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Enflurane Actions on Spinal Cords from Mice That Lack the β_3 Subunit of the $GABA_A$ Receptor

Shirley M. E. Wong, M.S., Gong Cheng, M.D.,† Gregg E. Homanics, Ph.D.,‡ Joan J. Kendig, Ph.D.§*

Background: **γ-Aminobutyric acid type A ($GABA_A$) receptors are considered important in mediating anesthetic actions. Mice lacking the β_3 subunit of this receptor ($\beta_3-/-$) have a higher enflurane minimum alveolar concentration (MAC) than wild types (+/+). MAC is predominantly determined in spinal cord.**

Methods: **The authors measured three population-evoked responses in whole spinal cords, namely, the excitatory postsynaptic potential (pEPSP), the slow ventral root potential (sVRP), and the dorsal root potential. Synaptic and glutamate-evoked currents from motor neurons in spinal cord slices were also measured.**

Results: **Sensitivity of evoked responses to enflurane did not differ between +/+ and −/− cords. The $GABA_A$ receptor antagonist bicuculline significantly ($P < 0.05$) attenuated the depressant effects of enflurane on pEPSP, sVRP and glutamate-evoked currents in +/+ but not −/− cords. The glycine antagonist strychnine elevated the pEPSP to a significantly greater extent in −/− than in +/+ cords, but the interactions between strychnine and enflurane did not differ between −/− and +/+ cords.**

Conclusions: **Similar enflurane sensitivity in spinal cords from −/− and +/+ mice was coupled with a decreased role for $GABA_A$ receptors in mediating the actions of enflurane in the former. This finding implies that other anesthetic targets substitute for $GABA_A$ receptors. Increase in glycine receptor-mediated inhibition was found in −/− cords, but the glycine receptor does not appear to be a substitute anesthetic target. This mutation thus led to a quantitative change in the molecular basis for anesthetic depression of spinal neurotransmission in a fashion not predicted by the mutation itself. The results argue against an immutable dominant role for $GABA_A$ receptors in mediating spinal contributions to MAC.**

γ-AMINOBUTYRIC acid type A ($GABA_A$) receptors are considered to be important target sites for the actions of volatile and many intravenous anesthetic agents.[1] Deletion or alteration of an important part of this receptor might therefore be predicted to alter anesthetic requirement. At the molecular level, mutation of a single amino acid in the TM2–TM3 region is sufficient to remove $GABA_A$ receptor sensitivity to volatile anesthetics and ethanol.[2] Behaviorally, mice genetically engineered to lack the β_3 subunit of the $GABA_A$ receptor ($\beta_3-/-$) display hyperactivity and seizures.[3] They have a higher anesthetic requirement for halothane and enflurane to produce immobility in response to a noxious stimulus (minimum alveolar concentration [MAC]).[4] Because MAC is primarily determined at the spinal level,[5,6] we conducted studies to test the hypothesis that deletion of the β_3 subunit would decrease the potency of enflurane to depress evoked responses in spinal cords from mice *in vitro*. Experiments were conducted using extracellular recording from intact cords and patch clamp recording from motor neurons in spinal cord slices.

Figure 1 shows a diagram of the circuitry and the primary transmitter receptors that mediate and modulate the responses examined in the current study. In whole cord, these are the population excitatory postsynaptic potential (pEPSP), the slow ventral root potential (sVRP), and the dorsal root potential (DRP). $GABA_A$ receptors are involved in generating the early fast component of the DRP, and, therefore, a $GABA_A$ antagonist will depress this response. Both $GABA_A$ and glycine receptors inhibit the pEPSP and the sVRP; therefore, $GABA_A$ and glycine agonists will depress and antagonists will elevate these responses. In motor neurons, the current evoked by stimulation in the dorsal root entry area (excitatory postsynaptic current) has the same circuitry and pharmacology as the pEPSP. Directly evoking motor neuron currents by glutamate in the presence of tetrodotoxin bypasses the presynaptic circuitry.

Materials and Methods

A foundation colony was established at Stanford University with six breeding pairs of mice heterozygous (+/−) for the mutant gene incapable of expressing the $GABA_A$ receptor β_3 subunit. These breeding pairs were the F6–F7 generations of the mutant line originated at the University of Pittsburgh[3] and were derived on a mixed C57GL/6J X 129/Sv/SvJ genetic background. Null mutants (−/−) were identified phenotypically at birth by pink eye color. Wild-type and heterozygous mice have black eyes at birth. All mice were genotyped retrospectively using Southern blot techniques. For the most part, controls were littermates of the nulls; to increase the numbers of homozygous wild types (+/+) in the series, some control experiments were performed using offspring of +/+ pairs.

Protocols were approved by the Institutional Animal Care and Use Committee at Stanford University. Experiments were performed on 1–6-day-old mice, using methods similar to those previously described.[7,8] After anesthesia with halothane and decapitation, spinal

* Research Assistant, † Research Associate, § Professor, Department of Anesthesia, Stanford University School of Medicine. ‡ Assistant Professor, Departments of Anesthesiology, Critical Care Medicine, and Pharmacology, University of Pittsburgh, Pittsburgh, Pennsylvania.

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Address reprint requests to Dr. Kendig: Department of Anesthesia, Stanford University School of Medicine, Stanford, CA 94305-5117. Address electronic mail to: kendig@leland.stanford.edu.

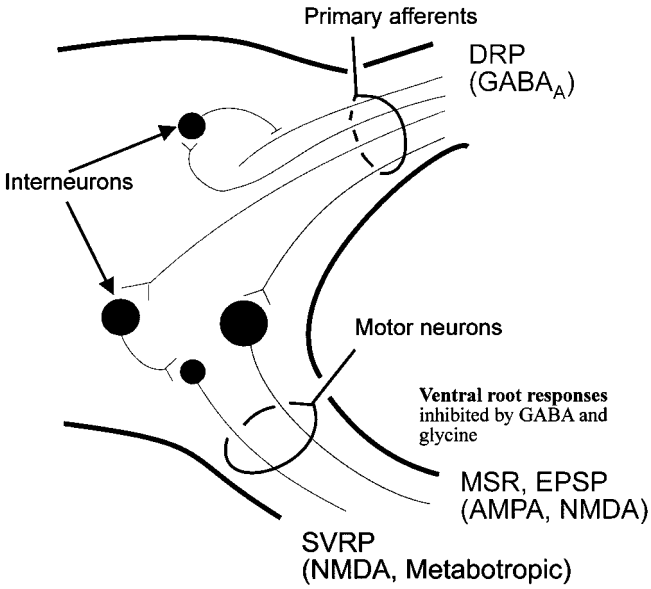

Fig. 1. Diagram of the pathways that mediate the evoked responses from spinal cord and the neurotransmitters that mediate and modulate them. The early part of the dorsal root potential (DRP) is generated in part by $GABA_A$ receptors on primary afferent nerve terminals responding to GABA released from interneurons. The excitatory postsynaptic potential (EPSP) that underlies the monosynaptic reflex (MSR) is mediated by glutamate receptors of both α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) subtypes located on the motor neurons. The slow ventral root potential is generated *via* a polysynaptic pathway; a relatively early component is sensitive to NMDA receptor antagonists, and a late component is sensitive to a variety of metabotropic receptor antagonists, including those for neurokinin 1 and 2 receptors and metabotropic glutamate receptor group I antagonists. In addition to the transmitters that mediate these responses, several others modulate them, including the inhibition of ventral root responses by $GABA_A$ and glycine. SVRP = slow ventral root potential.

cords were removed. For extracellular recording, the intact cord from midthoracic through sacral regions was mounted in a superfusion bath and arranged to permit stimulation of a dorsal root and recording from either a ventral root (pEPSP and sVRP) or an adjacent dorsal root (DRP). Stimulus duration was 0.2 ms. Stimulus intensity was adjusted in each experiment to minimize or eliminate the monosynaptic reflex compound action potential from the pEPSP. Intensity was nominally 9 V for the other potentials, which is supramaximal for sVRP and DRP; this intensity is sufficient to recruit small-diameter primary afferent fibers in the dorsal root.[9] Isolated spinal cords were perfused at 4 ml/min with artificial cerebrospinal fluid (ACSF) at 27–28°C equilibrated with 95% O_2–5% CO_2, pH 7.3–7.4. The pre-equilibrated ACSF was delivered from glass syringes mounted on an infusion pump set to a constant rate of approximately 4 ml/min. ACSF was of the following composition: 123 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, 2 mM $CaCl_2$, and 30 mM glucose. Single stimuli were delivered to the dorsal root at a constant frequency of 1/50 s throughout the experiment. Responses were digitized, averaged in groups of five, and stored for later analysis.

For patch clamp studies in spinal cord slices, 350-μm-thick slices were sectioned from the lumbar region on a Vibratome (Technical Products International, St. Louis, MO) and removed to oxygenated ACSF at room temperature for a 1-h recovery period. Individual slices were transferred to a chamber constantly superfused with oxygenated ACSF. All experiments were conducted at room temperature. Cells in the spinal cord slice were visualized on a closed-circuit television monitor using infrared illumination and a 40× water immersion objective. Studies were conducted in the large cell bodies in the ventral horn, most commonly seen in the ventral lateral or ventral medial area. In separate studies, these cells were identified as motor neurons by fluorescent labeling with Evans blue dye injected into the hind limb the day before animals were killed, as previously described.[10] Patch pipettes were pulled on a Flaming-Brown pipette puller (Sutter Instruments, Novato, CA) and filled with a solution of the following composition: 15 mM NaCl, 110 mM K gluconate, 10 mM HEPES, 2 mM $MgCl_2$, 11 mM EGTA, 1 mM $CaCl_2$, 2 mM MgATP, pH adjusted with KOH to 7.3. Pipettes typically had a tip resistance of 3–8 MΩ. Motor neuron responses were evoked by electrical stimulation of the dorsal horn *via* a concentric bipolar platinum electrode with tip diameter of 0.025 mm, using square wave stimuli 0.1 ms in duration, 1–20 V nominal intensity, with a frequency of 0.03–0.1 Hz. Excitatory postsynaptic currents were measured individually or analyzed statistically by averaging groups of 5–10. In separate experiments, responses were evoked by direct pressure application of glutamate from a pipette positioned near the cell body (Picospritzer, General Valve Division of Parker Hannefin Corporation, Fairfield, NJ). Pressure pulse was 9 psi, and the duration of the pulse was 200 ms. Glutamate concentration in the pipette was 5 mM. We found that these parameters consistently gave a reproducible inward current of good amplitude. Glutamate applications at 1-min intervals produced stable responses over the course of each experiment; receptor desensitization was not observed at this rate of application. Holding potential was −60 mV. The membrane potential value was not corrected for the junction potential, which was −13 mV. Experiments were conducted on a single cell in each slice.

Enflurane was prepared as a saturated stock solution and diluted to the desired concentration in ACSF immediately before use. Concentrations were measured by gas chromatography of samples from the perfusion chamber. Concentrations given with reference to MAC are based on adult rat MAC; MAC for volatile anesthetics in 1-week-old rats is approximately 20% higher than in adults, and a similar age-dependent potency difference probably occurs in mice.[11,12]

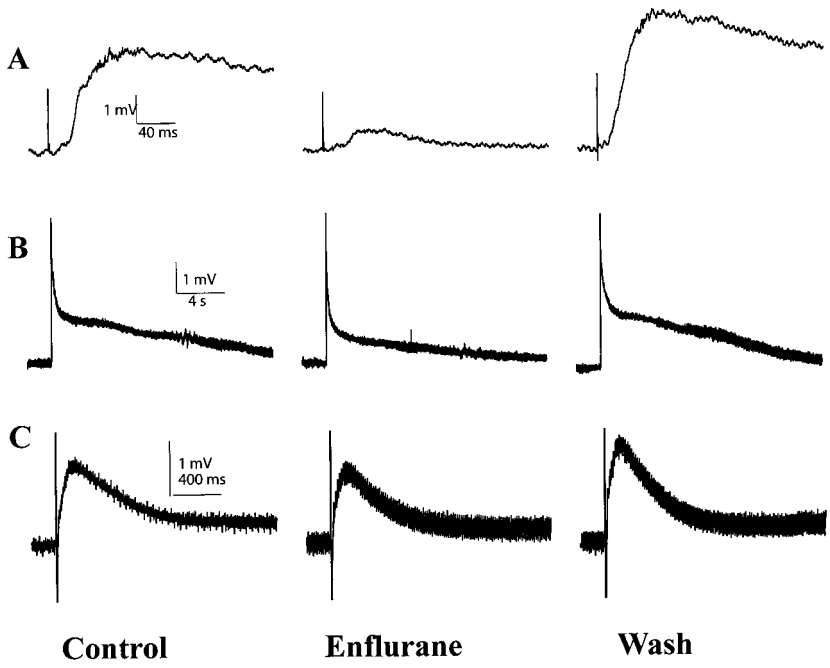

Fig. 2. Enflurane depressed all three evoked responses in −/− cords (shown here) as well as +/+ cords (not shown). (*A*) Population excitatory postsynaptic potential; (*B*) slow ventral root potential; (*C*) dorsal root potential. Records are averages of five responses. Enflurane concentrations were aproximately 0.7 MAC for population excitatory postsynaptic potential and dorsal root potential, 0.18 MAC for slow ventral root potential.

Statistical Analysis

In the whole cord studies, effects of enflurane, bicuculline, and strychnine on ventral root responses were measured as area under the curve at the data point corresponding to 30-min exposure and normalized to control values. pEPSP area encompassed the first 100 ms of the response, and SVRP the first 10 s. Effects on the dorsal root potential were measured as amplitudes of the early transient peak (latency determined in each experiment) and of the late steady state response 1 s after the stimulus. Significance of differences between cords from +/+ and −/− animals were tested by Student *t* test for unpaired data. Enflurane application was made first alone and then repeated in the presence of the antagonist in the same preparation, using the antagonist-modified response as the new control to control for the effect of the antagonist itself. The Student *t* test with paired data was used to analyze these results. In the patch clamp studies, enflurane was applied for 10 min. Enflurane effects were measured by taking the average of responses during the last 5 min after application compared with the average of responses in the 5 min immediately before the start of application. Enflurane effects were expressed as mean ± SEM, and differences among groups were tested with analysis of variance with the Dunnett test for multiple comparisons.

Results

Enflurane Actions

Enflurane exerted identical depressant effects on evoked responses in spinal cords from wild-type mice and mice lacking the β_3 subunit of the $GABA_A$ receptor.

Population-Evoked Responses in Whole Cords. Examples of the actions of enflurane on the pEPSP, sVRP, and DRP in cords from −/− mice are shown in figure 2. Comparisons of enflurane actions on these responses between +/+ and −/− animals are shown graphically in figure 3. Enflurane concentrations for pEPSP and DRP were approximately 0.7 MAC, and for the more sensitive sVRP enflurane concentrations were approximately 0.18 MAC. Enflurane depressant effects on all three responses were similar in extent; there were no significant differences in sensitivity between +/+ and −/− cords in either the ventral root responses (figs. 3A and B) or amplitudes of the early peak and late steady state DRP components (figs. 3C and D; $P > 0.05$).

Experiments on Motor Neurons in Spinal Cord Slices. The population-evoked responses in intact spinal cord reveal all the actions of an anesthetic on each of the pathways that generate them (fig. 1) but do not allow the possibility of discriminating among actions at different points along the pathway, *e.g.*, direct actions on motor neurons *versus* actions mediated by modulatory interneurons. To probe further for enflurane actions on motor neurons themselves that might be altered by the mutation, we conducted patch clamp studies on both synaptically evoked currents and glutamate-evoked currents in visually identified motor neurons during patch clamp conditions.

Synaptically Evoked Currents. Short latency currents evoked in motor neurons by stimulation in the dorsal root entry zone are the single-cell counterpart of the pEPSP in intact cord. Enflurane at approximately 1 MAC depressed synaptically evoked currents in cords from both +/+ and −/− mice. Examples are shown in figures 4A and B. As is the case with the pEPSP in intact

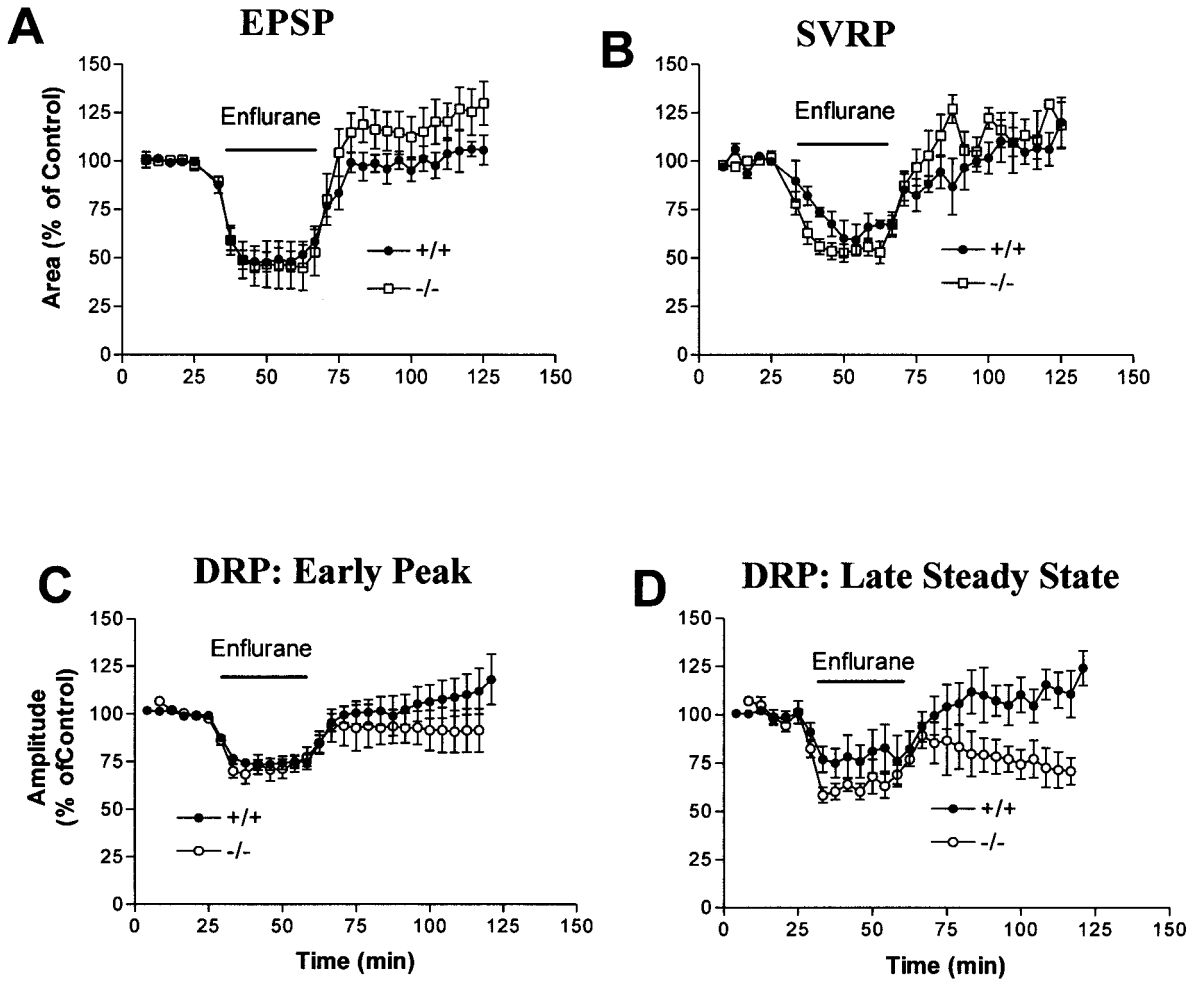

Fig. 3. Enflurane exerted equal depressant actions on population-evoked responses in spinal cords from +/+ and −/− mice. (*A* and *B*) Areas of the excitatory postsynaptic potential (EPSP) and slow ventral root potential (sVRP). (*C* and *D*) Amplitudes of the dorsal root potential (DRP) early peak (point of maximum upward deflection) and late steady state (measured 1 s after the stimulus). Enflurane was applied during the time shown by the bar. Enflurane concentrations were approximately 0.7 MAC for pEPSP, 0.18 MAC for sVRP, and 0.7 MAC for DRP. None of the responses displayed a significant difference in sensitivity to enflurane between wild-type and mutant cords. Data points are means of four to five individual experiments, and error bars are SEM.

cord, there were no differences in enflurane depressant effects on either amplitude or area of synaptically evoked currents between +/+ and −/− cords (figs. 4C–4F).

Currents Evoked by Glutamate Application. Brief pulses of glutamate were applied in the presence of tetrodotoxin (300 nM) to block impulse activity. Enflurane depressed these currents to a similar extent in both +/+ and −/− animals (fig. 5A). Data are shown in table 1. Figure 5 also shows examples of the interaction between $GABA_A$ receptors and enflurane depression (fig. 5B).

Evidence for Reduction of GABA-mediated Receptors in Mice with the Deletion

Similarity of the actions of enflurane on cords from +/+ and −/− mice led us to probe for evidence that GABA receptors were, in fact, fewer in mice with the β_3 subunit deletion. Two strategies were used: direct activation of $GABA_A$ receptors by agonists, and block of $GABA_A$ receptors by antagonists.

GABA Receptors on Motor Neurons. In intact spinal cord, activation of GABA receptors will depress the pEPSP by acting directly on the circuit from primary afferents to motor neurons (fig. 1). The $GABA_A$ agonist muscimol (1 μM) depressed the pEPSP much more in cords from +/+ than −/− mice (fig. 6A). Muscimol depressed the response in +/+ cords to 20.7 ± 3.80% of control *versus* 76.3 ± 3.63% in −/− cords (n = 5, $P < 0.0001$). This result clearly suggests that the expression of $GABA_A$ receptors is markedly reduced in mice with the deletion. A limited series of experiments was conducted in which currents in motor neurons were evoked by direct GABA application. Examples from +/+ and −/− mice are shown in figure 6B. Although there are GABA receptors on motor neurons in −/− mice, the currents evoked appear to be much smaller than in wild

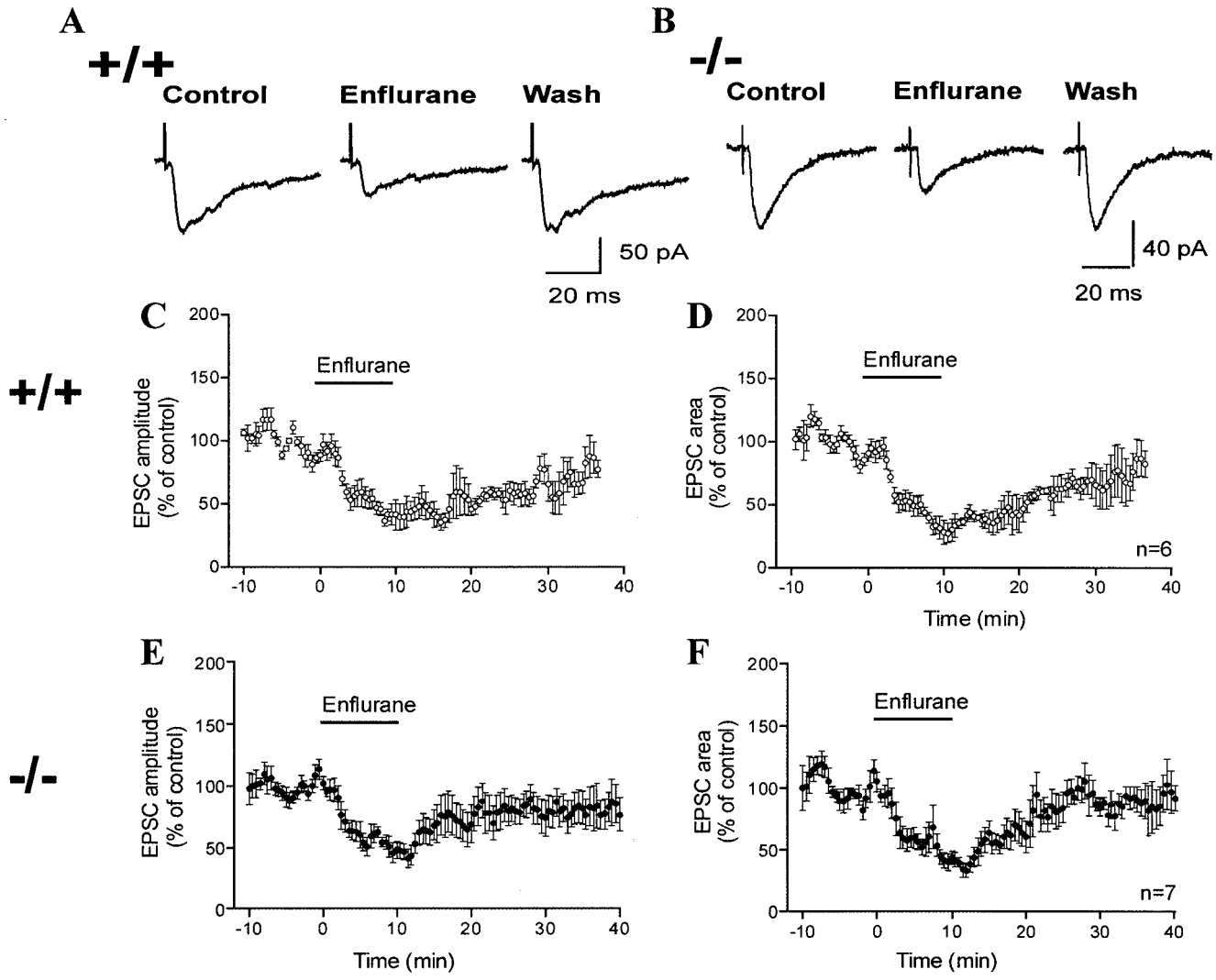

Fig. 4. Enflurane (1 MAC, 0.6 mM) exerted similar depressant effects on synaptically evoked currents in individual motor neurons in spinal cords from wild-type (+/+) and β_3 null (−/−) mice. (*A* and *B*) Examples of enflurane actions on currents in +/+ and −/− mice, respectively; stimuli to the dorsal root entry zone are indicated by the stimulus artifact preceding the response. Graphs show time course of enflurane actions on amplitude and area of synaptically evoked currents from +/+ (*C* and *D*) and −/− mice (*E* and *F*). There were no differences in either variable between the two groups. Holding potentials were −60 mV. Data points are means of six to seven cells, each in a different slice; error bars are SEM.

types. Because current amplitudes vary from neuron to neuron, no quantitative conclusions can be drawn from these experiments.

Tonic GABA-mediated Actions on Population Evoked Potentials. Examples of bicuculline actions on VRPs are shown in figures 7A and B and on the DRPs in figure 7C. The results are shown quantitatively in graphs for the VRPs in figures 8A and B and for two components of the DRP in figure 8C and D.

Ventral Root Potentials. In intact rodent spinal cord, $GABA_A$ receptor–mediated inhibitory tone is substantial; treatment with the $GABA_A$ receptor antagonists bicuculline (figs. 7A and B) or picrotoxin (data not shown) elevates VRPs and, at high concentrations, spontaneous waves of activity appear. Bicuculline (1 μM) increased the area of the pEPSP and sVRP in spinal cords from both null mutants (−/−) and wild-type (+/+) animals ($P < 0.01$; figs. 8A and B). There were no differences between −/− and +/+ in extent of bicuculline elevation of EPSP area ($P > 0.05$; fig. 8A); bicuculline increased sVRP area significantly ($P < 0.05$) more in −/− than in +/+ cords (fig. 8B). Inspection of the records suggests that this apparent difference is caused by greater distortion of the baseline by the sporadic unsynchronized activity induced by bicuculline in wild-type animals. A typical example of the actions of bicuculline in this respect is shown in figure 7B.

Dorsal Root Potential. The DRP is mediated, in part, by $GABA_A$ receptors on the primary afferent nerve terminals. The $GABA_A$ receptor antagonist bicuculline (10 μM) depressed the amplitude of the early

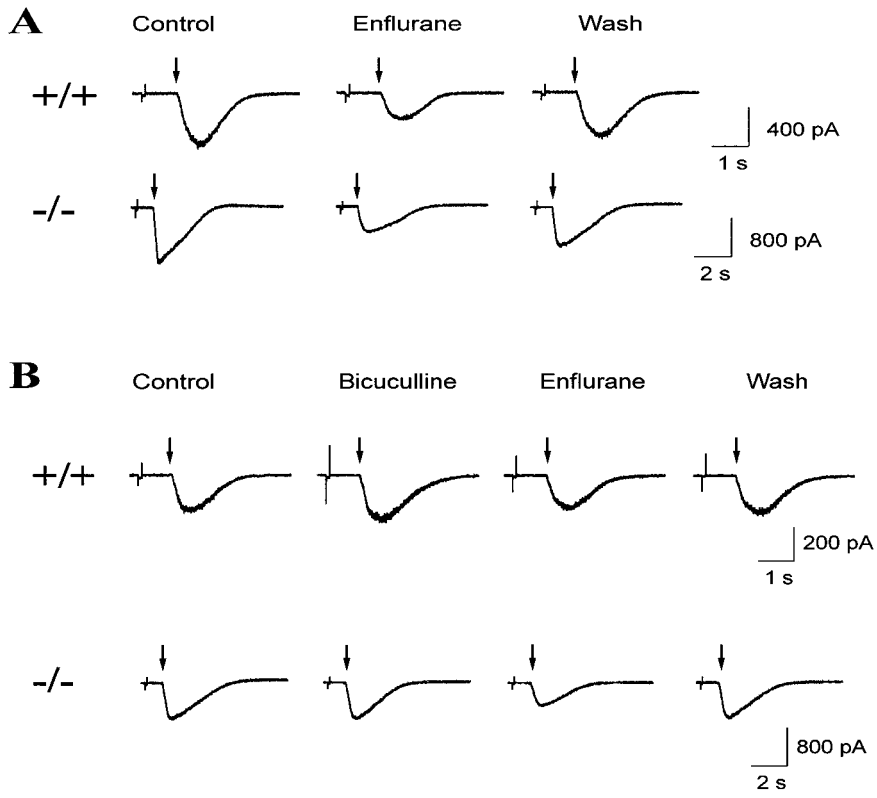

Fig. 5. (*A*) Currents evoked by glutamate (arrows) in the presence of tetrodotoxin (300 nM) to block presynaptic impulse activity in +/+ and −/− mice. (*B*) Experiments were conducted to test the role of $GABA_A$ receptors in enflurane effects on glutamate-evoked currents by comparing the effects of enflurane alone and in the presence of the $GABA_A$ receptor antagonist bicuculline (20 μM). Bicuculline significantly attenuated the effects of enflurane on the amplitudes of glutamate-evoked currents in wild-type cells but not in cells from mutant animals (table 1). Enflurane concentrations are approximately 1 MAC (0.6 mM). Holding potentials were −60 mV. Rectangular deflections at the start of each trace are hyperpolarizing voltage steps to monitor membrane resistance.

peak of the DRP in both −/− and +/+ cords (fig. 7C). Visually, the effects of bicuculline on the DRP resembled those of the mutation (fig. 7C), decreasing the amplitude of the fast-rising early component and leaving a relatively slow-rising late component insensitive to bicuculline. Bicuculline depressed the early peak of the DRP to a significantly ($P < 0.05$) greater extent in cords from +/+ than −/− mice (fig. 8C). Bicuculline elevated the amplitude of the late steady state component of this response to a similar extent ($P > 0.05$) in cords from +/+ and −/− animals (fig. 8D).

Role of $GABA_A$ Receptors in the Actions of Enflurane

We tested the part played by $GABA_A$ receptors in the depressant actions of enflurane by examining the extent to which bicuculline, by blocking these receptors, could attenuate the actions of enflurane.

Table 1. Actions of Enflurane Alone and with Bicuculline on Glutamate-evoked Currents in Motor Neurons

	Enflurane	Bicuculline	Enflurane + Bicuculline
+/+	67.04 ± 3.97 (9)	110.9 ± 11.61 (6)	81.44 ± 2.82 (5)*
−/−	64.33 ± 4.08 (7)	94.58 ± 3.67 (5)	71.08 ± 2.73 (5)†

During control conditions, there were no significant differences in enflurane sensitivity between +/+ and −/− mice. Bicuculline did not significantly increase current amplitudes. Bicuculline significantly attenuated the depressant actions of enflurane in motor neurons from +/+ but not −/− mice. When $GABA_A$ receptors were blocked with bicuculline, currents in motor neurons from −/− mice were more sensitive to enflurane than those from +/+ mice. Current amplitudes as percentage of control; mean ± SEM (N).

* Significantly different from enflurane alone ($P < 0.05$). † Significantly different from wild type ($P < 0.05$).

Experiments in Intact Cords. In intact cords, a protocol was designed to expose each spinal cord to enflurane alone and to enflurane in the presence of bicuculline, with controls for the effect of bicuculline alone (fig. 9). After an initial 30-min control period, preparations were exposed to a sequence of enflurane (30 min), wash (1 h), bicuculline (30 min), enflurane plus bicuculline (30 min), and wash with bicuculline (1 h). Controls for enflurane effects alone were the initial controls, and for enflurane with bicuculline were new controls at the end of bicuculline treatment. Bicuculline significantly ($P < 0.05$) attenuated the depressant effects of enflurane on both pEPSP and sVRP in cords from +/+ mice but not in cords from −/− mice (fig. 9).

Patch Clamp Studies in Motor Neurons. After the control period, bicuculline (20 μM) was applied, followed by enflurane. Examples for +/+ and −/− cells are shown in figure 5B. Because of the limited time over which cells could be held for each experiment, it was not possible to compare the actions of enflurane alone and in the presence of bicuculline in the same cell, as was performed in intact cords. Comparisons among the groups of cells exposed separately to enflurane or to enflurane plus bicuculline are shown in table 1. Bicuculline significantly ($P < 0.05$) attenuated the depressant effects of enflurane on glutamate-evoked currents in motor neurons from +/+ but not −/− cells (table 1).

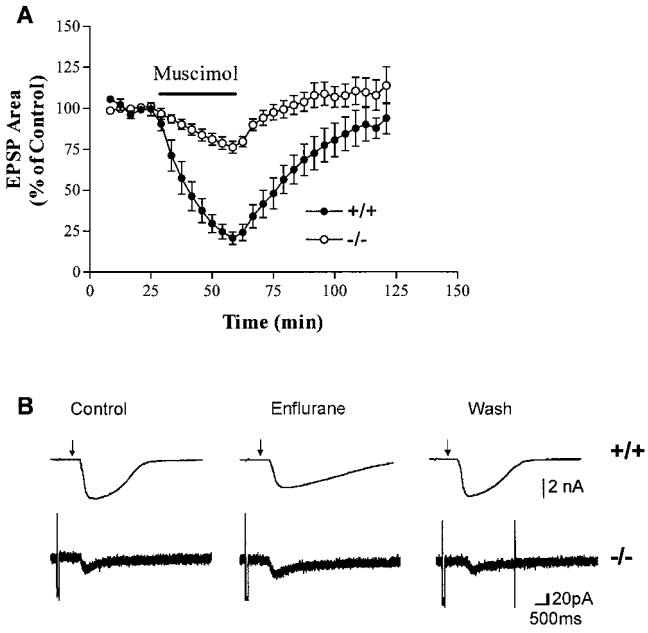

Fig. 6. Evidence that there are fewer $GABA_A$ receptors on motor neurons from −/− compared with +/+ mice. (*A*) A $GABA_A$ agonist, muscimol, exerts a far smaller depressant effect on the population excitatory postsynaptic potential (EPSP) evoked from cords of −/− than +/+ mice. Muscimol concentration, 1 μM; $P < 0.0001$; n = 5 of each type. (*B*) Relative amplitudes of currents evoked by GABA in a neuron from a +/+ animal compared with one from a −/− animal. Note the difference in calibration mark. Holding potential, −60 mV.

Developmental Compensation for the Missing Subunit in Mutant Animals

Because enflurane MAC is elevated in the mutants, we had predicted that enflurane would appear less potent in depressing motor neuron responses in spinal cords from −/− than +/+ mice. This was not the case, there being no difference between the groups. Experiments were conducted to test the hypothesis that the equal enflurane sensitivity of normal and mutant cords was caused by changes in other receptors that would compensate for the loss of $GABA_A$ receptors containing the β_3 subunit, both to restore excitability toward more normal levels and to serve as substitute targets for enflurane actions.

Glycine Receptors. A logical substitute for missing $GABA_A$ receptors is the related chloride channel, the glycine receptor, which plays an important inhibitory role in spinal cord. To test for developmental upregulation of glycine receptors, we examined the actions of the glycine antagonist strychnine on the pEPSP. Strychnine (400 nM) increased the area of the pEPSP significantly more ($P < 0.05$) in −/− than in +/+ cords, suggesting that glycinergic inhibition is, in fact, increased in −/− cords (fig. 10A). To test whether the depressant effects of enflurane were equalized by the increase in glycine receptors, we probed the interaction between glycine receptors and enflurane potency in a protocol similar to that used for $GABA_A$ receptor–enflurane interactions. If compensatory overexpression of the glycine receptor is responsible for equalizing the actions of enflurane, then strychnine is predicted to attenuate enflurane-depressant effects more in −/− than in +/+ cords. However, although there was a tendency for strychnine to reduce the apparent potency of enflurane in depressing the pEPSP, this was not significant ($P > 0.05$) in either +/+ or −/− cords, and there were no differences between the two populations in this respect (figs. 10B and C).

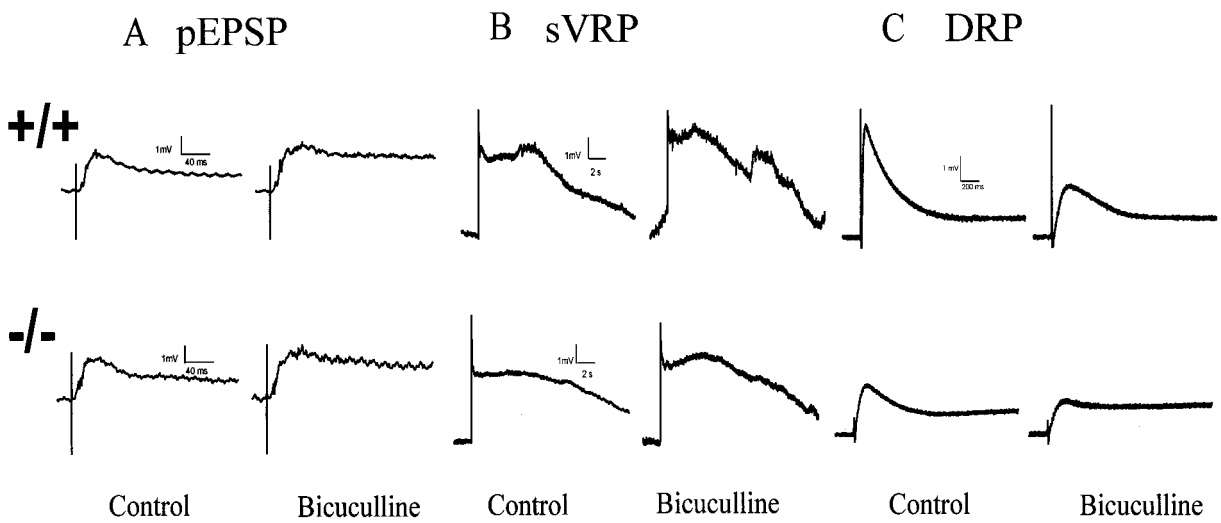

Fig. 7. Bicuculline actions on evoked responses in intact wild-type and mutant spinal cords. (*Top row*) Wild type (+/+); (*bottom row*) cords from animals lacking the β_3 subunit of the $GABA_A$ receptor (−/−). (*A*) Bicuculline increases the population excitatory postsynaptic potential (pEPSP) in both normal (+/+) and β_3 subunit–deficient (−/−) cords. (*B*) Bicuculline also increases the slow ventral root potential (sVRP) in −/− cords; in the wild-type animals, any such action on the stimulus-linked response is obscured by distortions in the baseline caused by unsynchronized wavelike activity induced by the convulsant as illustrated in this example. (*C*) The effects of bicuculline on the dorsal root potential (DRP) in wild-type animals resemble those of the mutation; an early fast-rising component is selectively reduced, leaving a slower bicuculline-insensitive component. Responses are averages of five records. Bicuculline concentrations were 1 μM for the pEPSP and the sVRP, 10 μM for the DRP.

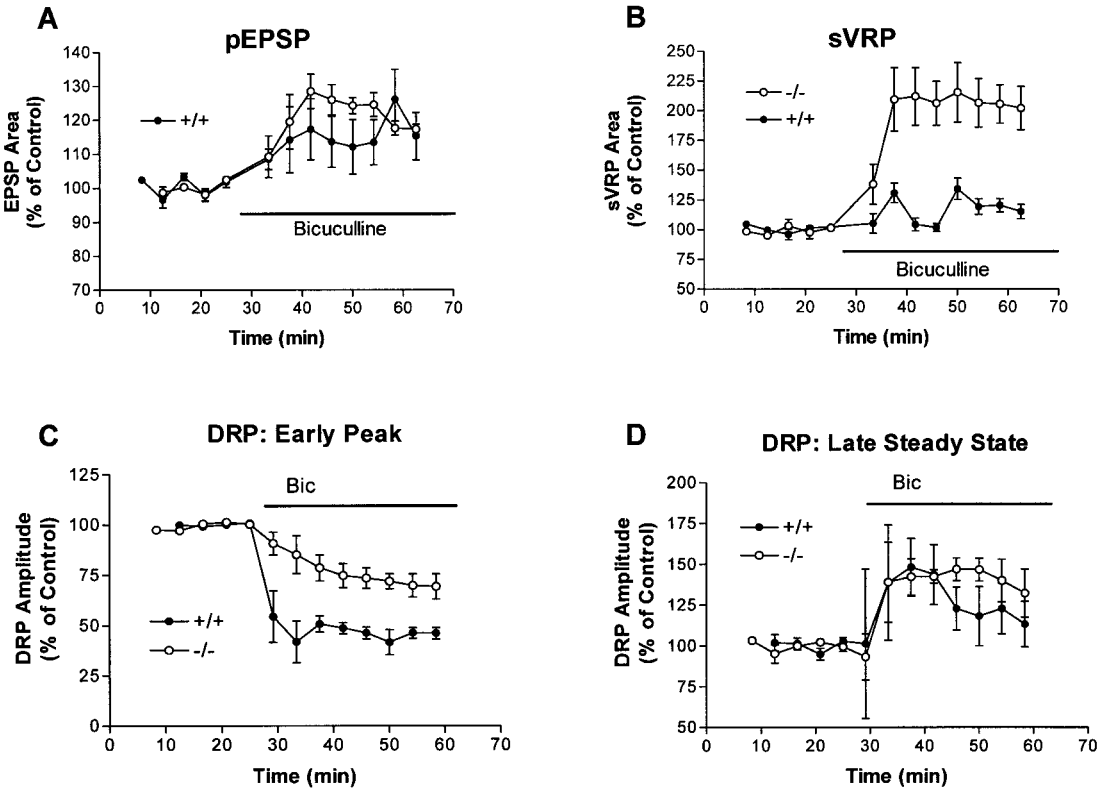

Fig. 8. Actions of bicuculline on the responses of intact spinal cord. (*A*) Bicuculline (1 μM) increased the area of the population excitatory postsynaptic potential (pEPSP) to a similar extent in both wild type and −/− cords. (*B*) Bicuculline appeared to increase slow ventral root potential (sVRP) area more ($P < 0.05$) in −/− cords than in +/+ cords. However, this appearance may be the result of greater unsynchronized wavelike activity induced by the convulsant $GABA_A$ antagonist in wild-type cords (fig. 7B), elevating the baseline and making analysis difficult. (*C*) Bicuculline (10 μM) depressed the amplitude of the early dorsal root potential (DRP) peak to a significantly greater extent in cords from +/+ mice than their −/− counterparts ($P < 0.05$). Early peak amplitude was taken as the point of maximum upward deflection from baseline in each experiment. (*D*) There are no significant differences between the two groups in bicuculline elevation of the late steady state response measured 1 s after the stimulus. Data points are means of four to five experiments; error bars are SEM.

Other Receptors. In the presence of bicuculline to block $GABA_A$ receptors, glutamate-evoked currents in mutant mouse spinal motor neurons are significantly ($P < 0.05$) more sensitive to enflurane than those of wild-type animals (table 1). This result suggests some change in glutamate receptors, perhaps a reduction in number, or in some cellular component that acts on glutamate receptors and may indirectly mediate an anesthetic effect.

Discussion

Enflurane Actions

Behavioral studies, in which enflurane MAC requirement was increased in mice lacking the β_3 subunit of the $GABA_A$ receptor,[4] led to the prediction that spinal cord sensitivity to enflurane would be decreased in β_3 −/− mice. Contrary to the prediction, enflurane exerted similar depressant effects in cords from −/− and +/+ mice. Behaviorally, the increased MAC in −/− mice is not dramatic, approximately 26% for enflurane and less for halothane.[4] Given the steepness of the dose–response curve for MAC, this is not a large difference and certainly does not support a dominant role for $GABA_A$ receptors containing the β_3 subunit in determining MAC. It is possible that the source of the increased enflurane requirement in −/− mice is supraspinal rather than at the spinal level. MAC is determined predominantly in spinal cord but with supraspinal influences.[6] In isolated spinal cord and spinal cord slices, supraspinal modulation is removed. The results of the current study thus suggest that the increase in enflurane requirement observed in intact −/− animals may be the result of a difference in supraspinal enflurane actions. Deficits in GABA-mediated inhibition have been observed in some supraspinal nuclei in −/− mice.[13]

Evidence for a Reduction in $GABA_A$ Receptors in Spinal Cords of Mice Lacking the β_3 Subunit

The $GABA_A$ agonist muscimol exerted much greater depressant effects on the pEPSP in wild-type mice than in mutants, a strong indication that there are fewer $GABA_A$ receptors in the circuit that mediates this response in −/− mice. The comparatively small currents

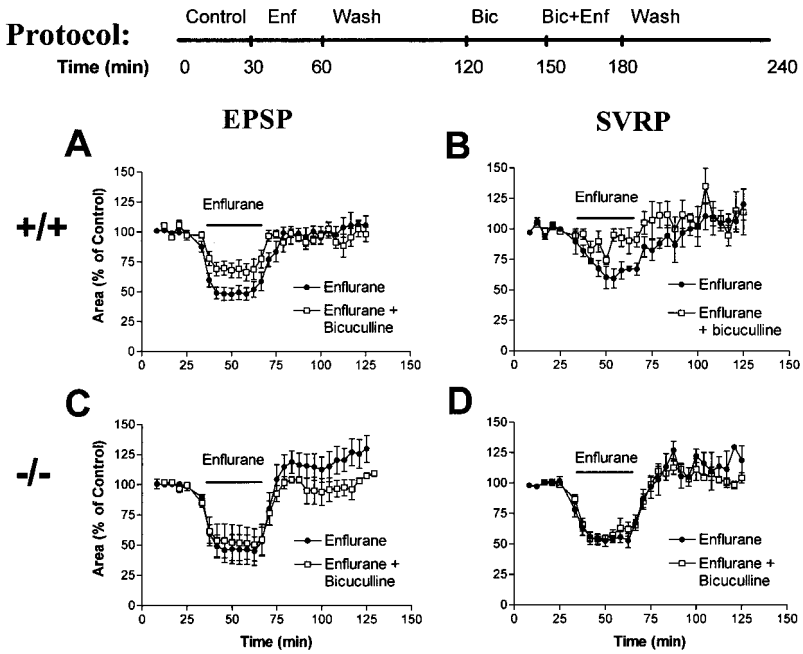

Fig. 9. $GABA_A$ receptors play a smaller role in enflurane actions in cords from −/− than +/+ mice. (*Top*) Protocol for assessing the interaction between enflurane and $GABA_A$ receptors. Data for enflurane alone are the same as those shown in figure 3. The excitatory effects of bicuculline were controlled for by making the responses in the presence of the antagonist the baseline against which enflurane effects were measured when bicuculline was present. The $GABA_A$ receptor antagonist bicuculline (1 μM) attenuated the actions of enflurane on the population excitatory postsynaptic potential (pEPSP; *A*) and the slow ventral root potential (sVRP; *B*) significantly ($P < 0.05$) in cords from +/+ animals but not from −/− mice (*C* and *D*). Enflurane concentrations were approximately 0.7 MAC for pEPSP, 0.18 MAC for sVRP. Data points are means of four to five experiments; error bars are SEM.

evoked by GABA in motor neurons of −/− mice are also suggestive of fewer receptors, although quantitative conclusions cannot be drawn from the limited number of experiments. However, the results with the antagonist bicuculline on ventral root potentials suggested equal tonic GABA-mediated inhibition in both populations. There are several possible explanations for the discrepancy. First, $GABA_A$ receptors of different subunit composition may be expressed in mutant animals. However, it is not likely that differences in bicuculline sensitivity among $GABA_A$ receptors of different subunit composition account for equal bicuculline sensitivity. $GABA_A$ receptors are, by definition, sensitive to bicuculline. The type of β subunit present in recombinant receptors expressed in nonneural cells does not greatly influence receptor pharmacology.[14–16] Second, and somewhat more likely, at concentrations greater than those used in the current study, bicuculline also blocks glycine receptors in rat motor neurons.[17] Because glycine receptors appear to be present in greater numbers in −/− mice, it is possible that the increased population of glycine receptors might be more sensitive to bicuculline than in normal animals. Finally, there is a difference in source of the agonist and location of the receptors between the muscimol and bicuculline experiments. Muscimol directly acts on the monosynaptic reflex circuit to depress the evoked response, whereas tonic GABA inhibition is caused by GABA release from interneurons. Muscimol will activate all accessible receptors, whereas the effects of bicuculline will be predominantly on receptors located at synapses. With respect to the DRP, the results with bicuculline clearly show a decrease in the early bicuculline-sensitive component of the response in −/− animals. This is in agreement with the finding that there

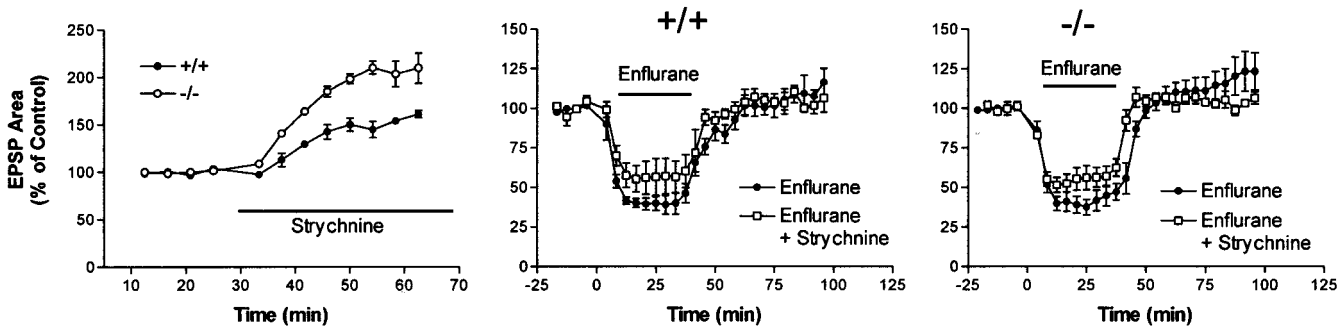

Fig. 10. Strychnine sensitivity is increased in cords from −/− mice. (*A*) Glycine inhibition is more pronounced in cords from −/− mice than +/+, as evidenced by a significantly ($P < 0.05$) greater increase in population excitatory postsynaptic potential (pEPSP) in the presence of strychnine (400 nM) in the former. (*B* and *C*) The interaction between glycine receptors and enflurane effects was probed by a protocol similar to that shown for $GABA_A$ receptors in figure 9, with strychnine (400 nM) in the place of bicuculline. However, glycine receptor block did not significantly attenuate the depressant effects of enflurane on this response in either population, although there was a tendency in that direction, and there were no differences between +/+ and −/− cords in this respect. Data points are means of four experiments; error bars are SEM.

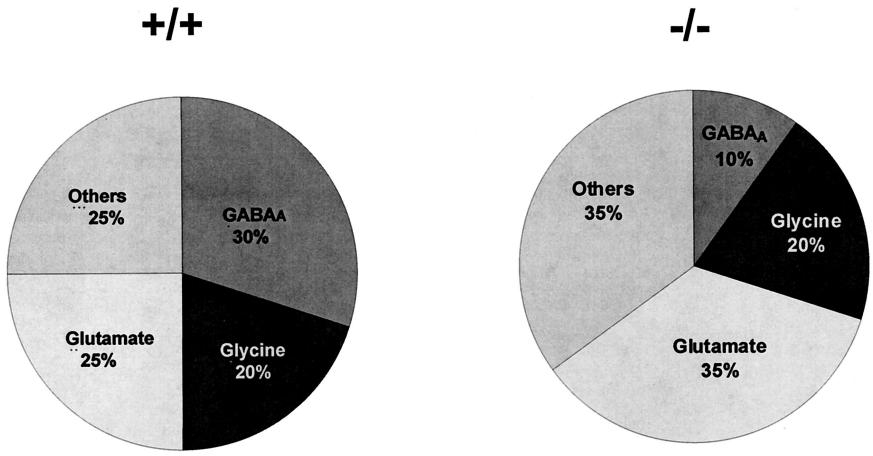

Fig. 11. Hypothetical actions of this mutation on the molecular basis for enflurane depression of spinal neurotransmission. It is assumed that multiple actions of enflurane on different targets contribute to its overall depressant effect. The actual proportions are not known, and the numbers are arbitrary. The data from the current study show that bicuculline significantly attenuates the actions of enflurane on ventral root responses in +/+ but not −/− cords, suggesting that the contribution of $GABA_A$ receptors is reduced by absence of the β_3 subunit. However, because the overall magnitude of depression remains the same, other targets must substitute for the role normally played by $GABA_A$ receptors. Glycine receptors do not appear to be the substitute; increased actions on glutamate receptors and other targets are hypothesized.

are fewer $GABA_A$ receptors on dorsal root ganglion cells in −/− mice than in wild-type mice.[18] Dorsal root ganglion cells are the cell bodies of the primary afferent sensory neurons whose terminals generate the DRP.

The role of $GABA_A$ receptors in Minimum Alveolar Concentration

***In Vivo* Studies.** Although the correlation between anesthetic potency and enhancement of $GABA_A$ receptor function is excellent,[19] it is not uniform. Some fluorinated alkanols abolish movement in response to a noxious stimulus[20] but do not act to increase current through $GABA_A$ receptors.[21] The inert gas xenon is a general anesthetic agent but has little effect on $GABA_A$ receptors.[22] Thus, although actions on $GABA_A$ receptors may be important for many inhalation agents, they are not essential for some, and for others, actions on other receptors may also contribute to MAC. In a recent study, intrathecal application of picrotoxin increased isoflurane MAC by a maximum of 43%.[23]

In Spinal Cord. We have previously shown in wild-type mice that enflurane depresses glutamate-evoked currents by direct actions, independent of actions on either $GABA_A$ or glycine receptors.[8] In the present studies, blockade of the $GABA_A$ receptor by bicuculline attenuated but did not block the depressant actions of enflurane on VRPs and on glutamate-evoked motor neuron currents in spinal cords from +/+ mice. This result suggests that $GABA_A$ receptors contribute to anesthetic actions in these mice but do not account for all of them. In −/− mice, there was no attenuation of the depressant actions of enflurane by bicuculline, suggesting little or no contribution of $GABA_A$ receptors to anesthetic actions in these animals.

The results with muscimol provide further evidence that $GABA_A$ receptors are not of dominant importance in determining spinal actions of anesthetics. Muscimol, which activates $GABA_A$ receptors, was clearly less effective in depressing a ventral root response in −/− animals. Muscimol is not an ideal control for a putative anesthetic action, since volatile anesthetic agents are thought to act by enhancing the effectiveness of GABA rather than by directly gating the receptor. However, it is probable that an anesthetic that exerted its effects predominantly by enhancing $GABA_A$ receptor activity should have also been less effective in these animals. Because the effects of enflurane alone were similar in the two populations, this result suggests that $GABA_A$ receptors are not dominant contributors to the effects of enflurane. Furthermore, loss of bicuculline attenuation coupled with identical enflurane actions suggest the molecular basis of spinal anesthetic sensitivity is shifted to another target than $GABA_A$ receptors in mice that lack β_3 subunits.

Strychnine Block of Glycine Receptors. The exaggerated effect of strychnine in cords from −/− mice suggests that glycinergic inhibition is more prominent in these animals than in wild-type controls, as a result of developmental compensation leading to overexpression of this other inhibitory chloride channel. It is plausible to suggest that glycine receptors could, in part, substitute for $GABA_A$ receptors. Glycine and GABA play similar inhibitory roles in spinal cord and are coreleased from the same interneuronal terminals onto motor neurons.[17] Tonic inhibition to rat spinal cord motor neurons is predominantly mediated by glycine rather than by $GABA_A$ receptors, although the latter also play a part.[24] Glycine receptors as well as $GABA_A$ receptors are subject to anesthetic enhancement.[2] However, block of the glycine receptor with strychnine did not significantly attenuate enflurane-depressant effects on the pEPSP in either +/+ or −/− cords, although there was a tendency in that direction. At least for this response, glycine receptors appear to play a relatively small role in anesthetic depressant actions, and there is no evidence that they become the substitute target for enflurane in −/− cords. Studies in rats using intrathecal application of strychnine suggest that, as is the case for a GABA antagonist, MAC cannot be increased by more than approximately 40%; furthermore, there was a plateau effect for both picrotoxin and strychnine such that intrathecal administration

of maximally effective amounts of both drugs together increased MAC by a maximum of 47%.[23]

Implications for theories of anesthesia.

The depressant actions of enflurane were identical in +/+ and −/− mice, yet the role of the $GABA_A$ receptor in enflurane actions is less in the −/− mice. A decrease in the role of the $GABA_A$ receptor in enflurane actions, combined with identical enflurane effects, forces the conclusion that the molecular basis for anesthetic depression has shifted as a result of the mutation. Some other receptor(s) or ion channels play a greater role in anesthetic depressant effects in −/− than in wild-type mice. The proportions of molecular substrates in the mix responsible for anesthetic actions have changed. We have shown that the substitute target is not the glycine receptor, although glycine inhibition is increased. Other possibilities include compensatory downregulation of excitatory glutamate receptors to maintain a balance between excitation and inhibition. In addition to changes in both excitatory and inhibitory amino acid–mediated neurotransmission in the mutant mice, there also may be changes in other less well-studied potential anesthetic targets, including ion channels modulated by second messenger-linked receptors or the poorly understood class of voltage-independent baseline potassium channels. Figure 11 illustrates this hypothetical shift in the basis for enflurane depression of spinal neurotransmission.

Genomic Strategies in Testing Hypotheses of Anesthetic Action

This study represents an early stage in the developing strategy of using genetically engineered animals to probe for target sites of anesthetic action. The results show that a volatile anesthetic agent may have a number of different target sites. Compensatory changes in expression of other receptors certainly occurred during development of this global knockout, and a shift in the contribution of these other receptors to the sum of anesthetic effects has also occurred. These changes were not a predictable result of the mutation. These studies illustrate some of the problems in interpreting results from such animals, a situation that will certainly improve as conditional knockouts and other types of mutations become available. They also illustrate the importance of testing hypotheses of anesthetic mechanisms in intact circuitry where all potential molecular targets are present, in addition to conducting detailed molecular studies on isolated receptors.

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References

1. Franks NP, Lieb WR: Molecular and cellular mechanisms of general anaesthesia. Nature 1994; 367:607–14
2. Mihic SJ, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, Mascia MP, Valenzuela CF, Hanson KK, Greenblatt EP, Harris RA, Harrison NL: Sites of alcohol and volatile anaesthetic action on GABA(A) and glycine receptors. Nature 1997; 389:385–9
3. Homanics GE, DeLorey TM, Firestone LL, Quinlan JJ, Handforth A, Harrison NL, Krasowski MD, Rick CE, Korpi ER, Makela R, Brilliant MH, Hagiwara N, Ferguson C, Snyder K, Olsen RW: Mice devoid of gamma-aminobutyrate type A receptor beta3 subunit have epilepsy, cleft palate, and hypersensitive behavior. Proc Natl Acad Sci U S A 1997; 94:4143–8
4. Quinlan JJ, Homanics GE, Firestone LL: Anesthesia sensitivity in mice that lack the beta3 subunit of the gamma- aminobutyric acid type A receptor. ANESTHESIOLOGY 1998; 88:775–80
5. King BS, Rampil IJ: Anesthetic depression of spinal motor neurons may contribute to lack of movement in response to noxious stimuli. ANESTHESIOLOGY 1994; 81:1484–92
6. Antognini JF: The relationship among brain, spinal cord and anesthetic requirements. Med Hypotheses 1997; 48:83–7
7. Savola MK, Woodley SJ, Maze M, Kendig JJ: Isoflurane and an alpha 2-adrenoceptor agonist suppress nociceptive neurotransmission in neonatal rat spinal cord. ANESTHESIOLOGY 1991; 75:489–98
8. Cheng G, Kendig JJ: Enflurane directly depresses glutamate AMPA and NMDA currents in mouse spinal cord motor neurons independent of actions on $GABA_A$ or glycine receptors. ANESTHESIOLOGY 2000; 93:1075–84
9. Lozier AP, Kendig JJ: Long-term potentiation in an isolated peripheral nerve-spinal cord preparation. J Neurophysiol 1995; 74:1001–9
10. Wang MY, Rampil IJ, Kendig JJ: Ethanol directly depresses AMPA and NMDA glutamate currents in spinal cord motor neurons independent of actions on GABAA or glycine receptors. J Pharmacol Exp Ther 1999; 290:362–7
11. Fang Z, Ionescu P, Gong D, Kendig J, Harris A, Eger EI 2nd: Maturation decreases ethanol minimum alveolar anesthetic concentration in mice as previously demonstrated in rats: There is no species difference. Anesth Analg 1997; 85:160–3
12. Fang Z, Gong D, Ionescu P, Laster MJ, Eger EI 2nd, Kendig J: Maturation decreases ethanol minimum alveolar anesthetic concentration (MAC) more than desflurane MAC in rats. Anesth Analg 1997; 84:852–8
13. Huntsman MM, Porcello DM, Homanics GE, DeLorey TM, Huguenard JR: Reciprocal inhibitory connections and network synchrony in the mammalian thalamus. Science 1999; 283:541–3
14. Krishek BJ, Moss SJ, Smart TG: A functional comparison of the antagonists bicuculline and picrotoxin at recombinant GABAA receptors. Neuropharmacology 1996; 35:1289–98
15. Luddens H, Korpi ER: GABA antagonists differentiate between recombinant GABAA/benzodiazepine receptor subtypes. J Neurosci 1995; 15:6957–62
16. Hadingham KL, Wingrove PB, Wafford KA, Bain C, Kemp JA, Palmer KJ, Wilson AW, Wilcox AS, Sikela JM, Ragan CI, Whiting, PJ: Role of the beta subunit in determining the pharmacology of human gamma-aminobutyric acid type A receptors [published erratum appears in Mol Pharmacol 1994 Jul;46:211]. Mol Pharmacol 1993; 44:1211–8
17. Jonas P, Bischofberger J, Sandkuhler J: Corelease of two fast neurotransmitters at a central synapse. Science 1998; 281:419–24
18. Krasowski MD, Rick CE, Harrison NL, Firestone LL, Homanics GE: A deficit of functional GABA(A) receptors in neurons of beta 3 subunit knockout mice. Neurosci Lett 1998; 240:81–4
19. Dildy-Mayfield JE, Mihic SJ, Liu Y, Deitrich RA, Harris RA: Actions of long chain alcohols on GABAA and glutamate receptors: Relation to in vivo effects. Br J Pharmacol 1996; 118:378–84
20. Eger EI 2nd, Ionescu P, Laster MJ, Gong D, Hudlicky T, Kendig JJ, Harris RA, Trudell JR, Pohorille A: Minimum alveolar anesthetic concentration of fluorinated alkanols in rats: Relevance to theories of narcosis. Anesth Analg 1999; 88:867–76
21. Ueno S, Trudell JR, Eger EI 2nd, Harris RA: Actions of fluorinated alkanols on GABA(A) receptors: Relevance to theories of narcosis. Anesth Analg 1999; 88:877–83
22. Franks NP, Dickinson R, de Sousa SL, Hall AC, Lieb WR: How does xenon produce anaesthesia? (letter). Nature 1998; 396:324
23. Zhang Y, Wu S, Eger EI, Sonner J: Neither GABAA nor strychnine-sensitive glycine receptors are the sole mediators of MAC for isoflurane. Anesth Analg 2001; 92:123–7
24. Cheng G, Kendig JJ: Enflurane enhances glycinergic synaptic transmission by both presynaptic and postsynaptic mechanisms in rat spinal cord (abstract). ANESTHESIOLOGY 2000; 93:A807