecular Research

A single nucleotide polymorphism in $GABA_A$ receptor isoforms is potentially responsible for isoflurane sensitivity in mice

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Genet. Mol. Res. 15 (2): gmr.15027340
Received July 29, 2015
Accepted December 14, 2015
Published June 10, 2016
DOI http://dx.doi.org/10.4238/gmr.15027340

ABSTRACT. $GABA_A$ receptors are chloride channels in the brain that are activated by binding with γ-aminobutyric acid (GABA). The cDNA sequences of $GABA_A$ receptor subunits from two strains of mice with different sensitivities to isoflurane were compared to identify nucleotide mutations. Included 80 mice from two strains with different sensitivities to isoflurane on C57BL/6 background. Forty mice were from an isoflurane-sensitive strain (S group) and 40 mice were from a resistant strain (R group). RNA was extracted from brains of the mice, and cDNA were reverse transcribed using AMV reverse transcriptase. The amplified products were processed, sequenced, and analyzed for differences between the two strains. Chi-square analysis was performed to compare differences in nucleotide mutation frequencies between the two strains. No differences were identified in the α1-6, β_2, β_3, or γ1-3 nucleotide sequences and no single nucleotide polymorphisms were found in the comparison with the GenBank sequence for the $GABA_A$ receptor subunit. A single nucleotide polymorphism (SNP) at the nucleotide position 462 (C/G) in the β_1 sequence was found. This SNP was observed in 5 mice from the sensitive strain and in 36 mice

from the resistant strain. The Fischer exact test ($P < 0.01$) was used to compare two strains of mice for SNP in the cDNA sequence of the β_1 subunit. Additional studies are required to understand whether the $GABA_A$ receptor is a specific target of inhaled anesthetic action or whether the identified SNP affects the action of the volatile anesthetic.

Key words: $GABA_A$ receptors; Isoflurane sensitivity; Median effective dose; Mice; SNP

INTRODUCTION

Inhaled anesthetics have been safely used in clinical practice for over 150 years, and most patients receive some form of inhaled anesthetic in the perioperative period (Fukazawa and Lee, 2014). Isoflurane is one of the most commonly utilized inhaled anesthetics, and has even demonstrated potential for use as an intravenous anesthetic during surgery (Zhou et al., 2006). Nevertheless, the molecular mechanisms underlying the action of isoflurane and other commonly utilized volatile anesthetics are still largely unknown. γ-Aminobutyric acid ($GABA_A$) receptors are the most numerous inhibitory neurotransmitter receptors in the central nervous system (CNS) (Belelli et al., 2009). Given the inhibitory nature of anesthetics that are critical for surgical application, the possible importance of $GABA_A$ receptors as targets of such anesthetics has long been postulated and investigated (Concas et al., 1992; Nelson et al., 2002; Davies, 2003). $GABA_A$ receptors are chloride channels activated by the binding of GABA (Virmani et al., 1990; Blarre et al., 2014), an inhibitory neurotransmitter, which is also widely distributed in the mammalian CNS. $GABA_A$ receptors are considered the leading candidate targets of volatile anesthetics owing to their role in reducing neuronal excitability.

In achieving an anesthetic effect, a protein-drug interaction is suggested to take place through direct receptor binding (Li et al., 2006; Chiara et al., 2013). Therefore, a mutation in the receptor sequence could induce conformational changes that modulate drug sensitivity. Human population-based studies have attempted to identify genetic polymorphisms responsible for altered response to volatile anesthetics. However, the time and large number of subjects needed to obtain conclusive data render these studies cumbersome. Given the similar genetic make-up of mice and humans, and the ease of obtaining desired traits in short periods by selective breeding, a mouse model could provide valuable insights into the molecular mechanisms underlying the action of anesthetics.

This study utilized two mouse strains, selectively bred to exhibit different sensitivities to isoflurane. We analyzed and compared the coding sequences for the $GABA_A$ receptor subunits α1-6, β1-3, and γ1-3 in the isoflurane-sensitive and isoflurane-resistant strains, with the goal of identifying nucleotide mutations that may account for differences in drug sensitivity. The results of this study shed light on the underlying genetic importance of proper receptor subunit binding to isoflurane for achieving an anesthetic effect in our mouse model.

MATERIAL AND METHODS

Isoflurane delivery

Isoflurane gas was administered as previously described (Wang et al., 2009). In brief,

mice were placed individually in an acrylic cylinder and both ends of the cylinder were fitted with rubber stoppers that held the tubing for circuit connectivity and a 16-gauge needle with a stopcock for gas sampling or for connecting to an infrared anesthetic gas analyzer. The stopcock was closed when not in use. An anesthetic gas scavenger (TA Medical Equipment Co, Ltd., Nanjing, China) was attached between the cylinder and the vacuum manifold, and was replaced hourly during anesthesia. Isoflurane (Abbott Laboratories Ltd., Queenborogh, Kent, UK; Series No. 11613Z8) was vaporized (MVP300 isoflurane vaporizer, Aeon Medical Instrument Co., Ltd.) in 2 L/min of oxygen, and this mixture was supplied continuously to the cylinder during anesthesia. An on-line infrared analyzer (90518 Multigas Analyzer, Spacelabs Medical, Inc., Redmond, WA, USA) was utilized to monitor isoflurane concentration in the cylinder. Isoflurane concentration was stabilized within 5 min of activating the vaporizer. Righting reflex and isoflurane inhalation median effective dose (ED_{50}) were measured as criteria for successful anesthesia.

Animal breeding and strain selection

ICR/CD-1 mice were selectively bred to establish strains with different sensitivities to isoflurane, as previously described (Wang et al., 2010). Based on their reaction to inhaled isoflurane, female and male mice were separated into sensitive and resistant groups and were randomly bred within the group. After 8 generations of selective breeding, two strains with different sensitivities to isoflurane were obtained and were labeled as ISO-S (sensitive) and ISO-R (resistant).

Righting reflex and isoflurane inhalation ED_{50} were used to measure anesthetic success. Male and female mice that were most sensitive or resistant to isoflurane were randomly mated to produce the two groups of mice for the next generation of breeding. The most sensitive and resistant animals were selected from ISO-S and ISO-R groups respectively when they were 65 to 70 days of age. The screening test was repeated for 8 generations of pressure screening, and within each group, the mice were randomly mated, and breeding was carried out for three generations for conservation of phenotype.

Tissue collection

Forty mice (20 males and 20 females) of similar age were selected from the same generation of each strain (ISO-S and ISO-R). The animals were decapitated, and the brain tissue was harvested and immediately stored in liquid nitrogen, for subsequent use. All procedures were performed in strict accordance with the Animal Care and Use Committee Guidelines at the Wuxi People's Hospital of Nanjing Medical University.

RNA extraction and cDNA construction

RNA was extracted from the isolated mouse brain tissues as previously described (Zaker et al., 2014). Total RNA was extracted from mouse brain tissues using an RNeasy® Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. In brief, 30 mg brain tissue was lysed in the designated lysis buffer supplied with the kit (β-mercaptoethanol and buffer). A total of 30 μL RNase-free H_2O was used to elute RNA. Eluted samples were aliquoted and stored at -80°C. Quantification of RNA was performed by measuring the

absorbance at A260 in a biophotometer (Eppendorf, Germany). RNA purity was determined by calculation of the A260/A280 ratio.

cDNA preparation

cDNA preparation was performed using a Reference RNA LA PCRMT Kit (AMV) Ver.1.1 according to the manufacturer's instructions as follows. Two 200-μL PCR tubes respectively for solution A and solution B were prepared on ice according to the following recipe. Solution A contained 0.75 μL RNase Free dH_2O, 0.5 μL Oligo dT-Adaptor Primer (2.5 pmol/μL), and 4 μL Total RNA (5.25 μL total). Solution B contained 2 μL $MgCl_2$ (25 mM), 1 μL 10X RNA PCR Buffer, 1 μL dNTP mixture (each 10 mM), 0.25 μL RNase inhibitor (40 U/μL), and 0.5 μL AMV reverse transcriptase (5 U/μL) (4.75 μL total).

Solution A was incubated in a 70°C water bath for 10 min, followed by rapid chilling on ice for 30 s. Solution B was then added to solution A and mixed to yield a 10-μL reverse transcription reaction mixture. The PCR tube with 10 μL reverse transcription mixture was placed in a thermocycler and the reaction was carried out with one cycle of 42°C for 40 min followed by 70°C for 15 min. The mouse brain cDNA thus prepared was stored at -20°C.

PCR amplification of brain tissue cDNA and sequencing

The primer premier 5.0 software was used to design primers to obtain full-length cDNA sequences of the target gene. Primers were synthesized by Shanghai Biological Engineering Co. The primer sequences are listed in Table 1. cDNA were amplified by PCR according to the conditions shown in Table 2. PCR products were analyzed using agarose gel electrophoresis and were cut out from the gels If the observed size of the band based on primer design was consistent with the identified molecular weight of the product, the isolated bands were sent to the Haibo Ho Gene Sequencing Company, along with sequencing primers for amplification.

Table 1. Genes and primers.

Gene	Forward primer	Reverse primer	Seq-1 primer	Seq-2 primer
$GABA_A\alpha_1$	5'-ATGAAGAAAAGTCGGGG-3'	5'-CTATTGATGGGGTGTGGGG-3'	5'-TCTTTCATCCTTCCAACT-3'	5'-CCAGAAAAGCCAAAGAAAGT-3'
$GABA_A\alpha_2$	5'-ATGAAGACAAAATTGAGCAC-3'	5'-TCATGGACTGACCCCTAA-3'	5'-TTGGCATTGTCATGTTATGG-3'	5'-AAAGAGGATGGGCTTGGGAC-3'
$GABA_A\alpha_2$	5'-ATGATAATCACACAAATGTG-3'	5'-TCTACTGTTTGCGGGATCATG-3'	5'-TATTCTCCTGTACTAGACCG-3'	5'-CACCACTGTTCTCACCATGA-3'
$GABA_A\alpha_4$	5'-TGGTTTCTGTCCAGAAGGTA-3'	5'-TACATTAGACTTTCTGATTT-3'	5'-AGGGCAGGCATGACCATCCA-3'	5'-ATGCCAATTTGAACATGAGG-3'
$GABA_A\alpha_5$	5'-CTTACTGGGAATGGACAAT-3'	5'-TTACTTTGGAGAGGTGGCCC-3'	5'-CCACCACCACAGACTTGGTG-3'	5'-AAAGTGGCCTATGCCACAGC-3'
$GABA_A\alpha_6$	5'-TGGTCTTGCTTCTCCCCTGG-3'	5'-CTACTCAACAGTACTGCTCA-3'	5'-AGGAGGCTAGAAGAATCTTC-3'	5'-TGGATTGGTTCATAGCTGTA-3'
$GABA_A\beta_1$	5'-ATGTGGACAGTACAAAATCG-3'	5'-TTAGTGTACATAGTAAAGCC-3'	5'-TGTCAAGGGTCAGATTTAAT-3'	5'-AAAAGGGAGCGAGCAAACAA-3'
$GABA_A\beta_2$	5'-ATGTGGAGAGTCCGGAAAAG-3'	5'-TTAGTTCACATAATAAAGCCA-3'	5'-GTGTCAGGCACCCAGAGTTG-3'	5'-AAGCAGTAATGGGACTTGGA-3'
$GABA_A\beta_3$	5'-TGTGGGGCTTTGCGGGAGG-3'	5'-TCAGTTAACATAGTACAGCCA-3'	5'-CCGCCACGCCAGTAAAATTC-3'	5'-GCTTTGTCTTTGTATTCCTG-3'
$GABA_A\gamma_1$	5'-TGGGTTCTGGGAAAGCCTTC-3'	5'-TTATAAGTATAGATACCCA-3'	5'-CCATAGGAAAATTATGAAGT-3'	5'-CTATTTCACGATTCAGACAT-3'
$GABA_A\gamma_2$	5'-ATGACGCTGTGGATTCTGCT-3'	5'-TTACAGATAAAGATAGGAGA-3'	5'-TCATTCCAAATTCTCAGCAT-3'	5'-CTGTCCTGGGTGTCCTTCTG-3'
$GABA_A\gamma_3$	5'-CGGAGAGCCATGGCTGCAAA-3'	5'-TTAAAGATACAGGTATCCAA-3'	5'-GCCTCCGCTGTTTTAGAATT-3'	5'-AATCGTGACAACATCTGCAG-3'

Table 2. Transcription reaction conditions and components.

Reaction conditions	Reaction mixture
94°C 30 s	10X PFU buffer 5 μL
95°C 5 min	H_2O 37.5 μL
55°C 50 s	dNTP 1 μL
72°C 2 min 30 s (35 cycles)	Primer F 2 μL
72°C 10 min	Primer R 2 μL
4°C hold	PFU 0.5 μL
	cDNA 2 μL
	Recover and sequence gel

Protein isolation and immunoblotting

Proteins were isolated from frozen brain tissues for western blot analysis according to a previously described procedure, with modifications (Hu et al., 2004; Liu et al., 2006; Chen et al., 2007). Briefly, frozen brain tissue was homogenized in ice-cold radio-immunoprecipitation assay (RIPA) lysis buffer supplemented with a protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA) for 30 min, and then centrifuged at 10,000 *g* for 15 min at 4°C. The supernatant was extracted and stored at -80°C for later analysis. Protein concentration was determined using a Bio-Rad assay system (Bio-Rad, San Francisco, CA, USA). Sample proteins were mixed with sample buffer (6.2 mM Tris-HCl, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.01% bromophenol blue and 50 mM 2-mercaptoethanol (2-ME) (Sigma Aldrich, St. Louis, MO, USA) and heated at 95°C for 5 min. Equal amounts of each protein sample were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad). The membranes were then blocked against non-specific binding with 5% nonfat milk in Tris buffered saline with Tween (50 Mm Tris, 1.5% NaCl, and 0.05% Tween 20 at pH = 7.5) for 1 h at room temperature.

After blocking, the membranes were incubated overnight at 4°C with primary antibodies against $GABA_A R\beta 1$ (1:1000), $GABA_A R\alpha$ (1:500), glycine receptor (1:1000), and β-actin (1:1000) as an internal loading control (Sigma Aldrich, MO, USA). Following this, the membranes were washed and incubated in alkaline phosphatase-conjugated secondary antibodies (1:10000, Sigma Aldrich, MO, USA) for 1 h at room temperature. Bands were detected using a chromogenic substrate, 5-bromo-4-chloro-3-indolyl phosphate in the presence of nitroblue tetrazolium (Sigma Aldrich, St. Louis, MO, USA). Densitometric analysis was performed on the bands using ImageJ software (NIH, Bethesda, MD) and was shown as percentages between groups as compared to β-actin.

Statistical analysis

Dose-response relationship data were analyzed by probit analysis. All statistical analyses were performed using SPSS 13.0 software (SPSS, Chicago, USA). Differences in nucleotide sequence between samples from isoflurane resistant and sensitive strains were compared using the χ^2 test. Fischer's exact test was used to make comparisons between the two strains of mice for the SNP found in the cDNA sequence of the β_1 subunit. Statistical significance was defined as $P < 0.05$.

RESULTS

cDNA analysis identified a SNP at nucleotide position 462 (C/G) in the β_1 subunit

The cDNA sequences from our two strains of mice for the $GABA_A$ receptor subunits α1-6, β2, β3, γ1-3 were identical. However, the gene sequence comparison for the β1 $GABA_A$ receptor subunit identified a SNP at the nucleotide position 462(C / G), with 36 mice from the resistant strain having C (cytosine) at nucleotide 462 while only 5 mice had C at this position in the sensitive strain. The remaining mice in both strains showed G (guanine) in this position on the cDNA of $GABA_A$ receptor β1 sequence (Figure 1). Application of a Chi-square test calculation determined that this difference was statistically significant ($P < 0.01$).

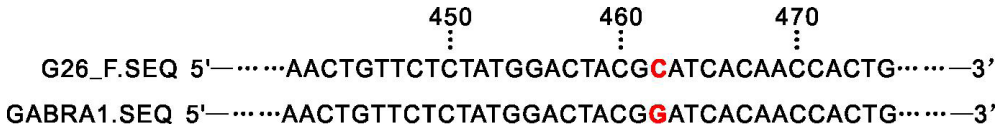

Figure 1. Common sequence modifications in isoflurane-resistant (ISO-R) mice. Identified SNP loci in the resistant mice are shown. Thirty-six ISO-R mice had a C/G SNP at nucleotide 462 of the $GABA_A$ β_1 receptor sequence as opposed to only 5 mice in the sensitive strain. The χ^2 test showed that this difference was statistically significant ($P < 0.01$).

Selective breeding produced isoflurane resistant mice

In primary mice, the isoflurane ED_{50} (95%CI) for females was 0.65% (0.58-0.72%) and that for males was 0.63% (0.56-0.69%). After four generations of breeding for resistance to isoflurane, ED_{50} was significantly higher than the sensitive groups ($P < 0.05$). The ED_{50} for male and female mice in the 11th generation of the selectively bred resistant populations were increased by 32 and 36% respectively, than those in the same generation of isoflurane sensitive mice (Figure 2).

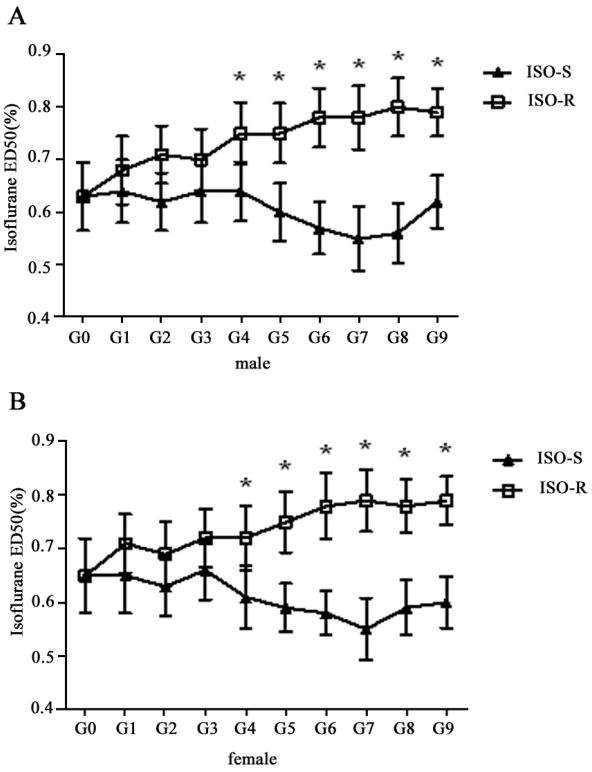

Figure 2. Generational differences in sensitivity to isoflurane. **A.** In the ISO-S sensitive strain of mice, the ED_{50} for isoflurane was similar between the 1st and the 11th generations (G1 = 64.0 *vs* G11 = 62.2%). **B.** In the ISO-R resistant strain of mice, the ED_{50} at G11 was 79.6 *vs* 67.5% at G1, showing increasing resistance through selective breeding.

Isoflurane-resistant mice exhibited reduced $GABA_A$ β_1 receptor subunit expression

Using western blot analysis for different $GABA_A$ receptor subunits and other receptors, the mice from the 11th generation expressed approximately 86% less $GABA_A$R β1 than isoflurane-sensitive mice (Figure 3). In general, other subunits of the $GABA_A$ receptor were similar between the two strains of mice. As the glycine receptor could play a role in mediating the drug's effects, its expression was examined. The expression of the glycine receptor was also similar between the two strains of mice. This evidence suggests that the identified SNP may play a role in altering the expression of the β1 subunit and may contribute to the differences in sensitivity noted between the two selectively bred strains of mice.

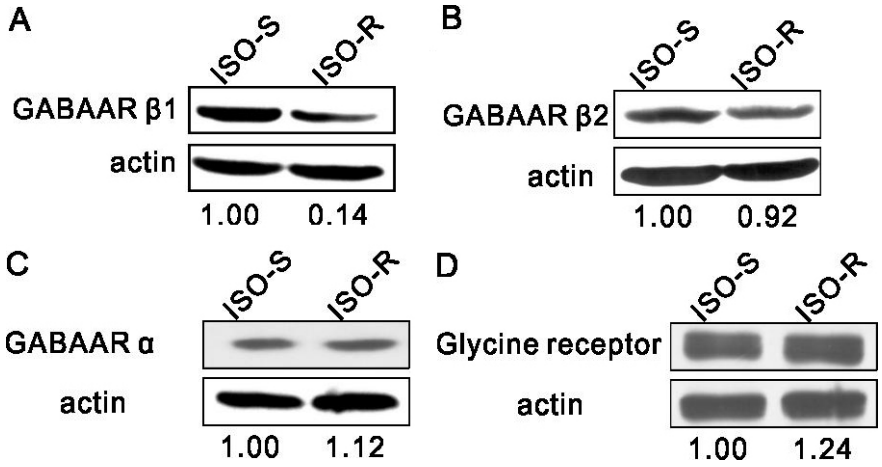

Figure 3. Receptor and receptor subunit expression in sensitive and resistant mice strains. As shown in the western blot images, ISO-R mice showed lower $GABA_A$R β1 expression than ISO-S sensitive mice. Densitometry revealed that the $GABA_A$R β1 expression in mice of ISO-R group accounted for approximately 86% of the expression in mice of ISO-S group. The expression of other $GABA_A$ receptor subunits and the glycine receptor was not markedly different between the two strains of mice.

DISCUSSION

The inhibitory neurotransmitter γ-amino butyric acid (γ-aminobutyric acid, GABA) is widely distributed in the mammalian CNS, and approximately 50% of CNS synapses use GABA in the regulation of neuronal excitability. $GABA_A$ receptors are the most common and important among the GABA receptors. Thus far, about 20 different $GABA_A$ subunits have been identified and categorized into eight subunit families named α1-6, β1-4, γ1-3, ρ1-3, δ1, ε1, π1, and θ1 (Barnard et al., 1998). The natural $GABA_A$ receptors in the mammalian brain are primarily comprised ofα, β, and γ subunits and many studies have shown that $GABA_A$ receptors maybe one of the targets of general anesthetics (Rudolph et al., 1999; McKernan et al., 2000).

Volatile general anesthetics have been applied for well over a century to induce corporeal paralysis for effective and humane surgical procedures. Interestingly, the mechanisms of how these, and other types of anesthetics, achieve their effects are still poorly understood.

In the present study, we used a novel model of mice that were sensitive or resistant to the volatile inhalational anesthetic, isoflurane, to provide evidence that modifications in the gene coding for the γ-amino butyric acid (γ-aminobutyric acid, $GABA_A$ receptor may play a role in isoflurane function. Thus, this study illuminates the mechanisms that may contribute to susceptibility of certain individuals to the drug as compared with others.

Various anesthetics have been shown to utilize different GABA receptor subtypes during GABA mediated paired pulse synaptic transmission, and that different subtypes exhibit differential expression in different regions of the brain and CNS (MacIver, 2014). In the present study, we have developed a mouse model that exhibits differing sensitivities to isoflurane; the cDNA of the $GABA_A$ receptor α1-6, β1-3, and γ1-3 subunits, from the sensitive and resistant mouse strains, were sequenced, analyzed, and compared. After obtaining the sequence results, the sequences were compared to those published in GenBank (http://www.ncbi.nlm.nih.gov/) and were found to be consistent. Our analysis of the cDNA of the $GABA_A$ receptor β1 subunit identified a single nucleotide polymorphism (SNP) locus consistent with GenBank results. In the resistant strain, the residue 462(C) had a greater frequency than the appearance of C in the sensitive group. Genomic SNPs, overall appeared to be uneven. In accordance with the location of the genomic SNP loci, their distribution can be divided into SNPs within the coding region of the genome (cSNP), the gene surrounding the SNP (pSNP), and the intergenic SNP (iSNP), as well as other categories. The probability of cSNP occurrence was small due to a low mutation rate of only 1/5 within the exon sequences; however, it has been important in the study of genetic diseases and breeding (Li and Sadler, 1991). The SNP loci found in this study are categorized as cSNPs. Mutations not directly affected by alterations in encoded amino acids, but with base sites located outside the first exon, are likely to result in RNA changes, and thus may affect protein expression. This can ultimately affect susceptibility of mice to inhaled anesthetics, which led to observed differences in sensitivity to inhaled anesthetics between the two mouse strains.

Mild sensory impairment has been shown to exist in the $GABA_A$ receptor β3 subunit knockout mice ($\beta3^{-/-}$) compared to wild type ($\beta3^{+/+}$). However, enflurane and halothane inhibit the righting reflex at similar concentrations in these mice. Halothane can elicit a slight resistance (10-20%) in $\beta3^{-/-}$ mice to tail clip (pain suppression and braking effect), suggesting that inhaled anesthetics may only have a mild impact on the braking action through the $GABA_A$ receptor β3 subunit (Quinlan et al., 1998). In another study, β2 subunit knockout mice exhibited decreased sensitivity to etomidate (Reynolds et al., 2003). Sekine et al. found that when mice were given isoflurane, propofol, midazolam, or other narcotics, mRNA expression of $GABA_A$ receptor α4 subunit increased in the brain (Sekine et al., 2006). Studies have shown that the anesthetic, etomidate achieves its effects through the β1 and β2 subunits of the $GABA_A$ receptor (Belelli et al., 1999; Yanovsky et al., 2012). Such studies show that the anesthetic propofol achieves joint anesthetic action through different $GABA_A$ receptor subunits. The $GABA_A$ receptor β3 subunit appears to be responsible for isoflurane-mediated amnesia during anesthesia in certain neuronal populations (Rau et al., 2011), suggesting that multiple subunits contribute to different functions necessary for achieving full anesthesia by treatment with inhalational isoflurane. Similar to these results, the findings of the present study strongly suggest that the $GABA_A$ receptor may a major target of inhaled anesthetics as well as other anesthetic drugs.

Despite this evidence, research supports a role for other receptors, such as NMDA receptors, in achieving full anesthesia using general anesthetics such as isoflurane. In particular, NMDA receptors may be important for achieving animal immobility in conditions

where GABAergic stimulation by anesthetics promotes unconsciousness, both of which are required for adequate surgical anesthesia. In conclusion as supported by previous studies, this study suggests that $GABA_A$ receptor is a possible site of action for general anesthetics and that modification of the B1 receptor subunit may alter the sensitivity of animals to isoflurane through modulation of its binding capability. However, this is an initial investigation and although the results are intriguing, further research is required to better understand the mechanisms by which such interactions and effects are achieved in the use of general anesthetics such as isoflurane in animal models.

ACKNOWLEDGMENTS

Research supported by the National Natural Science Funds for Young Scholar (#81100814).

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