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Anesthesia Sensitivity in Mice that Lack the β3 Subunit of the γ-Aminobutyric Acid Type A Receptor

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Background: **The mammalian γ-aminobutyric acid type A ($GABA_A$) receptor, a likely target of anesthetic action, exhibits remarkable subunit heterogeneity. *In vitro* expression studies suggest that there is subunit specificity to anesthetic responses at the $GABA_A$ receptor. The authors tested whether genetically engineered mice that lack the β3 subunit of the $GABA_A$ receptor differed in their sensitivities to several general anesthetic agents.**

Methods: **Median effective concentrations for loss-of-righting reflex and tail clamp/withdrawal for enflurane and halothane were determined in mice with and without the β3 gene and gene product. Sleep time was measured after intraperitoneal injection of pentobarbital, ethanol, etomidate, and midazolam.**

Results: **Null allele mice (β3 −/−) did not differ from wild-type mice (β3 +/+) in the obtunding response to enflurane and halothane but were significantly more resistant to enflurane (null allele half-effect concentrations [EC_{50}] of 2.59 ± 0.10 *vs.* wild-type EC_{50} of 2.06 ± 0.12 atm %, $P < 0.001$) and halothane (null allele EC_{50} of 1.73 ± 0.04 *vs.* wild-type EC_{50} of 1.59 ± 0.05 atm %, $P = 0.01$) as determined by tail clamp response. Wild-type and null allele mice exhibited divergent responses to other sedative agents active at the $GABA_A$ receptor. No differences were noted in sleep times after administration of pentobarbital and ethanol, but null allele mice were more resistant to etomidate (null allele EC_{50} of 17.8 ± 1.9 min *vs.* wild-type EC_{50} of 26.2 ± 2.4 min, $P < 0.02$) and midazolam (null allele EC_{50} of 14.2 ± 7.8 min *vs.* wild-type EC_{50} of 41.3 ± 10.4 min, $P < 0.05$).**

Conclusions: **The β3 subunit of the $GABA_A$ receptor appears to be important in the mediation of the immobilizing (tail clamp) but not obtunding (loss-of-righting reflex) effects of the volatile anesthetic agents enflurane and halothane. These data support the hypotheses that separate components of the anesthetic state are mediated *via* different central nervous system loci; that the $GABA_A$ receptor is a likely target for the immobilizing response to volatile anesthetic agents; and that the β3 subunit plays a direct or indirect role in the mediation of this response. Absence of the β3 subunit appears to attenuate the obtunding effect of midazolam and etomidate but appears not to alter the obtunding effect of pentobarbital, enflurane, and halothane, suggesting that these anesthetic agents produce hypnosis by different specific molecular mechanisms. (Key words: Benzodiazepine; enflurane; ethanol; etomidate; gene targeting; halothane; midazolam; pentobarbital.)**

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SIMILAR to other members of the superfamily of ligand-gated ion channels found in the nervous system, the γ-aminobutyric acid type A ($GABA_A$) receptor is a hetero-oligomeric protein composed of distinct polypeptide subunits.[1] These subunits have been grouped into several subtypes that share a high degree of amino acid sequence homology (α1-6, β1-3, γ1-3, δ, and ε). The identity of specific subunits found in a given receptor determines the pharmacology of that receptor; for example, the presence of the α6 subunit confers a high affinity for benzodiazepine inverse agonists and a low affinity for classical benzodiazepine agonists.[2] Multiple sources of data implicate the $GABA_A$ receptor as a likely target of anesthetic action,[3] and electrophysiologic studies suggest that subunit composition conditions $GABA_A$ receptor anesthetic responses.[4,5] For example, pentobarbital, etomidate, and propofol induce currents in GABA receptors constructed of β1 or β3 homomers.[6,7] Inclusion of certain subunits can render receptors insensitive to anesthetic effects; for example, modulation

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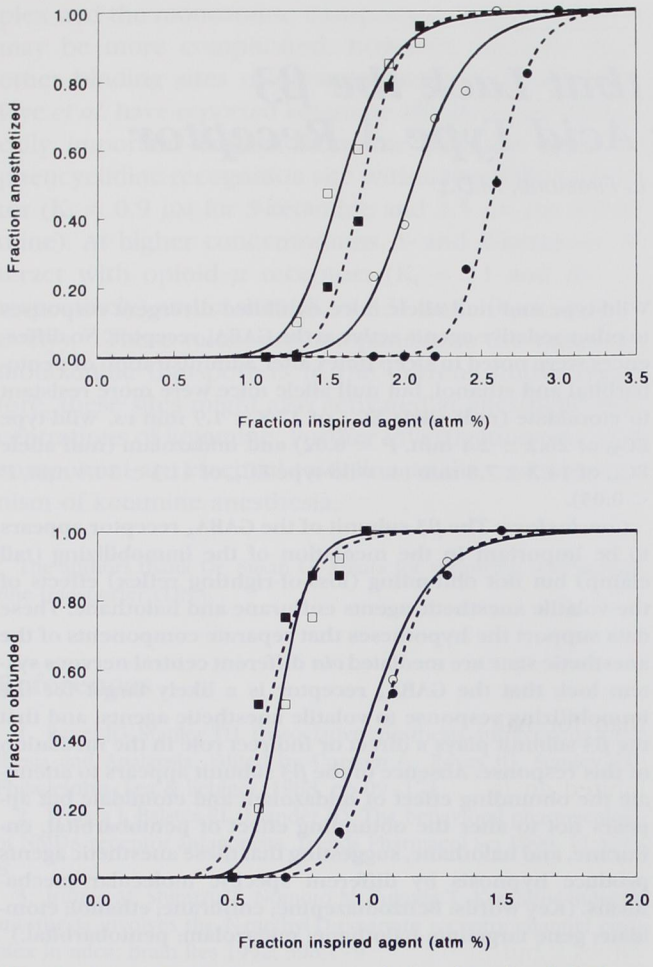

Fig. 1. Concentration–response relationship for halothane (*squares*) and enflurane (*circles*) in tail clamp (*top*) and loss-of-righting reflex (*bottom*) assays in wild-type (*open symbols*) and null allele (*filled symbols*) mice. Data were fit to a logistic equation using iterative, nonlinear regression (solid lines = wild-type mice; dotted lines = null allele mice).

of GABA-gated currents by the anesthetic neurosteroid 3α,21-dihydroxy-5α-pregnan-20-1 is markedly reduced in receptors containing the δ subunit.[8]

Recently, methods have been developed that permit the role of specific receptor subunits in behavioral anesthetic end points to be investigated. Specifically, the creation of mice that lack a specific gene ("null allele" or "knockout" mice, which cannot manufacture the corresponding gene product), offers a unique method to determine the importance of given $GABA_A$ receptor subunits to specific anesthetic responses. We recently created mice that lack the β3 subunit of the $GABA_A$ receptor.[9] The β3 subunit is widely expressed throughout the mammalian central nervous system, and therefore receptors that incorporate it are a likely target for anesthetic action. We compared the sensitivity of β3 null allele and wild-type mice to the obtunding and immobilizing actions of the volatile anesthetic agents enflurane and halothane and to the hypnotic effects of pentobarbital, ethanol, etomidate, and midazolam to determine whether this subunit is important to the actions of these anesthetic agents.

Materials and Methods

This work was approved by the animal care and use committee at the University of Pittsburgh (Pittsburgh, PA). Mice that lack the genes for the β3 subunit of the $GABA_A$ receptor (strain designation *Gabrb3*^{tm1Geh}) were created as previously described.[9] The β3 gene was disrupted by homologous recombination in mouse embryonic stem cells. The disrupted β3 gene was transmitted through the germ cell line, ultimately resulting in the production of wild-type (β3 +/+), heterozygous (β3 +/−), and null allele (β3 −/−) mice. Northern blot analysis confirmed that the disrupted β3 gene was not functional; the β3 protein was absent, and the β3 messenger ribonucleic acid was absent in null allele mice but present at 70% of wild-type levels in heterozygotes.

Volatile Agents

Halothane and enflurane requirements in adult mice were determined by using the end points of loss-of-righting reflex[10] and tail clamp/withdrawal.[11] For loss-of-righting reflex, mice were placed in individual wire mesh cages in a rotating carrousel in a sealed acrylic chamber. Carbon dioxide tension (maintained at < 1 atm %) and rectal temperature (maintained at 36.5 $\pm$ 1.0°C) were controlled to avoid effects on anesthetic requirement.[12,13] The atmosphere of the chamber was equilibrated for 15 min with a chosen concentration of halothane (atm %) or enflurane (atm %), confirmed by piezoelectric analysis (Siemens 120, Danvers, MA). A blinded observer scored the mice for loss-of-righting reflex in a quantal fashion, after which mice were allowed to recover in air for 30 min before a new trial. An enclosed chamber with similar characteristics was used to determine response to tail clamp. After equilibration at a given anesthetic concentration for 15 min, mice were scored in a quantal fashion by a blinded examiner for an organized motor withdrawal in response to clamping the tail, before recovery for 30 min and assay at another anesthetic concentration.

Table 1. Anesthetic Requirement in Mice with and without the β3 $GABA_A$ Receptor Subunit

Genotype	n (M/F)	Age (range) (wk)	Anesthetic	EC_{50} (atm % ± SE)	Slope (±SE)
Tail clamp					
β3 +/+	13 (5/8)	10.6 (11–16)	Enflurane	2.06 ± 0.12	11.1 ± 2.8
β3 +/−	11 (9/2)	13.2 (10–20)	Enflurane	2.21 ± 0.05	19.8 ± 5.2
β3 −/−	12 (2/10)	14.5 (8–20)	Enflurane	2.59 ± 0.10*	20.5 ± 5.6
β3 +/+	20 (10/10)	11.5 (8–16)	Halothane	1.59 ± 0.05	9.6 ± 1.8
β +/−	15 (7/8)	12.1 (9–16)	Halothane	1.80 ± 0.05†	15.5 ± 3.8
β3 −/−	23 (10/13)	11.3 (8–17)	Halothane	1.73 ± 0.04‡	14.0 ± 2.5
LORR					
β3 +/+	23 (14/9)	12.1 (9–14)	Enflurane	1.03 ± 0.06	9.4 ± 1.8
β3 −/−	15 (5/10)	10.5 (7–13)	Enflurane	1.08 ± 0.07	11.7 ± 2.6
β3 +/+	20 (15/5)	10.2 (9–13)	Halothane	0.68 ± 0.04	12.2 ± 2.7
β3 −/−	8 (2/6)	9.1 (8–10)	Halothane	0.64 ± 0.07	8.3 ± 2.5

GABA = γ-aminobutyric acid; LORR = loss of righting reflex.

* $P < 0.001$, null allele *versus* wild type.

† $P = 0.001$, heterozygote *versus* wild type.

‡ $P = 0.01$, null allele *versus* wild type.

Intravenous Agents

Sleep time (duration of the loss-of-righting reflex) was determined after intraperitoneal injection of pentobarbital (45 mg/kg), ethanol (3.5 mg/g), etomidate (20 mg/kg), or midazolam (60 mg/kg) as previously described.[14] Temperature was controlled and measured in a manner similar to the loss-of-righting reflex and tail clamp assays.

Concentration-response data for loss-of-righting reflex and tail clamp/withdrawal were fit to a logistic equation, yielding half-effect concentrations (EC_{50}), slopes, and estimates of their respective standard errors.[15] Null allele, heterozygous, and wild-type group responses were compared statistically by referring the variance ratio to a standard normal distribution.[16] Mean sleep times for each agent were compared in null allele and wild-type mice using an unpaired *t* test.

Results

The slopes of the concentration-response curves for tail clamp and loss-of-righting reflex with enflurane and halothane were statistically indistinguishable among the three groups, allowing valid comparisons of the EC_{50} values. The EC_{50} value for enflurane tail clamp in wild-type mice was similar to previously published values, whereas that for halothane was slightly higher than previous reports.[11,17] Null allele mice were significantly more resistant to enflurane and halothane (table 1 and fig. 1). The magnitude of the difference in sensitivity was almost three times greater for enflurane (EC_{50} in null allele mice was 26% greater than in wild-type mice) than for halothane (EC_{50} in null allele mice was 9% greater than in wild-type mice). Heterozygous mice exhibited an intermediate value for enflurane but were similar to null allele mice in their response to halothane (table 1). Median effective concentrations for loss-of-righting reflex with enflurane and halothane in wild-type mice were also similar to previously published values,[11] but null allele mice did not differ in their sensitivities to the two volatile agents in this assay (table 1 and fig. 1). The ratio of potency of enflurane in the loss-of-righting reflex and tail clamp assays for wild-type animals was almost identical to that previously reported.[11]

Although the sleep times of null allele and wild-type mice after administration of pentobarbital and ethanol were almost identical, null allele mice were significantly more resistant to etomidate and midazolam (table 2). The magnitude of the difference in sleep time was approximately twice as great with midazolam (65% less than in wild-type mice) than with etomidate (32% less than in wild-type mice).

Discussion

As we have previously reported, many mice lacking the β3 GABA receptor subunit gene have major abnormalities in craniofacial development and die as neonates. Null allele mice that survive behave abnormally,

Table 2. Sleep Time in Mice with and without the $\beta 3$ $GABA_A$ Receptor Subunit

Agent	Genotype	n (M/F)	Age (range) (wk)	Sleep Time (min) (mean ± SE)
Pentobarbital	$\beta 3$ +/+	19 (8/11)	15.4 (8–20)	38.5 ± 2.8
	β −/−	17 (6/11)	20.2 (15–28)	43.8 ± 3.9
Ethanol	$\beta 3$ +/+	25 (8/17)	12.7 (7–17)	58.1 ± 7.1
	$\beta 3$ −/−	28 (9/19)	13.5 (8–18)	60.1 ± 5.9
Midazolam	$\beta 3$ +/+	11 (3/8)	14.9 (11–22)	41.3 ± 10.4*
	$\beta 3$ −/−	12 (5/7)	15.9 (11–20)	14.2 ± 7.8*
Etomidate	$\beta 3$ +/+	12 (9/16)	18.4 (11–24)	26.2 ± 2.4†
	$\beta 3$ −/−	15 (5/10)	18.7 (12–24)	17.8 ± 1.9†

GABA = γ-aminobutyric acid.

* $P < 0.05$, null allele *versus* wild type.

† $P < 0.02$, null allele *versus* wild type.

exhibiting hyperactivity, incoordination, and epilepsy.[9] Lack of the $\beta 3$ gene is accompanied by grossly abnormal electroencephalographic activity and a marked reduction in the number of $GABA_A$ receptors, suggesting that the $\beta 3$ subunit is crucial in the assembly process for receptors and cannot simply be replaced by other available subunits.[9] The $\beta 3$ subunit is normally a frequent constituent of the $GABA_A$ receptor throughout the nervous system (especially in the cerebral cortex, hypothalamus, cranial nerve ganglia, and spinal cord),[18,19] and several lines of evidence suggest that its presence in a receptor may confer distinct pharmacologic properties. $GABA_A$ receptors containing $\beta 3$ subunits have a threefold greater affinity for GABA than do receptors with $\beta 1$ or $\beta 2$ subunits[20]; however, individual β subunits seem to differ little in their modulation by benzodiazepines, barbiturates, or steroids, at least when transiently expressed with α and γ subunits.[20] The mice in the current study, which lack $\beta 3$ subunits, offer a unique opportunity to investigate the role of these subunits in the behavioral end points of anesthetic action.

The absence of a difference in the potency of enflurane and halothane in the loss-of-righting reflex assay, in the face of a significant difference in the tail clamp assay, suggests that the $\beta 3$ subunit is important in the mediation of the immobilizing but not the hypnotic response to volatile agents. These data agree with recent data suggesting that separate elements of the anesthetic state are produced by different mechanisms. For example, anesthetic agents vary widely in the ratio of potency in producing amnesia to potency as surgical anesthetic agents,[21] and certain lipid-soluble volatile anesthetic agents predicted to act as such by the Meyer–Overton hypothesis fail to act as surgical anesthetic agents, yet can produce amnesia.[22] Such data support the hypothesis that anesthetic agents produce immobility and amnesia by disparate mechanisms or neural pathways; immobility may be mediated at a site possessing polar and nonpolar properties, such as an aqueous–protein interface of a membrane-bound receptor, whereas amnesia is produced at a nonpolar site.[23] Such a hypothesis predicts that the $GABA_A$ receptor is a more likely anesthetic target for the immobilizing response to a surgical stimulus than for the amnestic or sedative responses, as volatile anesthetic agents have their maximum effects on $GABA_A$ receptors at concentrations producing immobility.[23] Our data support the concept that the $GABA_A$ receptor is a likely target for the immobilizing response and suggest that the $\beta 3$ subunit may be important in this process, because of a direct interaction of volatile agents with $GABA_A$ receptor isoforms that contain the $\beta 3$ subunit, a marked general decrease in GABA-mediated inhibition associated with $\beta 3$ gene inactivation, or differences in neuronal circuitry due to development in the absence of the $\beta 3$ subunit. Because an analgesic component is integral to the tail clamp response, it is possible that the null allele mice have an altered threshold to pain, suggesting a role for $\beta 3$-containing subunits in neuronal analgesic pathways. We are currently collaborating on studies to examine pain responses in mice lacking the $\beta 3$ subunit. Further, the lack of a difference in the obtunding response as measured by the loss-of-righting reflex suggests that sedation by volatile anesthetic agents may be mediated at another site, similar to the amnestic response. Determining whether these wild-type and null allele mice differ in their amnestic response to volatile anesthetic agents would be a useful test of this "separate mechanisms" hypothesis.

The divergence in the sleep time response of the null

allele and wild-type mice to midazolam and etomidate *versus* pentobarbital, ethanol, and the volatile agents is unexpected because these agents are all believed to share the $GABA_A$ receptor as an important pharmacologic target. Although sensitivity to ethanol and pentobarbital are not correlated in heterogenous stock mice,[24] sensitivity to these drugs in lines of rodents bred for sensitivity to ethanol or diazepam has been parallel; *i.e.,* mice bred for sensitivity to ethanol were also sensitive to pentobarbital, benzodiazepines, and volatile anesthetic agents,[14,25] whereas mice bred for sensitivity to diazepam were also sensitive to ethanol, pentobarbital, and volatile anesthetic agents.[10,26] The current data suggest that these agents interact differently with receptors that possess or lack the β3 subunit. Absence of the β3 subunit appears to attenuate the obtunding effect of midazolam and etomidate but appears to have no result on the obtunding effects of pentobarbital, ethanol, enflurane, and halothane. Although there are few electrophysiologic data to suggest β subunit dependence of midazolam action, the efficacy of etomidate in the direct activation of chloride currents and modulation of GABA-evoked chloride currents of recombinant human GABA receptors shows a rank order of β subunit dependence of $\beta3 > \beta2 >>> \beta1$.[27] Because etomidate is most efficacious at β3-containing subunits, one might predict a higher etomidate requirement in their absence. Although we cannot exclude a pharmacokinetic basis for the difference in sleep times with midazolam and etomidate, this explanation seems unlikely because the mice share the same genetic background for all the enzymatic processes vital to drug inactivation and excretion.

In contrast to the α6 subunit of the $GABA_A$ receptor, whose absence does not change the anesthetic requirement for enflurane,[28] the β3 subunit of the GABA receptor seems to be involved integrally in the immobilizing response to volatile anesthetic agents. A lack of effect of the absence of the β3 subunit on the obtunding response to volatile anesthetic agents, pentobarbital, and ethanol, together with significant resistance to the obtunding effect of midazolam and etomidate, supports the hypothesis that anesthetic agents may modulate the $GABA_A$ receptor at separate sites *via* separate mechanisms of action, which depend on the composition of receptor subunits. Future studies using conditional β3 null allele mice may allow the brain regions most relevant to these mechanisms to be identified.

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