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**TITLE PAGE**

**Title:**

# Modulation of presynaptic β3-containing $GABA_A$ receptors limits the immobilizing actions of GABAergic anesthetics

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**RUNNING TITLE PAGE**

**Running Title:**

# Molecular mechanisms of GABAergic anesthetics in the spinal cord

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**List of non-standard abbreviations:**

| | |
|---|---|
| ACSF | Artificial cerebrospinal fluid |
| APV | DL-2-Amino-5-phosphonopentanoic acid |
| CNQX | 6-Cyano-7-nitroquinoxaline-2,3-dione |
| GABA | γ-Aminobutyric acid |
| IPSC | Inhibitory postsynaptic current |
| TTX | Tetrodotoxin |

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**Abstract**

Intravenous GABAergic anesthetics are potent hypnotics, but 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. As 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 larger 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 above 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 likely due to a presynaptic mechanism and that GABAergic agents with a specificity of post- versus presynaptic receptors would likely have much stronger immobilizing actions, pointing out novel avenues for drug development.

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## Introduction

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 (Smith and Thwaites, 1999; Ashworth and Smith, 1998; Watson and Shah, 2000; Grasshoff *et al.*, 2006b; Rudolph and Antkowiak, 2004). In clinical practice, these agents are routinely used for providing hypnosis and amnesia, but rarely 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. As 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 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 hindlimb 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 involve these receptor subtypes, recordings were carried out not only in slices derived from wild type, but additionally, in organotypic spinal slices prepared from β3(N265M) mutant mice. Our results suggest that intravenous GABAergic anesthetics like etomidate depress excitatory as well as 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.

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## 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 (Braschler *et al.*, 1989) and as previously reported (Grasshoff and Antkowiak, 2004). Briefly, 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 heparin-treated chicken plasma (Sigma, Taufkirchen, Germany) and coagulated by 13 µl of a thrombin solution (Sigma, Taufkirchen, Germany). The coverslips were inserted into plastic tubes containing 0.75 ml of nutrient fluid including 10 nM Neuronal Growth Factor (Sigma, Taufkirchen, Germany) and initially incubated in an atmosphere of 95%

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oxygen and 5 % carbon dioxide at 36.0 °C for 1-2 hours. 100 ml nutrient fluid consisted of 25 ml horse serum (Invitrogen, Karlsruhe, Germany), 25 ml Hanks' Balanced Salt Solution (Sigma, Taufkirchen, Germany), and 50 ml Basal Medium Eagle (Sigma, Taufkirchen, Germany). The roller tube technique described by Gähwiler was used to maintain the tissue (Gahwiler, 1981). After one day in culture, antimitotics (10 μM 5-fluoro-2-deoxyuridine, 10 μM cytosine-b-d-arabino-furanoside, 10 μM uridine [all from Sigma, Taufkirchen, Germany]) 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 and 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) in order to isolate action potential activity. The electrodes were advanced into the tissue until extracellular single or multi-unit spike activity (usually exceeding 100 μV in amplitude) could be clearly identified. Signals were digitized on a PC at 10 kHz via a Digidata 1200 analogue to digital/digital to analogue interface and Axoscope (Axon Instruments, Union City, California, USA).

**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

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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, Taufkirchen, Germany). Signals were acquired with an EPC 7 amplifier (List Medical, Darmstadt, Germany), lowpass filtered at 3 kHz and digitized on a PC at 10 kHz via a Digidata 1200 interface and Clampex (Axon Instruments). Patch pipettes were pulled from borosilicate capillaries (1.5 mm OD) and coated with Sylgard. Pipette resistances were between 1.5 and 2.5 MΩ after fire polishing. The pipette solution contained (in mM): 121 CsCl, 24 CsOH, 10 N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), 5 ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 1 $MgCl_2$, and 2 ATP, adjusted to pH 7.3 with 1M NaOH (all from Sigma, Taufkirchen, Germany).

**Preparation and Application of Test Solutions**

Test solutions were prepared by dissolving etomidate (Etomidat-®Lipuro, Braun, Melsungen, Germany) in ACSF equilibrated with 95% oxygen and 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 10-15 min after starting the perfusate change.

**Data Analysis**

Data were analyzed with in-house software written in OriginPro version 7 (OriginLab Corporation, Northampton, Massachusetts, USA) and MATLAB version 6.5 (The

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MathWorks Inc., Natick, Massachusetts, USA). 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 multi unit 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 previously described (Antkowiak and Helfrich, 1998). Estimated $EC_{50}$ values were derived from these fits.

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## 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). As 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 figure 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 figure 1B concentration-response relationships were fitted by Hill equations. In wild type animals, the estimated $EC_{50}$ value is 0.39±0.04 μM, and thereby within the relevant range for general anesthesia in rats (Dickinson *et al.*, 2003) and swine (Johnson *et al.*, 2003). Remarkably, 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.

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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 to 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 as to 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 post synaptic $GABA_A$ receptors. To test this hypothesis, we investigated the effects of etomidate on miniature inhibitory post synaptic currents (mIPSCs). GABAergic miniature post synaptic currents were pharmacologically isolated by adding CNQX (50 μM), APV (50 μM), strychnine (1 μM) and TTX (1 μM) to the medium. The effects of etomidate on mIPSCs are shown in figure 2. A representative example of the effects of etomidate on mIPSCs in spinal neurons is given in figure 2A. Original recordings display a prolonging of half-decay time by 2.5 μM etomidate. As demonstrated in figure 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. 2C and D). As 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

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depressing spinal network activity could not be explained by a limited effect on postsynaptic $GABA_A$ receptors.

**Effects of etomidate on spontaneous inhibitory post synaptic currents in ventral horn interneurons**

As demonstrated above, the limited efficacy of etomidate in depressing spinal neurons can not be explained by a saturation of post synaptic $GABA_A$ 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 post synaptic sIPSCs. Therefore, the corresponding hypothesis is that etomidate neither affects amplitudes, nor frequencies of spontaneous IPSCs. The effects of etomidate on sIPSCs are displayed in figure 3. Spontaneous GABAergic post synaptic 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 figure 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 while it did not affect amplitudes or frequencies of GABAergic sIPSCs. Figure 3B demonstrates a

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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 post synaptic 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. While 2.5 μM etomidate induces a three-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 can 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. While 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 above 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-response curve measured from extracellular recordings of spinal network activity as depicted in figure 1.

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## 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 as well as on GABAergic inhibitory post synaptic 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 figure 1, the concentration response curve plateaued to an uppermost limit of about 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; Grasshoff and Antkowiak, 2006). Compared to intravenous anesthetics, volatile anesthetics are known to affect, besides $GABA_A$ receptors, a multitude of molecular targets in the spinal cord (Campagna *et al.*, 2003; Cheng and Kendig, 2003; Cheng and Kendig, 2000; Cheng and Kendig, 2002; Wong *et al.*, 2001). 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 where intravenous GABAergic anesthetics were far less effective in depressing involuntary movements compared to volatile anesthetics (Smith and Thwaites, 1999; Ashworth and Smith, 1998; Watson and Shah, 2000). However, in clinical study settings, immobility can also be achieved with intravenous anesthetics at concentrations

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approximately five-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 previously been demonstrated 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 as to 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 since etomidate reduces action potential-dependent GABA release in a concentration dependent manner (Figure 3B). Anesthetic-induced depression of GABA release onto

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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 identify of the β-subunit in the receptor complex (Jurd *et al.*, 2003; Siegwart *et al.*, 2002). 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). Most strikingly, etomidate failed to cause loss of hindlimb-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 etomidate in spinal slices derived from wild type and mutant mice. Within a range of concentrations commonly assumed to

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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. As the mutation also abolished the ability of etomidate to ablate the hindlimb withdrawal reflex in knock in mice (Jurd *et al.*, 2003), our results are clearly supporting 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 β3 subunits, a finding which is quite different in the brain, where β3 subunits are present in only 20-30% of all $GABA_A$ receptors (Benke *et al.*, 1994). As mentioned in the above paragraph, etomidate exerts opposing actions on spinal network activity via pre- and postsynaptic $GABA_A$ receptors in the ventral horn, raising the question as to which of these two groups of receptors are able to be modulated by etomidate. As not only the prolonging effect on IPSCs but also the reduction of the frequency of spontaneous IPSCs was completely abolished by the β3(N265M) mutation, it is clear that both functional groups of $GABA_A$ receptors express β3-subunits. Thus, intravenous GABAergic anesthetics like etomidate and propofol, acting via β3-containing $GABA_A$ receptors, act on both excitatory and inhibitory neurons in the ventral horn. This opposing effect therefore limits the effectivity 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 as to 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,

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but also to depress GABAergic interneurons, thus reducing the release of GABA in the same spinal region. As 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 β3(N265M) mutant mice revealed that the release of GABA in the ventral horn is regulated by $GABA_A$ receptors incorporating a β3 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 β3 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.*, 2006a), opening the opportunity to develop subtype selective agents which can be used clinically as potent muscle relaxants or immobilizers in the future.

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**Figure legends**

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 (filled circles) or β3 N265M mutant mice (blank circles). For each concentration, the mean value and standard error were obtained from 8-12 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 grey vertical bar.

Fig. 2: Effects of etomidate on action potential-independent GABAergic inhibitory post synaptic currents (miniature IPSCs) in spinal ventral horn interneurons in organotypic slices of wild-type mice. In order to isolate the currents pharmacologically they were measured in the presence of CNQX 50 μM, AP5 50 μM, strychnine 1 μM, and TTX 1 μM. 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).

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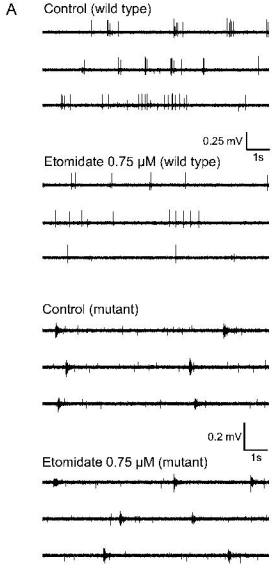
Fig. 3: Effects of etomidate on spontaneous GABAergic inhibitory post synaptic currents (spontaneous IPSCs) in spinal ventral horn interneurons in organotypic slices. In order to isolate the currents pharmacologically they were measured in the presence of CNQX 50 μM, AP5 50 μM, and strychnine 1 μM. Neurons were held at a membrane potential of -70 mV. For each concentration, the mean value and standard error were obtained from 8-10 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 on 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 on 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* (*; $p<0.05$, ***; $p<0.001$).

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.


A
Control (wild type)
0.25 mV
1s
Etomidate 0.75 μM (wild type)
Control (mutant)
0.2 mV
1s
Etomidate 0.75 μM (mutant)

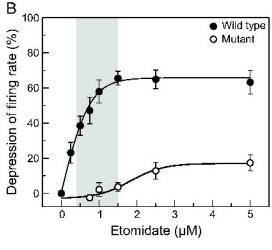

B
Depression of firing rate (%)
Wild type
Mutant
Etomidate (μM)

Fig. 1

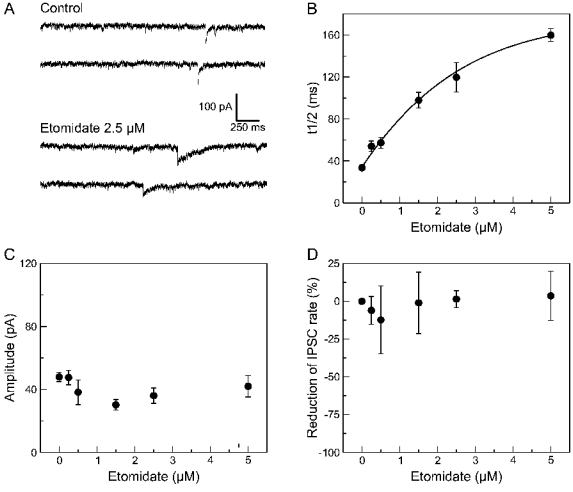

A
Control
100 pA
250 ms
Etomidate 2.5 μM
B
t1/2 (ms)
Etomidate (μM)
C
Amplitude (pA)
Etomidate (μM)
D
Reduction of IPSC rate (%)
Etomidate (μM)

Fig. 2

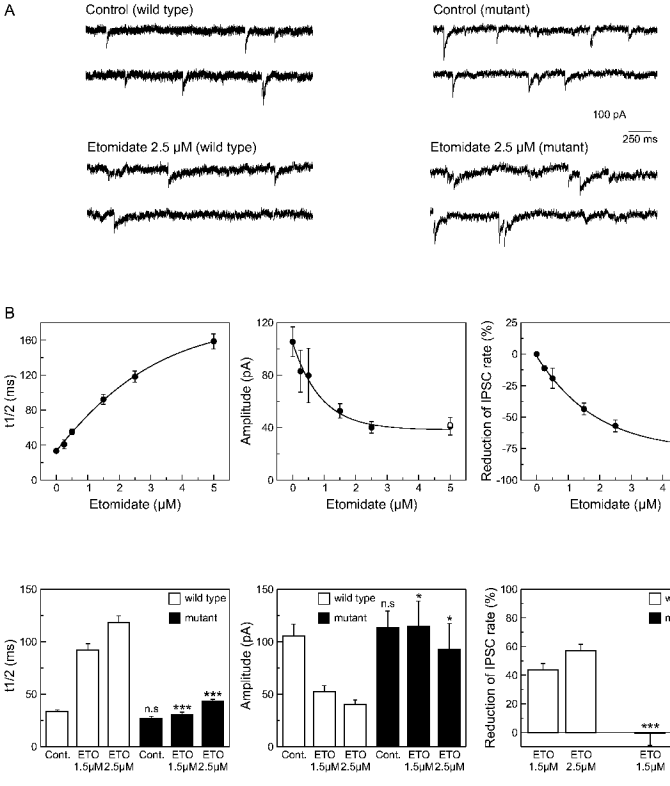

A
Control (wild type)
Control (mutant)
100 pA
250 ms
Etomidate 2.5 μM (wild type)
Etomidate 2.5 μM (mutant)
B
t1/2 (ms)
Etomidate (μM)
Amplitude (pA)
Etomidate (μM)
Reduction of IPSC rate (%)
Etomidate (μM)
wild type
mutant
t1/2 (ms)
Cont.
ETO 1.5μM
ETO 2.5μM
n.s
***
Amplitude (pA)
n.s
*
Reduction of IPSC rate (%)

Fig. 3

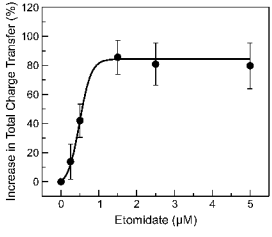

Increase in Total Charge Transfer (%)
120
100
80
60
40
20
0
0
1
2
3
4
5
Etomidate (μM)

Fig. 4