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Modulation of Presynaptic β3-Containing $GABA_A$ Receptors Limits the Immobilizing Actions of GABAergic Anesthetics

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ABSTRACT

Intravenous GABAergic anesthetics are potent hypnotics but are rather ineffective in depressing movements. Immobility is mediated, in part, by the ventral horn of the spinal cord. We hypothesized that the efficacy of these anesthetics in producing immobility is compromised by the activation of $GABA_A$ receptors located presynaptically, which modulate GABA release onto neurons in the ventral horn. Because anesthetics acting by modulation of $GABA_A$ receptor function require GABA to be present at its binding site, a decrease in GABA release would abate their efficacy in reducing neuronal excitability. Here we report that in organotypic spinal cord slices, the efficacy of the intravenous anesthetic etomidate to depress network activity of ventral horn neurons is limited to approximately 60% at concentrations greater than 1 μM that produce immobility. Depression of spinal network activity was almost abolished in spinal slices from β3(N265M) knock-in mice. In the wild type, etomidate prolonged decay times of $GABA_A$ receptor-mediated inhibitory postsynaptic currents (IPSCs) and concomitantly reduced the frequency of action potential-dependent IPSCs. Etomidate prolonged the decay time of $GABA_A$ receptors at all tested concentrations. At concentrations greater than 1.0 μM, anesthetic-induced decrease of GABA release via modulation of presynaptic $GABA_A$ receptors and enhancement of postsynaptic $GABA_A$ receptor-function compensated for each other. The results suggest that the limited immobilizing efficacy of these agents is probably due to a presynaptic mechanism and that GABAergic agents with a specificity for post- versus presynaptic receptors would probably have much stronger immobilizing actions, pointing out novel avenues for drug development.

General anesthetics like etomidate and propofol, which act almost exclusively via $GABA_A$ receptors, are potent hypnotics, but their efficacy in depressing spontaneous and evoked movements is clearly limited (Ashworth and Smith, 1998; Smith and Thwaites, 1999; Watson and Shah, 2000; Rudolph and Antkowiak, 2004; Grasshoff et al., 2006a). In clinical practice, these agents are routinely used for providing hypnosis and amnesia but rarely are administered to achieve immobility. This difference in efficacy reported in vivo was also observed in neuronal networks from spinal ventral horn interneurons in vitro (Grasshoff and Antkowiak, 2004). Ventral horn interneurons control the timing and excitability of motoneurons, the output structure of the spinal cord (Kiehn, 2006). Immobility, defined as the anesthetic-induced ablation of spontaneous and evoked movements in response to a noxious stimulus, is primarily mediated by molecular targets in the spinal cord (Collins et al., 1995; Campagna et al., 2003). However, the reasons for the low effectiveness of GABAergic anesthetics in producing immobility are not known.

To explain the low immobilizing capacity of intravenous GABAergic anesthetics, we hypothesized that besides depressing neuronal excitability in the ventral horn of the spinal cord, these agents concomitantly reduce GABA release. Because anesthetics acting predominantly by modulation of $GABA_A$ receptor function require GABA to be present at its binding site (Orser et al., 1994; Banks and Pearce, 1999), a decrease in GABA release can be expected to abate the efficacy of such therapeutics in reducing neuronal excitability. To answer the question of whether this mechanism accounts for the limited immobilizing properties of GABAergic anesthetics, the effects of etomidate on neuronal network activity were investigated in organotypic spinal slices. We

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ABBREVIATIONS: ACSF, artificial cerebrospinal fluid, APV, DL-2-amino-5-phosphonopentanoic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; IPSC, inhibitory postsynaptic current; mIPSC, miniature inhibitory postsynaptic current; sIPSC, spontaneous inhibitory postsynaptic current.

quantified the impact of the anesthetic on the time course of $GABA_A$ receptor-mediated synaptic events, on GABA release onto neurons in the ventral horn, and on the activity of the intact spinal network. Previous work on β3(N265M) knock-in mice has demonstrated that etomidate causes immobility measured as the loss of hind limb withdrawal reflexes predominantly via $GABA_A$ receptors harboring β3 protein subunits (Jurd et al., 2003; Rudolph and Antkowiak, 2004). To find out which of the effects of etomidate examined in spinal slices involves these receptor subtypes, recordings were carried out not only in slices derived from wild type but also in organotypic spinal slices prepared from β3(N265M) mutant mice. Our results suggest that intravenous GABAergic anesthetics like etomidate depress excitatory and GABAergic interneurons in the ventral horn of the spinal cord. The modulation of presynaptic β3-containing $GABA_A$ receptors reduces the release of GABA and limits thereby the efficacy of etomidate to cause profound overall inhibition in the spinal cord.

Materials and Methods

Animals. Embryos for the preparation of spinal slice cultures were obtained from homozygous wild-type mice (statistically 87.5% 129/SvJ; 12.5% 129/Sv) or mutant mice homozygous for an asparagine to methionine point mutation at position 265 of the $GABA_A$ receptor β3 subunit (N265M) on the same genetic background (Jurd et al., 2003). All procedures were approved by the animal care committee (Eberhard-Karls-University, Tuebingen, Germany) and were in accordance with the German law on animal experimentation.

Spinal Slice Cultures. Preparation of spinal cord slices from embryos obtained from either pregnant wild-type or mutant mice (E 15) was performed according to the method originally described by Braschler and coworkers (1989) and as reported previously (Grasshoff and Antkowiak, 2004). In brief, embryos were rapidly decapitated and placed in ice-cold Gey's balanced salt solution consisting of 1.5 mM $CaCl_2$, 5 mM KCl, 0.22 mM KH_2PO_4, 11 mM $MgCl_2$, 0.3 mM $MgSO_4$, 137 mM NaCl, 0.7 mM $NaHCO_3$, and 33 mM D-glucose (all from Sigma, Taufkirchen, Germany). Spinal columns were freed from inner organs and limbs and were cut transversely into 300 μm thick slices using a vibratome. The slices were placed on a coverslip and embedded in a plasma clot consisting of 13 μl of heparin-treated chicken plasma (Sigma) and coagulated by 13 μl of a thrombin solution (Sigma). The coverslips were inserted into plastic tubes containing 0.75 ml of nutrient fluid including 10 nM neuronal growth factor (Sigma) and initially incubated in an atmosphere of 95% oxygen/5% carbon dioxide at 36.0°C for 1 to 2 h. Nutrient fluid (100 ml) consisted of 25 ml of horse serum (Invitrogen, Karlsruhe, Germany), 25 ml of Hanks' balanced salt solution (Sigma), and 50 ml of basal medium Eagle (Sigma). The roller tube technique described by Gähwiler (1981) was used to maintain the tissue. After 1 day in culture, antimitotics (10 μM 5-fluoro-2-deoxyuridine, 10 μM cytosine-β-D-arabino-furanoside, and 10 μM uridine; all from Sigma) were added to reduce proliferation of glial cells. The slices were used after 14 days in vitro for whole-cell patch-clamp and extracellular recordings.

Extracellular Recordings. The spinal cord slices were continuously perfused with an artificial cerebrospinal fluid (ACSF) consisting of 120 mM NaCl, 3.3 mM KCl, 1.13 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1.8 mM $CaCl_2$, and 11 mM D-glucose. The ACSF was bubbled with 95% oxygen/5% carbon dioxide. Glass electrodes filled with ACSF (resistance, approximately 2–5 MΩ) were used for recording extracellular signals, which were bandpass-filtered (passband 200-5000 Hz) to isolate action potential activity. The electrodes were advanced into the tissue until extracellular single or multiunit spike activity (usually exceeding 100 μV in amplitude) could be clearly identified. Signals were digitized on a personal computer at 10 kHz via a Digidata 1200 analog-to-digital/digital-to-analog interface and Axoscope (Molecular Devices, Sunnyvale, CA).

Whole-Cell Voltage-Clamp Recordings. Whole-cell voltage-clamp recordings were performed on visually identified ventral horn interneurons at room temperature (20–24°C). The cultures were perfused with ACSF as specified above, with the addition of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 50 μM), DL-2-amino-5-phosphonopentanoic acid (APV, 50 μM), and 1 μM strychnine (all from Sigma). Signals were acquired with an EPC 7 amplifier (List Medical, Darmstadt, Germany), low pass-filtered at 3 kHz, and digitized on a personal computer at 10 kHz via a Digidata 1200 interface and Clampex (Axon Instruments). Patch pipettes were pulled from borosilicate capillaries (1.5 mm outside diameter) and coated with Sylgard. Pipette resistances were between 1.5 and 2.5 MΩ after fire-polishing. The pipette solution contained 121 mM CsCl, 24 mM CsOH, 10 mM HEPES, 5 mM EGTA, 1 mM $MgCl_2$, and 2 mM ATP, adjusted to pH 7.3 with 1 M NaOH (all from Sigma).

Preparation and Application of Test Solutions. Test solutions were prepared by dissolving etomidate (Etomidat-Lipuro; Braun, Melsungen, Germany) in ACSF equilibrated with 95% oxygen/5% carbon dioxide. Etomidate was administered via the bath perfusion using gas-tight syringe pumps (ZAK Medicine Technique, Marktheidenfeld, Germany), which were connected to the experimental chamber via Teflon tubing (Lee, Frankfurt, Germany). The flow rate was approximately 1 ml/min. To ensure steady-state conditions, recordings during anesthetic treatment were carried out 10 to 15 min after starting the perfusate change.

Data Analysis. Data were analyzed with in-house software written in OriginPro version 7 (OriginLab Corp, Northampton, MA) and MATLAB version 6.5 (The MathWorks Inc., Natick, MA). IPSC current decays were fitted with monoexponential functions. Data analysis of extracellular recordings was performed as described previously (Grasshoff and Antkowiak, 2004). After close inspection of the raw data, action potentials were detected by setting a threshold well above baseline noise. The mean firing rate was obtained from single or multiunit activity; it is defined as the number of detected action potentials divided by the recording time of 180 s. The natural firing mode of spinal ventral horn neurons in culture consisted of bursts of action potentials separated by silent periods. Comparative statistics were performed with a two-tailed Student's t test for paired data. Unless otherwise stated, results are given as mean ± S.E.M. Concentration-response curves were fitted by Hill equations, as described previously (Antkowiak and Helfrich, 1998). Estimated EC_{50} values were derived from these fits.

Results

The Impact of $GABA_A$ Receptors Containing β3 Subunits on Etomidate Effects on Spinal Network Activity. It has been reported that the suppression of noxious-evoked movements in response to etomidate is completely abolished in mice harboring a point mutation (N265M) in the second transmembrane region of the β3 subunit of $GABA_A$ receptors (Jurd et al., 2003). Because the ablation of spontaneous or stimulus-induced movements by general anesthetics is known to be primarily spinally mediated (Collins et al., 1995; Campagna et al., 2003), this leads to the hypothesis that the depression of spinal neurons by etomidate is largely mediated by $GABA_A$ receptors containing a β3 subunit. We tested the hypothesis by comparing the effects of etomidate on spinal network activity in cultured slices from wild-type and β3(N265M) knock-in mice. Network activity was measured by extracellular recordings of ventral horn interneurons, which were visually identified in the slices. Concentration-dependent effects of etomidate on average discharge

rates of spinal neurons are summarized in Fig. 1. Figure 1A displays original recordings under control conditions and in the presence of 0.75 μM etomidate measured in slices from wild-type and β3(N265M) mutant mice. In Fig. 1B, concentration-response relationships were fitted by Hill equations. In wild-type animals, the estimated EC_{50} value is 0.39 $\pm$ 0.04 μM and thereby within the relevant range for general anesthesia in rats (Dickinson et al., 2003) and swine (Johnson et al., 2003). It is remarkable that full depression of spontaneous activity could not be achieved. The concentration-response curve of etomidate showed an upper limit close to 60%. Etomidate displayed no depressant effects on network activity in organotypic slices from β3(N265M) mutant mice within the clinically relevant range. The deletion of etomidate's depressant effects by the β3(N265M) point mutation strongly supported our hypothesis that the depression of spinal neurons is predominantly mediated by $GABA_A$ receptors containing a β3 subunit. At higher concentrations, etomidate diminished network activity slightly in slices from the mutant mice (Fig. 1B) but was still less effective compared with the lowest concentration (0.25 μM) tested in the wild type.

The Effects of Etomidate on Postsynaptic $GABA_A$ Receptors in Ventral Horn Interneurons. The observation that the concentration-response curve of etomidate plateaued at a maximum of 60% of the total depression of firing, even at very high concentrations of the anesthetic, raises the question of how this limited efficacy in depressing spinal neurons can be explained. One hypothesis that is consistent with this observation is that the limitation of etomidate on the network level resembles a saturation of postsynaptic $GABA_A$ receptors. To test this hypothesis, we investigated the effects of etomidate on miniature inhibitory postsynaptic currents (mIPSCs). GABAergic miniature postsynaptic currents were pharmacologically isolated by adding CNQX (50 μM), APV (50 μM), strychnine (1 μM), and tetrodotoxin (1 μM) to the medium. The effects of etomidate on mIPSCs are shown in Fig. 2. A representative example of the effects of etomidate on mIPSCs in spinal neurons is shown in Fig. 2A. Original recordings display a prolonging of half-decay time by 2.5 μM etomidate. As demonstrated in Fig. 2B, this prolonging in half-decay times is concentration-dependent and shows no limitation. Neither amplitudes nor frequencies of mIPSCs were affected by etomidate (Fig. 2, C and D). Because the concentration-response curves for half-decay times showed no limitation, as was observed for the depression of network activity, the limited efficacy of etomidate in depressing spinal network activity could not be explained by a limited effect on postsynaptic $GABA_A$ receptors.

Effects of Etomidate on Spontaneous Inhibitory Postsynaptic Currents in Ventral Horn Interneurons. As demonstrated above, the limited efficacy of etomidate in depressing spinal neurons cannot be explained by a saturation of postsynaptic $GABA_A$ receptors. Thus, the next question that we asked was whether presynaptic effects of etomidate, especially a depression of GABA-releasing interneurons, accounts for the limitation. In this context, it is important to state that, at clinically relevant concentrations, intravenous anesthetics like etomidate are modulators of GABA-induced chloride currents requiring the presence of GABA at the $GABA_A$ receptor. Direct (i.e., GABA-independent) actions of etomidate have only been observed at higher concentrations of etomidate. A decrease in presynaptic release of GABA would result in reduced amplitudes and frequencies of postsynaptic sIPSCs. Therefore, the corresponding hypothesis is that etomidate affects neither amplitudes nor frequencies of spontaneous IPSCs. The effects of etomidate on sIPSCs are displayed in Fig. 3. Spontaneous GABAergic postsynaptic currents were measured in the presence of CNQX (50 μM), APV (50 μM), and strychnine (1 μM). Original recordings of the effects of 1.5 and 2.5 μM etomidate on sIPSCs in organotypic slices from wild-type and β3 knock-in mice are shown in Fig. 3A. In slices obtained from wild-type mice, etomidate prolongs half-decay times and concurrently reduces the number and amplitudes of GABAergic sIPSCs. In slices from β3 knock-in mice, 1.5 and 2.5 μM etomidate induce only a minimal increase in half-decay times, whereas it did not affect amplitudes or frequencies of GABAergic sIPSCs. Figure 3B demonstrates a concentration-dependent increase of half-decay times resembling the effects of etomidate on mIPSCs. In contrast to the actions on mIPSCs, etomidate diminishes amplitudes and reduces frequencies of spontaneous GABAergic IPSCs. This result suggests that etomidate decreases the presynaptic release of GABA.

We next set out to address the question of whether $GABA_A$ receptors involved in pre- and postsynaptic actions of etomidate contain a β3 subunit by comparing the effects of 1.5 and 2.5 μM etomidate on sIPSCs in cultured spinal slices of wild-type and β3 knock-in mice. Figure 3C summarizes the results from the corresponding experiments. Although 2.5 μM etomidate induces a 3-fold prolongation of the half-decay time of GABAergic IPSCs in the wild type, it exerts only a negligible prolonging effect in the mutant, consistent with the hypothesis that $GABA_A$ receptors mediating postsynaptic effects of etomidate contain a β3 subunit. Regarding presynaptic effects of etomidate, 2.5 μM etomidate neither reduces amplitudes of GABAergic sIPSCs nor depresses the IPSC rate in the mutant, whereas the anesthetic at a concentration of 2.5 μM diminished the amplitude by half and exerted a 60% depression of IPSC rate in the wild type. From these results, we deduce that not only postsynaptic but also presynaptic $GABA_A$ receptors mediating spinal effects of etomidate incorporate a β3 subunit.

Effects of Etomidate on Total Charge Transfer of GABAergic sIPSCs in Ventral Horn Interneurons. The results so far indicate that the limited efficacy of etomidate in depressing spinal neurons can be explained by concomitant effects on pre- and postsynaptic $GABA_A$ receptors exerting opposing effects on network activity. Although the prolonging of half-decay times by etomidate increases the charge transfer, the reduction of IPSC amplitudes and IPSC rates decreases the impact of etomidate on charge transfer. Assuming that the changes in total charge transfer of $GABA_A$ receptors reflect the effects of etomidate on the network level, we expect a limitation in total charge transfer at concentrations greater than 1.5 μM. The increase in total charge transfer of spontaneous GABAergic IPSCs was calculated as the product of the integral of averaged spontaneous IPSCs with the frequency of the events. Figure 4 displays the effects of etomidate on the total charge transfer. The curve demonstrates a concentration-dependent increase in total charge transfer and a ceiling starting at a concentration of 1.5 μM etomidate, thereby mirroring the run of the concentration-

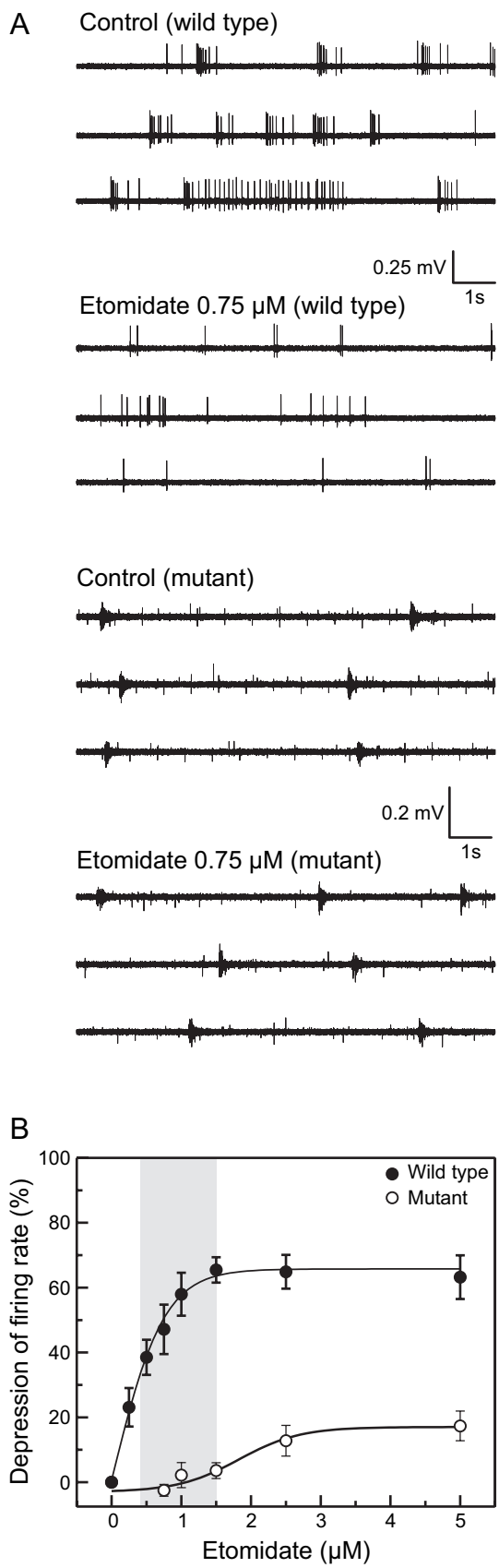

Fig. 1. Effects of etomidate on neuronal network activity in organotypic spinal slice cultures. A, excerpts of original recordings under control conditions and in the presence of 0.75 μM etomidate measured in slices from wild-type and β3(N265M) mutant mice. B, comparison of concentration-response relationships of etomidate-induced depression of spinal neuronal network activity in organotypic slices obtained from either wild-type (●) or β3(N265M) mutant mice (○). For each concentration, the mean value and standard error were obtained from 8 to 12 cells. The curve represents a Hill fit to the data (R^2 = 0.966). The half-maximal effect (EC_{50}) occurred at 0.39 ± 0.04 μM, and the maximal depression of action potential activity was 65.6 ± 2.5% in wild-type mice. The clinically relevant concentration range is marked by a gray vertical bar.

response curve measured from extracellular recordings of spinal network activity as depicted in Fig. 1.

Discussion

GABAergic Drugs Display a Limited Efficacy in Depressing Ventral Horn Neurons. In the current study, we investigated the effects of the intravenous GABAergic anesthetic etomidate on the network activity and on GABAergic inhibitory postsynaptic currents in organotypic spinal slice cultures obtained from either wild-type or β3(N265M) mutant mice. The striking result is that the effects of etomidate on action potential activity of ventral horn neurons saturated at concentrations exceeding 1.0 μM, which are known to cause immobility. As displayed in Fig. 1, the concentration-response curve plateaued to an uppermost limit of approximately 60% depression of the firing rate, thereby resembling the concentration-response curve of another GABAergic anesthetic, propofol, in the ventral horn of the spinal cord (Grasshoff and Antkowiak, 2004). In sharp contrast to intravenous GABAergic drugs, volatile anesthetics like sevoflurane, isoflurane, or enflurane do not display a limitation in their effectiveness in depressing ventral horn neurons (Grasshoff and Antkowiak, 2004, 2006). Compared with intravenous anesthetics, volatile anesthetics are known to affect, besides $GABA_A$ receptors, a multitude of molecular targets in the spinal cord (Cheng and Kendig, 2000, 2002, 2003; Wong et al., 2001; Campagna et al., 2003). Although the experimental conditions in vitro are different from the in vivo measurement of immobility, our results reflect closely those obtained from studies in humans in whom intravenous GABAergic anesthetics were far less effective in depressing involuntary movements compared with volatile anesthetics (Ashworth and Smith, 1998; Smith and Thwaites, 1999; Watson and Shah, 2000). However, in clinical study settings, immobility can also be achieved with intravenous anesthetics at concentrations approximately 5-fold higher than those required for hypnosis (Smith et al., 1994). At these high concentrations, intravenous anesthetics like propofol frequently require the application of ephedrine to keep the blood pressure stable. The capability of GABAergic anesthetics to produce immobility, despite their limited efficacy in reducing neuronal excitability in the spinal cord, can be explained by the involvement of supraspinal sites. It has been demonstrated previously that local administration of pentobarbital, another anesthetic acting predominantly via $GABA_A$ receptors (Zeller et al., 2007), into the mesopontine tegmental anesthesia locus can cause deep anesthesia (Devor and Zalkind, 2001; Sukhotinsky et al., 2005). This result, together with findings of the present study, allows the conclusion that the immobilizing properties of GABAergic anesthetics are mediated in part via the ventral horn of the spinal cord and, in part, via supraspinal structures, including the mesopontine tegmental anesthesia locus. In summary, these results lead to the conclusion that general anesthetics acting via

positive modulation of $GABA_A$ receptors display a restricted capacity in depressing the excitability of neurons in the ventral horn of the spinal cord, leading to the question of which mechanism is responsible for this limitation.

Depression of GABA Release Is Limiting the Efficacy of Etomidate in the Ventral Horn. Observing that GABAergic anesthetics display a limited efficacy in reducing spinal network activity, we hypothesized that besides depressing neuronal excitability, these agents simultaneously inhibit GABA release in the ventral horn of the spinal cord via presynaptic $GABA_A$ receptors. The results of the current study support this hypothesis because etomidate reduces action potential-dependent GABA release in a concentration-dependent manner (Fig. 3B). Anesthetic-induced depression of GABA release onto ventral horn neurons, caused by a presynaptic mechanism of action, is expected to increase the excitability of these cells. In contrast, anesthetic actions mediated via postsynaptic $GABA_A$ receptors decreases the excitability of ventral horn neurons. Thus, pre- and postsynaptic actions of etomidate affect neurons in the ventral horn in opposing ways. As demonstrated by the experiments on the total charge transfer the pre- and postsynaptic actions of etomidate largely balance out between 1 and 5 μM, thereby explaining the limited efficacy of etomidate in reducing spinal network activity.

Involvement of β3-Containing $GABA_A$ Receptors in the Actions of Etomidate. The ability of the intravenous anesthetics etomidate and propofol to modulate $GABA_A$ receptors is uniquely dependent on the identity of the β subunit in the receptor complex (Siegwart et al., 2002; Jurd et al., 2003). Receptors containing β2 or β3 but not β1 subunits are highly susceptible to both anesthetics. Moreover, point mutations in the β subunits have dramatic effects on the sensitivity of $GABA_A$ receptors to anesthetics (Belelli et al., 1997; Mihic et al., 1997) For example, the β3(N265M) mutation was shown to largely abolish the modulatory effects of etomidate at $GABA_A$ receptors (Siegwart et al., 2002). Subsequent work on β3(N265M) knock-in mice showed that the immobilizing properties of etomidate almost exclusively depend on β3 subunits (Jurd et al., 2003). It is most striking that etomidate failed to cause loss of hind limb withdrawal reflexes in β3 knock-in mice, even when delivered at very high concentrations close to the lethal dose. In contrast, a similar mutation in the β2 subunit did not alter the ability of etomidate to ablate motor reflexes (Reynolds et al., 2003).

In the present study, we characterized the effects of etomi-

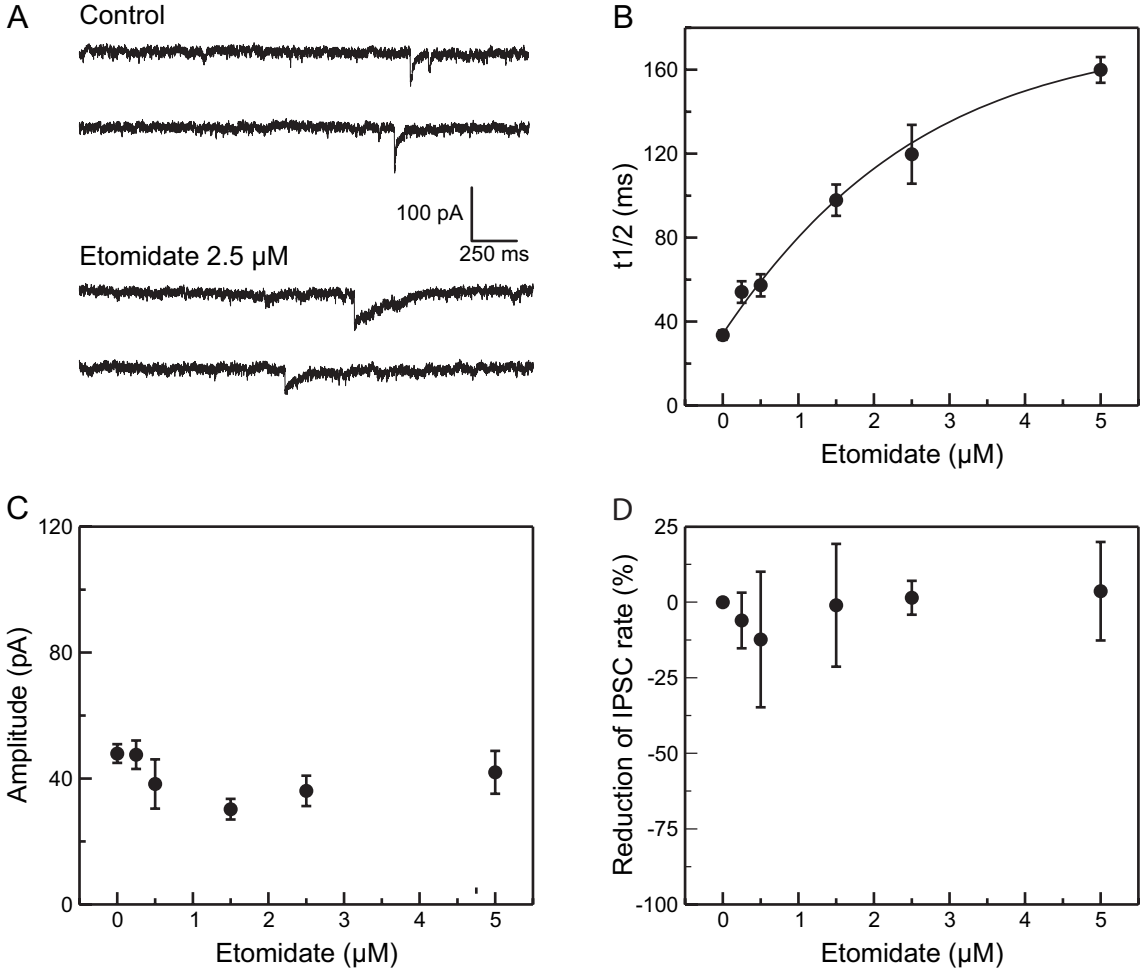

Fig. 2. Effects of etomidate on action potential-independent GABAergic inhibitory postsynaptic currents (miniature IPSCs) in spinal ventral horn interneurons in organotypic slices of wild-type mice. To isolate the currents pharmacologically, they were measured in the presence of 50 μM CNQX, 50 μM AP5, 1 μM strychnine, and 1 μM tetrodotoxin. Neurons were held at a membrane potential of -70 mV. For each concentration, the mean value and standard error were obtained from six cells. A, original recordings of the effects of 2.5 μM etomidate on miniature IPSCs. Effects of etomidate on half-decay times of miniature IPSCs are displayed in B, on amplitudes in C, and on the reduction of miniature IPSC rates in D.

date in spinal slices derived from wild-type and mutant mice. Within a range of concentrations commonly assumed to produce immobility in vivo (1.0–1.5 μM) (Dickinson et al., 2003), effects of etomidate at $GABA_A$ receptors and on action potential firing were almost completely abolished by the β3(N265M) mutation. Because the mutation also abolished the ability of etomidate to ablate the hind limb withdrawal reflex in knock-in mice (Jurd et al., 2003), our results clearly

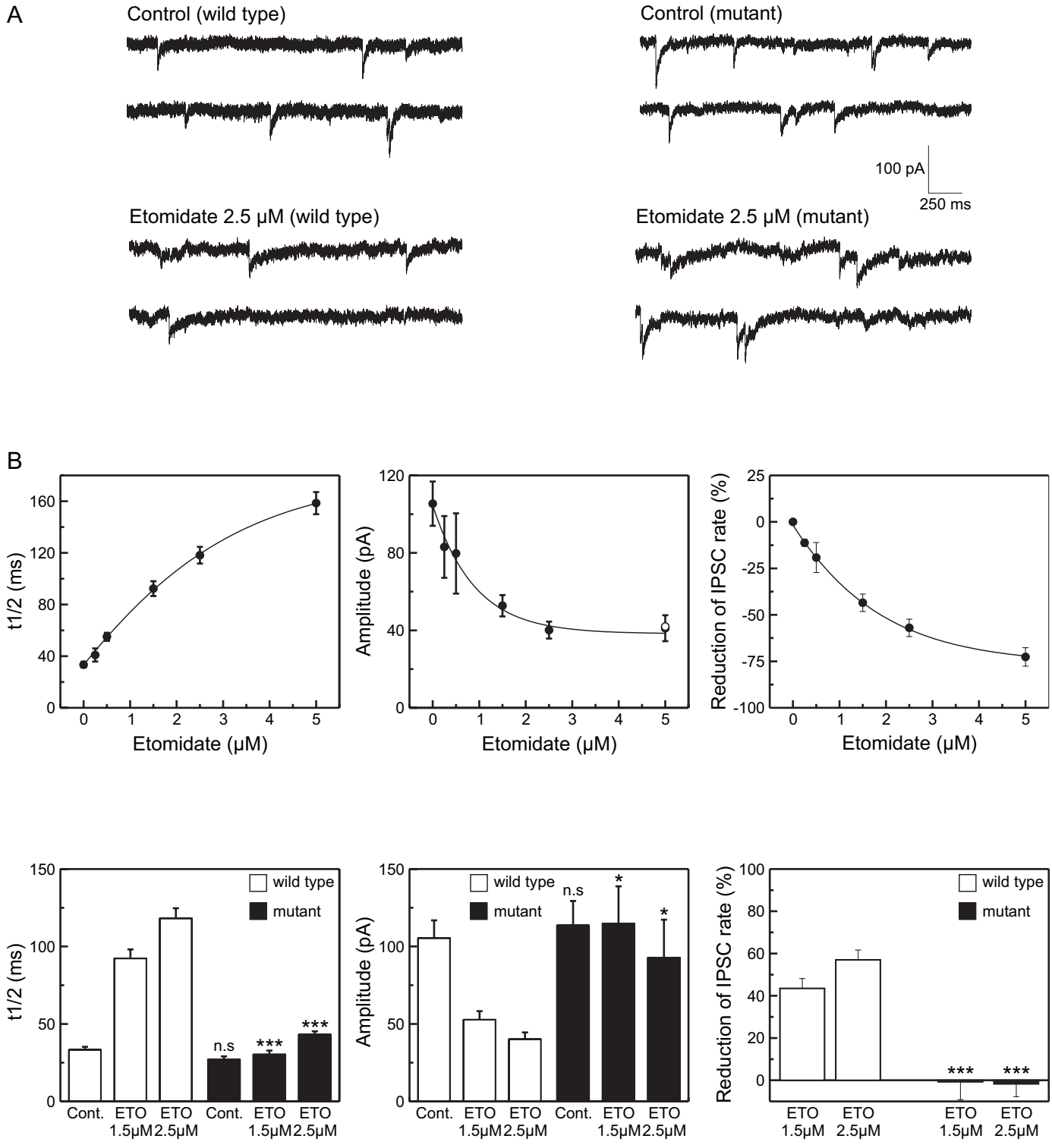

Fig. 3. Effects of etomidate on spontaneous GABAergic inhibitory postsynaptic currents (spontaneous IPSCs) in spinal ventral horn interneurons in organotypic slices. To isolate the currents pharmacologically, they were measured in the presence of 50 μM CNQX, 50 μM AP5, and 1 μM strychnine. Neurons were held at a membrane potential of −70 mV. For each concentration, the mean value and standard error were obtained from 8 to 10 cells. A, original recordings of the effects of 2.5 μM etomidate on spontaneous IPSCs in organotypic spinal cord slices obtained from wild-type and β3(N265M) mutant mice. B, effects of etomidate on half-decay times, amplitudes, and the reduction of spontaneous IPSCs rates in wild-type mice. C, comparison of the effects of 1.5 and 2.5 μM etomidate on half-decay times, amplitudes, and the reduction of spontaneous IPSC rates in slices from wild-type and β3(N265M) mutant mice. Statistical analysis was performed by means of a two-tailed Student's t test (*, $p < 0.05$; ***, $p < 0.001$).

support the idea that the ventral horn of the spinal cord presents a major contribution to etomidate-induced immobility. In addition, the current results suggest that the majority of $GABA_A$ receptors in the ventral horn harbor $\beta3$ subunits, a finding that is quite different in the brain, where $\beta3$ subunits are present in only 20 to 30% of all $GABA_A$ receptors (Benke et al., 1994). As mentioned above, etomidate exerts opposing actions on spinal network activity via pre- and postsynaptic $GABA_A$ receptors in the ventral horn, raising the question of which of these two groups of receptors is able to be modulated by etomidate. Because not only the prolonging effect on IPSCs but also the reduction of the frequency of spontaneous IPSCs was completely abolished by the $\beta3$(N265M) mutation, it is clear that both functional groups of $GABA_A$ receptors express $\beta3$ subunits. Thus, intravenous GABAergic anesthetics like etomidate and propofol, acting via $\beta3$-containing $GABA_A$ receptors, act on both excitatory and inhibitory neurons in the ventral horn. This opposing effect therefore limits the effectiveness of etomidate to cause profound overall inhibition in the spinal cord and thus limits the immobilizing capability of this intravenous GABAergic anesthetic.

Future Prospects for the Design of Intravenous Anesthetics to Produce Immobility. In summary, the results provide one explanation of why intravenous GABAergic anesthetics are poor immobilizers. We demonstrate that etomidate has the ability to simultaneously depress not only excitatory neurons in the ventral horn of the spinal cord but also GABAergic interneurons, thus reducing the release of GABA in the same spinal region. Because general anesthetics potentiate the effect of GABA at $GABA_A$ receptors, they require the neurotransmitter to exert their depressant effects. This action counteracts the depressant effects on spinal network activity and causes thereby a limitation of the anesthetic efficacy. Furthermore, the experiments using $\beta3$(N265M) mutant mice revealed that the release of GABA in the ventral horn is regulated by $GABA_A$ receptors incorporating a $\beta3$ subunit. However, the localization of these functionally presynaptic receptors is unclear. They can be present either at the presynaptic terminal or at presynaptic inhibitory neurons. Apart from these presynaptic $GABA_A$ receptors, those mediating the depressant anesthetic effects possess a $\beta3$ subunit as well. Thus, GABAergic anesthetics in current clinical use act unspecifically at both pre- and postsynaptic receptors in the spinal cord. Immunohistochemical experiments have demonstrated that $GABA_A$ receptors located on spinal interneurons harbor different types of α subunits (Bohlhalter et al., 1996). Electrophysiological experiments using cultured spinal slices from α-knock-in mice revealed first evidence that these $GABA_A$ receptors are located on excitatory and inhibitory interneurons (Grasshoff et al., 2006b), opening the opportunity to develop subtype-selective agents that can be used clinically as potent muscle relaxants or immobilizers in the future.

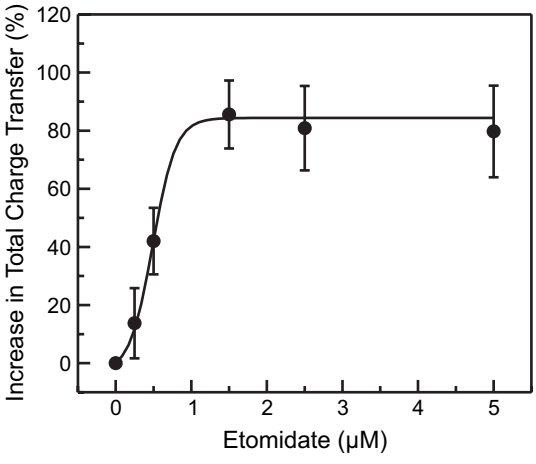

Fig. 4. Concentration-response curve of the changes of the total charge transfer in a given time interval. The total charge transfer was calculated as the product of the integral of averaged spontaneous IPSCs with the frequency of the events. The curve demonstrates a concentration-dependent increase in total charge transfer and a ceiling starting at a concentration of 1.5 μM etomidate, thereby mirroring the run of the concentration-response curve measured from extracellular recordings of spinal network activity, as depicted in Fig. 1B.

Acknowledgments

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References

Antkowiak B and Helfrich FC (1998) Effects of small concentrations of volatile anesthetics on action potential firing of neocortical neurons in vitro. *Anesthesiology* **88:**1592–1605.
Ashworth J and Smith I (1998) Comparison of desflurane with isoflurane or propofol in spontaneously breathing ambulatory patients. *Anesth Analg* **87:**312–318.
Banks MI and Pearce RA (1999) Dual actions of volatile anesthetics on $GABA_A$ IPSCs: dissociation of blocking and prolonging effects. *Anesthesiology* **90:**120–134.
Belelli D, Lambert JJ, Peters JA, Wafford K, and Whiting PJ (1997) The interaction of the general anesthetic etomidate with the γ-aminobutyric acid type A receptor is influenced by a single amino acid. *Proc Natl Acad Sci U S A* **94:**11031–11036.
Benke D, Fritschy JM, Trzeciak A, Bannwarth W, and Mohler H (1994) Distribution, prevalence, and drug binding profile of γ-aminobutyric acid type A receptor subtypes differing in the β-subunit variant. *J Biol Chem* **269:**27100–27107.
Bohlhalter S, Weinmann O, Mohler H, and Fritschy JM (1996) Laminar compartmentalization of GABAA-receptor subtypes in the spinal cord: an immunohistochemical study. *J Neurosci* **16:**283–297.
Braschler UF, Iannone A, Spenger C, Streit J, and Luscher HR (1989) A modified roller tube technique for organotypic cocultures of embryonic rat spinal cord, sensory ganglia and skeletal muscle. *J Neurosci Methods* **29:**121–129.
Campagna JA, Miller KW, and Forman SA (2003) Mechanisms of actions of inhaled anesthetics. *N Engl J Med* **348:**2110–2124.
Cheng G and Kendig JJ (2000) Enflurane directly depresses glutamate AMPA and NMDA currents in mouse spinal cord motor neurons independent of actions on GABAA or glycine receptors. *Anesthesiology* **93:**1075–1084.
Cheng G and Kendig JJ (2002) Pre- and postsynaptic volatile anaesthetic actions on glycinergic transmission to spinal cord motor neurons. *Br J Pharmacol* **136:**673–684.
Cheng G and Kendig JJ (2003) Enflurane decreases glutamate neurotransmission to spinal cord motor neurons by both pre- and postsynaptic actions. *Anesth Analg* **96:**1354–1359.
Collins JG, Kendig JJ, and Mason P (1995) Anesthetic actions within the spinal cord: contributions to the state of general anesthesia. *Trends Neurosci* **18:**549–553.
Devor M and Zalkind V (2001) Reversible analgesia, atonia, and loss of consciousness on bilateral intracerebral microinjection of pentobarbital. *Pain* **94:**101–112.
Dickinson R, Awaiz S, Whittington MA, Lieb WR, and Franks NP (2003) The effects of general anaesthetics on carbachol-evoked gamma oscillations in the rat hippocampus in vitro. *Neuropharmacology* **44:**864–872.
Gähwiler BH (1981) Organotypic monolayer cultures of nervous tissue. *J Neurosci Methods* **4:**329–342.
Grasshoff C and Antkowiak B (2004) Propofol and sevoflurane depress spinal neurons in vitro via different molecular targets. *Anesthesiology* **101:**1167–1176.
Grasshoff C and Antkowiak B (2006) Effects of isoflurane and enflurane on GABAA and glycine receptors contribute equally to depressant actions on spinal ventral horn neurones in rats. *Br J Anaesth* **97:**687–694.
Grasshoff C, Drexler B, Rudolph U, and Antkowiak B (2006a) Anaesthetic drugs: linking molecular actions to clinical effects. *Curr Pharm Des* **12:**3665–3679.
Grasshoff C, Rudolph U, and Antkowiak B (2006b) Switch of diazepam effects in the spinal cord by a mutation in alpha3-containing $GABA_A$ receptors. *Anesthesiology* **105:**A775.
Johnson KB, Egan TD, Layman J, Kern SE, White JL, and McJames SW (2003) The influence of hemorrhagic shock on etomidate: a pharmacokinetic and pharmacodynamic analysis. *Anesth Analg* **96:**1360–1368.
Jurd R, Arras M, Lambert S, Drexler B, Siegwart R, Crestani F, Zaugg M, Vogt KE, Ledermann B, Antkowiak B, et al. (2003) General anesthetic actions in vivo strongly attenuated by a point mutation in the $GABA_A$ receptor beta3 subunit. *FASEB J* **17:**250–252.
Kiehn O (2006) Locomotor circuits in the mammalian spinal cord. *Annu Rev Neurosci* **29:**279–306.
Mihic SJ, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, Mascia MP, Valenzuela CF, Hanson KK, Greenblatt EP, et al. (1997) Sites of alcohol and volatile anaesthetic action on $GABA_A$ and glycine receptors. *Nature* **389:**385–389.

Orser BA, Wang LY, Pennefather PS, and MacDonald JF (1994) Propofol modulates activation and desensitization of GABAA receptors in cultured murine hippocampal neurons. *J Neurosci* **14:**7747–7760.
Reynolds DS, Rosahl TW, Cirone J, O'Meara GF, Haythornthwaite A, Newman RJ, Myers J, Sur C, Howell O, Rutter AR, et al. (2003) Sedation and anesthesia mediated by distinct $GABA_A$ receptor isoforms. *J Neurosci* **23:**8608–8617.
Rudolph U and Antkowiak B (2004) Molecular and neuronal substrates for general anaesthetics. *Nat Rev Neurosci* **5:**709–720.
Siegwart R, Jurd R, and Rudolph U (2002) Molecular determinants for the action of general anesthetics at recombinant $alpha_2beta_3gamma_2$gamma-aminobutyric $acid_A$ receptors. *J Neurochem* **80:**140–148.
Smith C, McEwan AI, Jhaveri R, Wilkinson M, Goodman D, Smith LR, Canada AT, and Glass PS (1994) The Interaction of Fentanyl on the Cp50 of Propofol for Loss of Consciousness and Skin Incision. *Anesthesiology* **81:**820–828.
Smith I and Thwaites AJ (1999) Target-controlled propofol vs. sevoflurane: a double-blind, randomised comparison in day-case anaesthesia. *Anaesthesia* **54:**745–752.
Sukhotinsky I, Hopkins DA, Lu J, Saper CB, and Devor M (2005) Movement suppression during anesthesia: neural projections from the mesopontine tegmentum to areas involved in motor control. *J Comp Neurol* **489:**425–448.
Watson KR and Shah MV (2000) Clinical comparison of 'single agent' anaesthesia with sevoflurane versus target controlled infusion of propofol. *Br J Anaesth* **85:**541–546.
Wong SM, Cheng G, Homanics GE, and Kendig JJ (2001) Enflurane actions on spinal cords from mice that lack the beta3 subunit of the $GABA_A$ receptor. *Anesthesiology* **95:**154–164.
Zeller A, Arras M, Jurd R, and Rudolph U (2007) Identification of a molecular target mediating the general anesthetic actions of pentobarbital. *Mol Pharmacol* **71:**852–859.

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