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Opposing Actions of Etomidate on Cortical Theta Oscillations Are Mediated by Different γ-Aminobutyric Acid Type A Receptor Subtypes

Berthold Drexler, M.D., Claire L. Roether, M.Sc.,† Rachel Jurd, Ph.D.,‡ Uwe Rudolph, M.D.,§ Bernd Antkowiak, Ph.D.‖*

Background: **Cortical networks generate diverse patterns of rhythmic activity. Theta oscillations (4–12 Hz) are commonly observed during spatial learning and working memory tasks. The authors ask how etomidate, acting predominantly *via* γ-aminobutyric acid type A ($GABA_A$) receptors containing β_2 or β_3 subunits, affects theta activity *in vitro*.**

Methods: **To characterize the effects of etomidate, the authors recorded action potential firing together with local field potentials in slice cultures prepared from the neocortex of the β_3(N265M) knock-in mutant and wild type mice. Actions of etomidate were studied at 0.2 μM, which is approximately 15% of the concentration causing immobility (~1.5 μM).**

Results: **In preparations derived from wild type and β_3(N265M) mutant mice, episodes of ongoing activity spontaneously occurred at a frequency of approximately 0.1 Hz and persisted for several seconds. Towards the end of these periods, synchronized oscillations in the theta band developed. These oscillations were significantly depressed in slices from β_3(N265M) mutant mice ($P < 0.05$). In this preparation etomidate acts almost exclusively *via* β_2 subunit containing $GABA_A$ receptors. In contrast, no depression was observed in slices from wild type mice, where etomidate potentiates both β_2- and β_3-containing $GABA_A$ receptors.**

Conclusions: **At concentrations assumed to cause sedation and amnesia, etomidate depresses theta oscillations *via* β_2-containing $GABA_A$ receptors but enhances these oscillations by acting on β_3 subunit containing receptors. This indicates that the overall effect of the anesthetic reflects a balance between enhancement and inhibition produced by different $GABA_A$ receptor subtypes.**

RECENTLY, brain imaging and electrophysiological studies on human subjects provided evidence that general anesthetics predominantly affect cortical neurons when applied at low concentrations causing sedation and amnesia but not hypnosis or immobility.[1,2] Furthermore, Veselis *et al.*[3] have shown that the sedative and amnestic properties of intravenous anesthetics can be separated experimentally. These observations raise the possibility that different molecular substrates and different types of cortical population activities might be involved in anesthetic-induced amnesia and sedation.

* Research Assistant, † Graduate Student, ‖ Professor of Experimental Anesthesiology, Department of Anesthesiology, Section of Experimental Anesthesiology, University of Tuebingen, Tuebingen, Germany; ‡ Postdoctoral Fellow, § Assistant Professor of Molecular Neuropharmacology, Institute of Pharmacology and Toxicology, University of Zurich, Zurich, Switzerland.

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Address reprint requests to Dr. Drexler: Department of Anesthesiology, Section of Experimental Anesthesiology, University of Tuebingen, Schaffhausenstr. 113, D-72072 Tuebingen, Germany. Address electronic mail to: berthold.drexler@uni-tuebingen.de. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

In the past few years considerable progress has been achieved in understanding how γ-aminobutyric acid (GABA)-releasing interneurons participate in hippocampal and neocortical information processing. Specific classes of electrically and synaptically coupled interneurons form different types of inhibitory networks.[4–6] Moreover, these networks participate in different types of oscillatory activity.[7] Fast spiking γ-aminobutyric acid-mediated (GABAergic) basket cells, mainly innervating the soma of pyramidal cells, give rise to fast gamma oscillations. In the neocortex another class of interneurons, making synaptic contacts predominantly on the apical dendrites of pyramidal cells, generate theta oscillations.[5] Neocortical theta oscillations are prominent during delayed working memory tasks and during sensorimotor integration.[8–10] The hippocampal theta rhythm has been shown to be important for spatial learning.[11,12]

The anesthetic properties of etomidate are determined almost exclusively by actions on γ-aminobutyric acid type A ($GABA_A$) receptors containing β_2 or β_3 subunits. $GABA_A$ receptors with the $\alpha_1\beta_2\gamma_2$ combination make up more than 60% of $GABA_A$ receptors in the brain, whereas β_3 subunits are present in only 15–20% of all $GABA_A$ receptors. Experimental work elucidating possible roles of these receptor subtypes for general anesthesia *in vivo* has been reviewed recently.[13] We have previously reported that at small, subanesthetic concentrations etomidate reduces spontaneous action potential firing of cortical neurons in excised brain slices. Making use of β_3(N265M) knock-in mice we found that β_3-containing and—assuming that other potential targets of etomidate are irrelevant in this respect—β_2-containing $GABA_A$ receptors equally contribute to this effect.[14] Although this finding indicated that both $GABA_A$ receptor subtypes mediate relevant actions on the network level, their specific contribution remains to be elucidated. Here we focus on cortical theta oscillations, which have shown to be a neural correlate of memory formation and learning. We conclude from our results that $GABA_A$ receptors containing β_2 and β_3 subunits mediate opposing effects of etomidate on this type of cortical network activity.

Materials and Methods

Animals

Mice of both sexes homozygous for an asparagine to methionine point mutation at position 265 of the $GABA_A$ receptor β_3 subunit (N265M) and homozygous wild type controls on the same genetic background as described

previously (statistically 87.5% 129/SvJ; 12.5% 129/Sv) were used for this study.[14] 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.

Organotypic Slice Cultures

Neocortical slice cultures were prepared from 2- to 5-day-old mice as described by Gähwiler *et al.*[15,16] In brief, for the preparation of somatosensory cortex, animals were deeply anesthetized with halothane and decapitated. Cortical hemispheres were aseptically removed and stored in ice-cold Gey's solution. After removal of the meninges, 300-μm thick coronal slices were cut. Slices were transferred onto clean glass coverslips and embedded in a plasma clot. The coverslips were transferred into plastic tubes (Nunc) containing 750 μl of nutrition medium and incubated in a roller drum at 37°C. After 1 day in culture, antimitotics were added. The suspension and the antimitotics were renewed twice a week. Cultures were used after 2 weeks *in vitro*.

Electrophysiology

Extracellular recordings were performed in a recording chamber mounted on an inverted microscope. Slices were perfused with an artificial cerebrospinal fluid consisting of (in mM) NaCl 120, KCl 3.3, NaH_2PO_4 1.13, $NaHCO_3$ 26, $CaCl_2$ 1.8, and glucose 11. Artificial cerebrospinal fluid was bubbled with 95% oxygen and 5% carbon dioxide. Artificial cerebrospinal fluid-filled glass electrodes with a resistance of approximately 3 to 5 MΩ were positioned on the surface of the slices and advanced into the tissue until extracellular spikes exceeding 100 μV in amplitude were visible. All experiments were conducted at 34°C.

Preparation and Application of Test Solutions

Test solutions were prepared by dissolving etomidate (Janssen-Cilag, Neuss, Germany) in the artificial cerebrospinal fluid to yield the desired concentration. A closed, air-free system was used to prevent evaporation of the anesthetics.

Etomidate was applied *via* bath perfusion using syringe pumps (ZAK, Marktheidenfeld, Germany), connected to the experimental chamber *via* Teflon tubing (Lee, Frankfurt, Germany). The flow rate was approximately 1 ml/min. When switching from artificial cerebrospinal fluid to drug-containing solutions, the medium in the experimental chamber was replaced by at least 95% within 2 min. Effects on the spike patterns were stable approximately 5 min later. To ensure steady state conditions, recordings during anesthetic treatment were carried out 10 min after commencing the change of the perfusate.

Data Analysis

Data were acquired on a personal computer with the digidata 1200 AD/DA interface and Axoscope 9 software (Axon Instruments, Foster City, CA). The recorded signal was composed of fast action potentials and slow local field potentials (LFP)[17,18] separated from each other by digital band-pass filtering (200–2000 Hz for action potentials and 1–10 Hz for LFP). Extracellularly recorded spikes were counted offline using self-written programs in OriginPro7 (OriginLab Corporation, Northampton, MA). The mean of spikes occurring during a recording period of 180 s was used as the average spike rate. As firing occurred typically in bursts (episodes of ongoing activity, EOAs), we furthermore calculated the spike rate within a time window 200–1000 ms after the onset of the EOA.

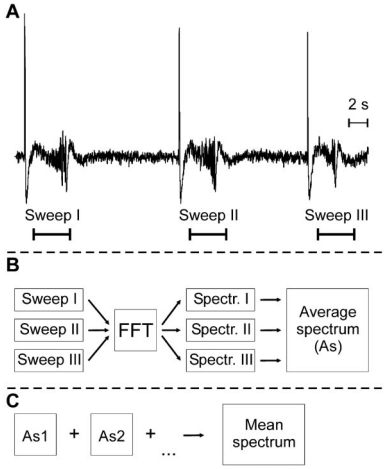

Fig. 1. Schema of analysis steps involved in calculating power-density spectra from local field potential (LFP) recordings. (*A*) First, epochs of fixed length (minimum 1 s) from the continuous LFP recording are cut, corresponding to episodes of ongoing activity. (*B*) The power-density spectrum for each epoch is calculated by fast Fourier transform and the individual spectra for a single 180-s recording are averaged to obtain the average spectrum (As) of this recording. (*C*) The mean spectrum is finally computed from all the average spectra obtained under the same condition (control, etomidate).

Computation of power-density spectra from the LFP is schematized in figure 1. First, EOAs were cut from the continuous LFP recording, starting where the first peak had decayed to zero. The appropriate window length (1–6 s) was chosen for the control recording of each culture to contain as much as possible of and only the EOA (fig. 1A). Power density between zero and 30 Hz in the EOAs was then determined by fast Fourier transform using the Pwelch method under Matlab 6.5 (MathWorks Inc., Natick, MA). The spectrum was first computed for individual Hanning windows at width 1 fitted to each sweep, with zero padding. The average over these windows was then taken as the power-density spectrum for each EOA. Subsequently an average spectrum for each 180 s recording was obtained by averaging the spectra of the individual EOAs (fig. 1B).

The mean spectrum was then calculated from all the spectra obtained under the same experimental condition (fig. 1C). We observed that small variations in electrode positioning resulted in pronounced differences in the

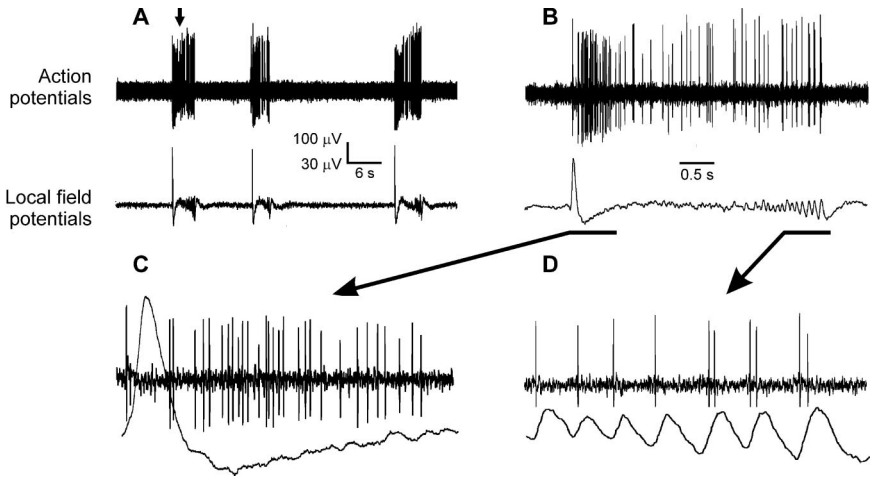

Fig. 2. Correlation of action potential firing (upper traces) and local field potentials (lower traces) shown at different temporal resolutions. (*A*) Three episodes of ongoing activity (EOAs) spontaneously occurring within 60 s of recording time. (*B*) The first EOA (arrow) is displayed at a higher time resolution. (*C*) Early phase of this EOA. Note the peak in the local field potential (LFP). (*D*) Late phase of the same EOA. Note the action potential firing at regular intervals and the corresponding oscillations in the LFP.

amplitudes of the recorded signal, making it difficult to compare the absolute amplitudes between different recordings. Therefore, all spectra were normalized to the total power density in their spectrum under control conditions. Data acquired in the presence of etomidate were processed in the same way.

Difference spectra were finally obtained by subtracting the mean control spectrum from the mean drug spectrum. We used analysis of variance and *post hoc* Student *t* test for statistical testing. All results are given as mean ± SEM.

Results

Patterns of Spontaneous Neuronal Activity in the Absence of Etomidate

Actions of etomidate were investigated in cultured brain slices derived from the somatosensory cortex of postnatal wild type and β_3(N265M) mutant mice. As in our previous studies, spontaneous neuronal activity was induced by removing Mg^{2+} ions from the extracellular solution.[19,20] In figure 2, data from a typical recording are presented: EOAs lasting approximately 3 to 7 s were separated by periods of neuronal silence (fig. 2A). During EOAs spontaneous action potential firing was accompanied by changes in the LFP (fig. 2B). EOAs exhibited a characteristic time structure: at the onset, neurons discharged at a high rate. Simultaneously a biphasic signal occurred in the LFP, exhibiting a large positive and a smaller negative peak (fig. 2C). In the following seconds neurons discharged at lower rates while the LFP was close to baseline, indicating the presence of uncorrelated action potential activity. Towards the end of EOAs an oscillatory component in the LFP developed, arising from progressive synchronization of neuronal activity. During this phase, action potentials appeared time locked to the rising phase of the oscillations in the LFP (fig. 2D).

In slices derived from wild type mice, EOAs occurred at a frequency of 0.128 ± 0.018 Hz (n = 51). In cultures from mutant mice the frequency of EOAs was 0.145 ± 0.017 Hz (n = 66). These mean values were not statistically different ($P > 0.5$). Similarly, the corresponding discharge rates, calculated from action potential activity during a time period of 180 s in slices from wild type and mutant mice, were not significantly different (7.88 ± 1.06 Hz and 10.29 ± 0.85 Hz, n = 49/67, $P > 0.05$). In summary, activity patterns monitored in slices from wild type and β_3(N265M) mutant mice did not differ under control conditions.

Effects of Etomidate on Action Potential Firing

In a recent study we have reported that etomidate depresses spontaneous action potential activity in slices prepared from wild type mice to a significantly larger extent than in slices derived from β_3(N265M) mutant mice.[14] Here, a more detailed analysis is provided on specifically how the anesthetic affects the discharge patterns. Throughout these studies, etomidate was applied at 0.2 μM. At this concentration neuronal activity was depressed in slices from wild type animals by 65% compared with 31% in slices derived from mutant mice.[14] Example recordings of the effect of etomidate are displayed in figure 3. Etomidate did not change the rate of EOAs (wild type: −11.92 ± 11.36%; mutant: −0.25 ± 6.45%; $P > 0.05$) but decreased action potential activity by reducing the discharge rate within EOAs.

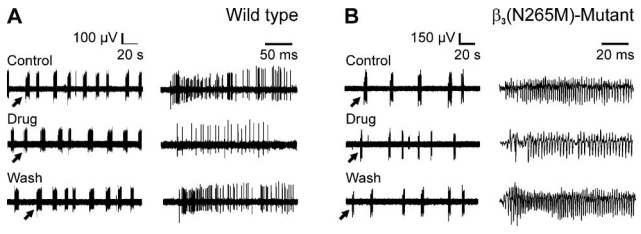

Fig. 3. Extracellular recordings of spontaneous action potential firing in slices from wild type (*A*) and β_3(N265M) mutant mice (*B*). Effect of 0.2 μM etomidate on the occurrence of episodes of ongoing activity (EOA, left section) and action potential firing within an EOA (right section). The number of EOAs was not changed by etomidate. Within a single EOA etomidate reduces action potential firing in the mutant to a lesser degree than in the wild type.

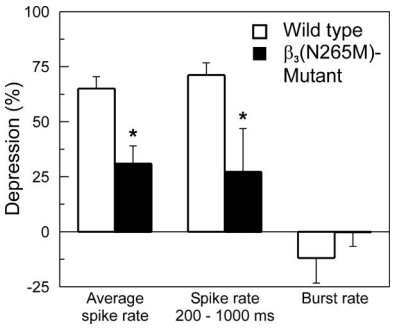

Fig. 4. Depression of spontaneous action potential firing by 0.2 μM etomidate in wild type (white) and β_3(N265M) mutant (black) preparations. Average spike rate (wild type 65.39 ± 5.42%; mutant 30.86 ± 8.15%; $P < 0.05$) and burst rate (wild type: −11.92 ± 11.36%; mutant: −0.25 ± 6.45%; $P > 0.05$) are counted over 180 s. Spike rate 200 to 1000 ms (wild type 71.24 ± 5.54% *versus* mutant 27.25 ± 19.6%; $P < 0.05$) is the discharge rate between 200 and 1000 ms after the beginning of a burst.

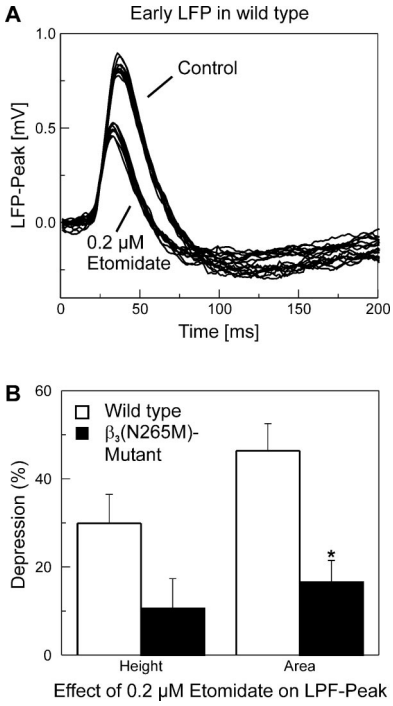

Fig. 5. (*A*) Recordings of the early local field potential (LFP) in wild type mouse preparations under control conditions and in the presence of 0.2 μM etomidate (n = 9). (*B*) Depression of the peak of the LFP at the beginning of an episode of ongoing activity. Height (not significant) and area under curve ($P < 0.05$, n = 9/11) for wild type (white) and β_3(N265M) mutant (black) preparations.

We further analyzed the time course of depressant action of etomidate within EOAs to find out whether synaptic inhibition mediated by β_3-containing $GABA_A$ receptors is homogeneous within EOAs. As described above the firing pattern is characterized by high firing rates at the immediate onset of an EOA. Therefore we quantified the etomidate-induced depression within the time window 200–1000 ms after the onset of EOAs. Similar values to the average spike rate were obtained when restricting the analysis to this time window (wild type 71.24 ± 5.54% *versus* mutant 27.25 ± 19.6%; $P < 0.05$; n = 9; fig. 4).

Effects of Etomidate on the Local Field Potential

Next we investigated the effect of etomidate on local field potentials separately for the onset (early LFP) and towards the end of EOAs. Etomidate decreased the peak amplitude of the early LFP in slices from wild type mice by 30% and in slices from mutant mice by approximately 10%. However, this difference did not reach statistical significance ($P > 0.05$). Furthermore, the anesthetic shortened the duration of the early LFP. To quantify this effect, we computed the area under the early LFP curve where it deviated from the baseline. This area was reduced by etomidate in slices from wild type mice to a larger extent than in slices from β_3(N265M) mutant mice ($P < 0.05$, n = 9/11; fig. 5).

Etomidate also had a prominent effect on the oscillatory activity developing towards the end of EOAs. To quantify these actions, power-density spectra of the LFP were computed as described above. In figure 6A the power spectra obtained from LFP recordings in slices from wild type (n = 9) and mutant mice (n = 8) in the absence of etomidate are displayed. A peak close to 6 Hz is observable in both types of preparations. Across all computed frequencies the averaged power densities were not significantly different ($P > 0.05$). This result provides further evidence that neuronal activity patterns monitored in slices derived from wild type and mutant mice did not differ under control conditions.

In preparations from wild type mice, etomidate is predicted to modulate $GABA_A$ receptors containing β_2 or β_3 subunits. The effect of etomidate on oscillatory activity in the LFP of the wild type is shown in figure 6B: the anesthetic enhanced power densities between 3 and 8 Hz. However, drug-induced amplification of oscillations did not reach statistical significance ($P > 0.05$). It is remarkable that in the wild type, but not in the β_3(N265M) mutant, effects of etomidate displayed a large variability, as indicated by the error bars in figure 6B. Possible variations in the expression of β_3 subunits, making up only 15–20% of all $GABA_A$ receptors could serve as an explanation.

In slices from β_3(N265M) mutant mice the effects of etomidate are largely restricted to $GABA_A$ receptors containing β_2 subunits, assuming that other potential targets are not relevant in this respect. The corresponding spectra are displayed in figure 6C: here, etomidate exhibited the opposite effect and significantly decreased power densities to between 3 and 8 Hz less than control values ($P < 0.05$).

To provide a direct comparison of the effects of etomidate observed in slices from wild type and mutant mice, difference spectra are displayed in figure 6D. The difference between the computed traces, most promi-

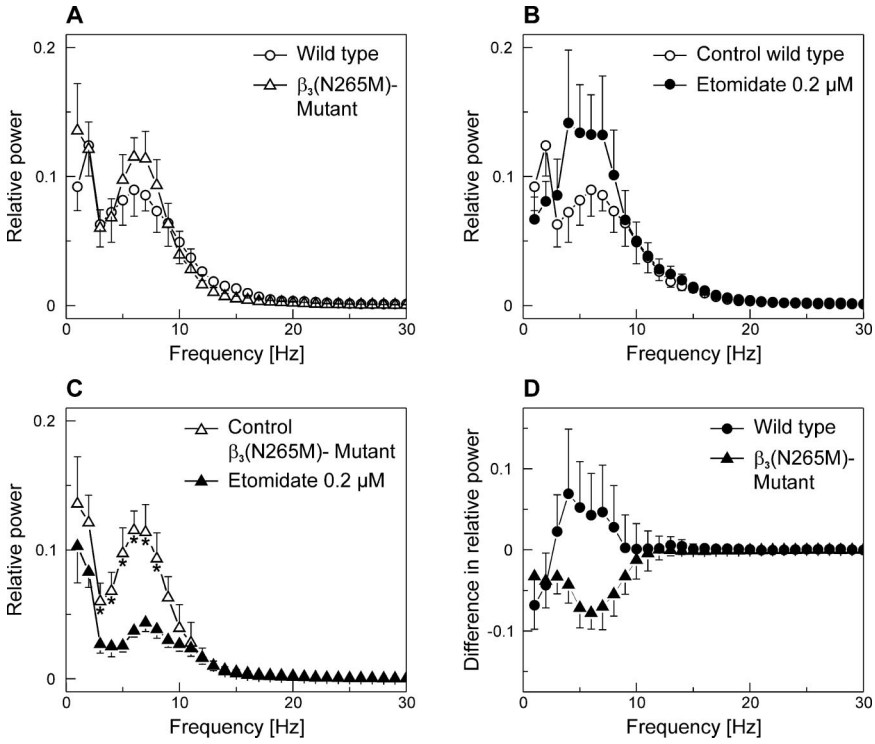

Fig. 6. Power-density spectrum of the local field potential; sample sizes are n = 9 for the wild type and n = 8 for β_3(N265M) mutant. (*A*) Power spectra for wild type (open circle) and β_3(N265M) mutant (open triangle) mouse preparations under control conditions. The averaged power densities with a peak close to 6 Hz were not significantly different between both types of preparation ($P > 0.05$). (*B*) The effect of 0.2 μM etomidate (solid circle) compared with control (open circle) in the wild type: etomidate enhances power between 3 and 8 Hz; however, statistical significance is not reached ($P > 0.05$). Note the large standard errors of the mean compared with the mutant preparation. (*C*) The effect of etomidate (solid triangle) compared with control (open triangle) in the β_3(N265M) mutant: the anesthetic decreases power between 3 and 8 Hz ($P < 0.05$). (*D*) The difference spectra are obtained by subtracting control from drug condition for both wild type and mutant. Etomidate exhibits opposing actions on theta range oscillations in wild type (solid circle) and β_3(N265M) mutant (solid triangles), most prominent between 3 and 8 Hz. Note the different scale bar compared with (*A*)–(*C*).

nent between 3 and 8 Hz, is apparently produced exclusively by $GABA_A$ receptors containing β_3 subunits.

Discussion

Estimates of Clinically Relevant Concentrations

In the current study we characterized subunit specific actions of 0.2 μM etomidate on neocortical neurons. To judge the relevance of our results for clinical anesthesia, it is necessary to answer the question of whether the anesthetic concentration tested here falls within the clinically relevant range.[21] Dickinson *et al.*[22] proposed that an aqueous concentration of 1.5 μM etomidate should be close to the EC_{50} value for lack of responses to painful stimuli. This concentration represents an uppermost limit because amnesia, sedation, and hypnosis—concordant with a significant depression of cortical neurons *in vivo*—are achieved with considerably smaller concentrations. Unfortunately, quantitative estimates on etomidate blood concentrations producing amnesia, sedation, and hypnosis are lacking. However, we hypothesize that at 0.2 μM, which is approximately 15% of the concentration causing immobility, the drug might produce amnesia and sedation *in vivo*.

Comparison to Related In Vitro *Studies on Gamma and Theta Oscillations*

Dickinson *et al.*[22] quantified the effects of etomidate on gamma oscillation in hippocampal brain slices. These investigators report a 30% decrease in oscillation frequency at 2 μM, which is 10-fold higher than the concentration used in the current work. At first glance, this difference in the effective concentrations is surprising. However, the effects of etomidate on different forms of network activity, namely γ-and θ-range oscillations, have been investigated in the study of Dickinson *et al.*[22] and the current work. The frequency of hippocampal gamma oscillations is largely determined by the decay time of $GABA_A$ receptor mediated inhibitory postsynaptic currents.[23,24] Interestingly, Lukatch and MacIver have demonstrated that this is also true for slower neocortical theta oscillations.[18] An important difference between these rhythms is defined by the kinetic properties of $GABA_A$ receptor-mediated inhibitory postsynaptic currents: inhibitory postsynaptic currents involved in gamma oscillations have a fast decay time of 10–15 ms compared with inhibitory postsynaptic currents involved in theta oscillations (approximately 100–150 ms).[18,23] Obviously, different subtypes of the $GABA_A$ receptor participate in γ- and theta oscillations. It seems possible that subtypes of the $GABA_A$ receptor, highly sensitive to etomidate, are part of the mechanism producing theta oscillations but of minor importance for gamma oscillations.

Time Course of $GABA_A$ Receptor-mediated Inhibition

Cortical GABAergic interneurons do not exhibit unique firing properties. Although low-threshold-spiking neurons are activated by moderate depolarizing inputs, fast-spiking neurons require a considerable stronger excitation to generate action potentials.[5,25] Therefore, the latter cells are expected to be active predominantly

during the early phase of EOAs when cortical neurons discharge at high rates. Because $GABA_A$ receptor subtypes show specific patterns of subcellular distribution, activation of specific $GABA_A$ receptor subtypes should be coupled to the firing of specific types of GABAergic interneurons, which selectively project onto the dendrite, the soma, or axon initial segment of pyramidal cells.[26] Thus it seemed possible that β_2-containing and β_3-containing $GABA_A$ receptors are predominantly activated within specific time windows during EOAs. Therefore we quantified the effect of the drug on the discharge rate during the early phase of EOAs separately. However, we found that the fraction of inhibition mediated *via* β_3 subunits in the early phases of EOAs compared to the average depression did not differ (fig. 4). Thus from our current results no conclusions can be drawn regarding what type of interneurons activate β_2- or β_3-containing $GABA_A$ receptors.

Impact of β_3 Subunit Containing Receptors in Mediating the Depressant Effects of Etomidate

The data summarized in figures 4 and 5 clearly indicate that approximately 50% of the overall depressant effect of etomidate on action potential firing and the early peak in LFP are mediated by β_3-containing $GABA_A$ receptors. This result is somewhat surprising because β_3 subunit-containing receptors clearly constitute a minor fraction of all $GABA_A$ receptors. However, their high impact on neuronal activity can be explained by the slower decay kinetics of functional $GABA_A$ receptors containing β_3 subunits compared with receptors possessing β_2 subunits.[27,28] Furthermore, there is evidence that β_3 receptors are predominantly located on pyramidal cells whereas β_2 receptors are also found on GABAergic interneurons.[26,29] Inhibition of GABAergic neurons *via* β_2-containing receptors should decrease GABA release on pyramidal cells and therefore increase their excitability. Finally, the location at strategically important sites such as the axonal hillock should make a considerable difference with regard to the impact of specific $GABA_A$ receptor subtypes. In conclusion, physiologic impact and relative abundance of diverse $GABA_A$ receptor subtypes may largely differ.

Opposing Action of β2 and β3 Subunit Containing Receptors in Mediating the Depressant Effects of Etomidate

A large body of evidence indicates that theta activity has a central role in hippocampal learning.[12,30] More recently, it has been shown that neocortical neurons display theta activity during working memory tasks.[8–10] However, although data suggesting that cortical theta activity is an important neuronal correlate of memory and learning processes are continuously accumulating, causal relationships are far from being understood. This is also true for the effects of anesthetic agents on learning and memory, although interest in this area of research is increasing. Surprisingly, anesthetic drugs diminish working memory performance when administered during a learning task, whereas post-training exposure to anesthetic agents may improve learning.[31,32] Obviously, the effect of a single anesthetic strongly depends on the particular neuronal process that is affected. It is therefore important to investigate how specific types of network activity are affected by anesthetic agents.

In the current study we have provided evidence that β_2-containing and β_3-containing $GABA_A$ receptors exhibit opposing actions on theta activity in neocortical slice cultures. Overall enhancement of this kind of network activity results from balanced activation of these $GABA_A$ receptor subtypes, as indicated by the results displayed in figure 6. The involvement of different $GABA_A$ receptor subtypes in mediating specific actions on cortical neurons might also help to explain the finding of Veselis *et al.*[3] that at concentrations producing a similar degree of sedation, anesthetic drugs acting predominantly *via* $GABA_A$ receptors display different potencies in affecting working memory performance of human subjects. Taken together, these results raise the possibility that different subtypes of cortical $GABA_A$ receptors contribute in different ways to the sedative and amnestic properties of intravenous anesthetics.

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