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Determination of the EC_{50} Amnesic Concentration of Etomidate and Its Diffusion Profile in Brain Tissue

Implications for In Vitro *Studies*

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Background: **Etomidate is a widely used general anesthetic that has become a useful tool to investigate mechanisms of anesthetic action *in vivo* and in brain slices. However, the free aqueous concentration of etomidate that corresponds to amnesia *in vivo* and the diffusion profile of etomidate in brain slices are not known.**

Methods: **The authors assessed the effect of intraperitoneally injected etomidate on contextual fear conditioning in mice. Etomidate concentrations in brain tissue were obtained by high-performance liquid chromatography. Uptake studies in 400-μm-thick brain slices were used to calculate the diffusion and partition coefficients of etomidate. A diffusion model was used to calculate the expected concentration profile within a brain slice as a function of time and depth. The predicted rate of drug equilibration was compared with the onset of electrophysiologic effects on inhibitory circuit function in recordings from hippocampal brain slices.**

Results: **Etomidate impaired contextual fear conditioning with an ED_{50} dose of 11.0 ± 0.1 mg after intraperitoneal injection, which corresponded to an EC_{50} brain concentration of 208 ± 9 ng/g. The brain:artificial cerebrospinal fluid partition coefficient was 3.35, yielding an $EC_{50,amnesia}$ aqueous concentration of 0.25 μM. The diffusion coefficient was approximately 0.2×10^{-6} cm^2/s. The development of etomidate action in hippocampal brain slices was compatible with the concentration profile predicted by this diffusion coefficient.**

Conclusions: **The free aqueous concentration of etomidate corresponding to amnesia, as defined by impaired contextual fear conditioning in mice, is 0.25 μM. Diffusion of etomidate into brain slices requires approximately an hour to reach 80% equilibration at a typical recording depth of 100 μm. This information will be useful in designing and interpreting *in vitro* studies using etomidate.**

ETOMIDATE [R-1-ethyl-1-(a-methylbenzyl)-imidazole-5-carboxylate] is an ultrashort-acting nonbarbiturate intravenous anesthetic that is widely used as an induction agent for general anesthesia. The standard induction dose rapidly produces hypnosis and immobility,[1] and lower doses provide sedation.[2,3] The ultrashort action of etomidate results from redistribution from the brain to other tissues and its high clearance by ester hydrolysis in the liver and the plasma.[4–6]

A large body of evidence suggests that etomidate acts by enhancing the response of γ-aminobutyric acid (GABA) type A receptors ($GABA_A$Rs) to GABA, or by direct activation of these receptors.[7–9] $GABA_A$Rs are the major inhibitory neurotransmitter receptors in the brain and are considered to be prime anesthetic target sites.[7–9] In recombinant $GABA_A$Rs, etomidate acts as a positive modulator at low concentrations (0.1–1 μM), directly activates receptors at intermediate concentrations (> 5–10 μM),[10–13] and inhibits receptor function at yet higher concentrations (> 50 μM).[10,14] Similar findings of potentiation at low concentrations and direct activation and block at higher concentrations of etomidate were recently published for native $GABA_A$Rs in the spinal cord.[15,16] These effects, in conjunction with its parallel *in vitro* and *in vivo* enantioselectivity, and its reduced effects in gene-targeted mice carrying mutations that render some $GABA_A$Rs insensitive to etomidate, strongly implicate the $GABA_A$R as the molecular target responsible for its anesthetic actions.[17,18]

Modulation and direct activation of $GABA_A$Rs by etomidate depend on subunit composition. Etomidate displays functional selectivity and greater potency and efficacy for β_2 and β_3 subunits over β_1 subunits.[10,12–14] This characteristic separates etomidate from many other anesthetics, in particular volatile agents, which are far more promiscuous with respect to their molecular target sites. Despite this considerable body of knowledge, we have yet to determine how the molecular interactions of etomidate with β_2 and β_3 subunit–containing $GABA_A$Rs alter cellular function and integrative properties of neural networks to cause sedation and amnesia, hypnosis (unconsciousness), and immobility.[19]

The hippocampal brain slice is commonly used to study anesthetic effects on integrative electrophysiologic function. Beyond its use as a model system, anesthetic modulation of hippocampal function may itself contribute to their ability to impair learning and memory.[20–22] In addition to mechanical stability and intact circuitry, this preparation offers several other advantages over *in vivo* electrophysiologic recordings. These include the ability to precisely control the ionic milieu and to deliver pharmacologic agents at known concentra-

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tions. For studies of anesthetic effects, drug concentrations at tissue sites of action would ideally be the same as those that produce behavioral effects *in vivo*. To accomplish this, the drug should be applied at the appropriate free concentration and for a duration that assures equilibration of brain tissue with the superfusion medium. In contrast to other anesthetics,[23,24] few studies have measured the *free* etomidate concentrations that correspond to specific behavioral endpoints,[25] and no studies have determined the *free* concentrations that produce amnesia. In addition, the diffusion coefficient for etomidate within brain slice tissue, and thus the duration for which etomidate must be applied before steady state is achieved, remain unknown. We recently showed that concentration–time–depth profiles within brain slices can vary substantially for different types of anesthetics because of their distinct physicochemical properties and that these details must be considered when designing or interpreting *in vitro* experiments.[26,27]

We therefore performed the current study to answer two questions: (1) What free aqueous concentration of etomidate *in vitro* corresponds to amnesia *in vivo*? (2) What is the rate of diffusion of etomidate within a brain slice? The amnesic properties of etomidate were assessed using contextual fear conditioning. Tissue uptake measurements, extraction, high-performance liquid chromatography, and computational modeling allowed us to determine the partition and diffusion coefficients of etomidate, and thus to determine the $EC_{50,amnesia}$ of etomidate and calculate the expected tissue concentration as a function of time and depth in a brain slice preparation during drug application. We compared these predictions with electrophysiologic measurements of the effect of etomidate on hippocampal inhibitory network properties in acute brain slice preparations.

Materials and Methods

All experiments were conducted according to the guidelines laid out in the *Guide for the Care and Use of Laboratory Animals* [28] and were approved by the Committee on Animal Research of the University of California, San Francisco, California, and by the University of Wisconsin Animal Care and Use Committee, Madison, Wisconsin.

Mice

Animals used in the current study were all hybrid wild-type mice. For behavioral analyses, determination of brain etomidate concentrations, and brain slice uptake studies, 129/SvJ × C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME). Electrophysiologic recordings were performed with mice that either had the same genetic background (129/SvJ × C57BL/6J), kindly provided by Gregg Homanics, Ph.D. (Associate Professor of Anesthesiology and Pharmacology, University of Pittsburgh, Pittsburgh, PA), or were 129/SvJ × 129/Sv hybrid mice, kindly provided by Uwe Rudolph, Ph.D. (Assistant Professor of Molecular Neuropharmacology, University of Zurich Medical School, Zurich, Switzerland). In the latter case, the embryonic stem cell was a 129/SvJ × 129/Sv hybrid and crossed with 129/SvJ mice. The theoretical contribution of the 129SvJ substrain is therefore 87.5% and that of the 129/Sv substrain is 12.5% in the second generation. Breeding pairs resulting from two generations of homozygous breedings were transferred from Zurich to Madison. The first generation offspring of these pairs provided the subjects for the current studies. Analysis of electrophysiologic recordings did not reveal any differences between the two hybrid wild-type strains, and data were therefore pooled. Animals were housed in the animal care facility under 12-h cycles of light and dark and had continuous access to standard mouse chow and water.

Anesthetic Behavioral Analysis I: Determination of Drug Equilibrium after a Single Injection of Etomidate

A single injection of etomidate results in a rapid peak in brain concentration followed by a rapid redistribution to the other tissues and then a steady slow decline in plasma levels due to ester hydrolysis.[6] During this slow decline, the brain and blood compartments are in quasi-equilibrium, so brain concentrations also change slowly during this time.[5,29] We desired to test the amnesic potential of etomidate when brain concentrations were changing only slowly but were still sufficient to produce substantial neurologic impairment. To identify the appropriate time window, we tested the effect of etomidate administration on open field activity after an intraperitoneal injection. For these experiments, 3- to 4-month-old animals (male and female) were placed in an open plastic chamber and allowed to move freely. All experiments were performed between 08:00 and 14:00 h. After an initial observation period of 30 min, animals were injected either with a single dose of etomidate (10 mg/kg) or saline (sham-injected controls). Afterward, mice were returned to the same chamber and observed for 60 min after injection. For behavioral analysis, they were videotaped, and their behavior was scored either off-line or in parallel with the recording. An independent observer classified the behavior as "exploring" (locomotor activity), "immobile," "grooming" (including face and paw washing), or "undefined" (none of the previous categories). Data analysis was performed with custom routines written in Matlab (The MathWorks Inc., Natick, MA).

Anesthetic Behavioral Analysis II: Contextual Fear Conditioning

Experiments were performed on 3- to 4-week-old animals (male and female) and conducted between 09:00 and 15:00 h. Using a pavlovian approach, we determined the dose of etomidate administered intraperitoneally required to reduce freezing to context by 50% as a measure for impaired learning and memory. Techniques applied for the determination of the effect of anesthetics on learning and memory have been described previously.[30,31] Mice were injected only once with a single dose of etomidate ranging from 5 to 15 mg/kg. Control animals (n = 8) received injections of the vehicle used to deliver etomidate (35% propylene glycol in saline). Training for contextual fear conditioning took place 25–30 min after injection, which observations of open field activity had demonstrated to be during the middle of the slow recovery phase. Therefore, 25 min after injection of a single dose of etomidate, each animal was rapidly transferred to a training chamber and allowed to explore the chamber for 3 min before training began. Subsequently, animals received three 2-s electric foot shocks (11-Hz bipolar square waves) that were 90 s apart. Within 60 s after the last shock, animals were returned to their home cages. The shock currents were 1 mA at all etomidate doses. The next day, we assessed freezing to context. Each mouse was transferred to the identical chamber in which it was trained the previous day and was observed for 8 min. Shocks were not administered. Four animals were observed simultaneously, one in each of the four chambers *via* a video camera. No personnel were in the context testing room during testing. To score freezing to context, an observation of one of the four animals was made every 2 s. Therefore, each animal was scored once every 8 s. Behavior was judged as freezing if there was no visible movement except for breathing.[32] The percentage of time an animal froze during the 8-min observation period was calculated as the number of observations judged to be freezing divided by the total number of observations in 8 min, *i.e.*, 60 observations.[32] For each group score at a given etomidate dose, the mean and SEM were calculated. Nonlinear regression was performed to calculate a 50% effective dose (ED_{50}), the Hill coefficient (n), and the maximal value (A) of the dose–response curve for fear conditioning according to the equation

$$\text{Freeze Score} = A \times \left(1 - \frac{\text{etomidate}^n}{ED_{50}^n + \text{etomidate}^n}\right).$$

Goodness of fit was evaluated by using the Levenberg-Marquardt algorithm, and X^2 minimization in combination with the R^2 value. Learning was defined as impaired if the group score differed from the corresponding unanesthetized (vehicle-injected) trained group.

Brain Slice Tissue Preparation

For electrophysiologic recordings, mice were decapitated under deep isoflurane and ketamine anesthesia, and the heads were immediately immersed in ice-cold (1°–2°C) dissection buffer consisting of 127 mM NaCl, 1.88 mM KCl, 1.21 mM KH_2PO_4, 26 mM $NaHCO_3$, 1.44 mM $MgSO_4$, 2.17 mM $CaCl_2$, 2.5 mM ascorbic acid, 5 mM kynurenic acid, and 10 mM glucose saturated with 95%O_2–5%CO_2. With the brain immersed, a block of tissue containing both hippocampi was dissected out at an angle of approximately 15° off the frontal plane to allow for optimal visualization of the cortical laminae, and the tissue was fixed to a tray with cyanoacrylate glue. Transverse slices (400 μm) were cut with a vibrating microtome (Leica VT 1000S; Leica Microsystems Nussloch GmbH, Nussloch, Germany) and allowed to recover in dissection buffer at room temperature (22°–24°C) for at least 1 h. Subsequently, slices were held submerged for another hour at room temperature in carbogen-saturated artificial cerebrospinal fluid (ACSF) (pH 7.4, 290–300 mOsm), before transfer to the recording chamber. ACSF was identical to dissection buffer, except that ascorbic acid and kynurenic acid had been omitted.

Determination of Brain Etomidate Concentrations by Extraction and High-performance Liquid Chromatography

To determine the etomidate concentration present in the brain 30 min after injection (thus at the time during which mice were subjected to training to contextual fear conditioning), another set of animals was injected each with a single bolus of the same etomidate doses used for the behavioral analyses, and brain etomidate concentrations were measured by high-performance liquid chromatography.[33] For these experiments, 30 min after injection, animals were decapitated, and the brains were quickly removed and weighed. Brain tissue was immediately homogenized in pentane (2 ml) after the addition of sodium fluoride (60 μl aqueous solution, 10 mg/ml). After separation of pentane, the extraction was repeated twice, each time with 2 ml pentane. Pooled pentane phase was evaporated under nitrogen. The residue was dissolved in 100 μl of the eluent, and 40 μl of the eluent was injected into the column (Agilent Zorbax Eclipse XDB-C18; 2.1 × 150 mm; 3.5 μm) of an Agilent 1100 Series LC system equipped with a quaternary pump and an ultraviolet detector (Agilent Technologies, Palo Alto, California). The detection wavelength was 242 nm. An isocratic mobile phase consisting of 25:25:50 (vol/vol) of acetonitrile:methanol:25 mM phosphate buffer (pH 8.1) was used at a flow rate of 0.25 ml/min. Standard curves were generated by spiking blank tissue samples with known amounts of etomidate and plotting the peak area against the concentration. We separately determined in spiked samples of brain that we had full recovery of

etomidate, so it was not necessary to use an internal standard in quantifying etomidate concentrations. Data were analyzed using Microsoft Excel (Microsoft, Redmond, WA) and Origin 6.1 (MicroCal, Northampton, MA).

Etomidate Uptake Experiments

Isoflurane-anesthetized mice were decapitated and the brains rapidly removed and placed in iced ACSF. Slices were prepared with Tissue Slicer (Siskiyou, Inc., Grants Pass, OR). Single 400-μm-thick brain slices were placed in 200-ml flasks containing ACSF plus 0.8 mg/l (3.27 μM) etomidate that had been preequilibrated with carbogen gas. Flasks were placed in a covered container with a humidified carbogen atmosphere, and the assembly was gently agitated using a clinical rotator (Fisher Scientific, Pittsburgh, PA) to ensure that the slices remained suspended in solution so that etomidate diffused into both tissue surfaces. Slices remained in solution 0.67–360 min. At a given test interval, a slice was removed from solution, blotted to remove excess ACSF, weighed, and transferred to a test tube for extraction and analysis as described above for whole brain.

Modeling Concentration–Time–Depth Profiles

The concentration–depth profile for diffusion into a brain slice was modeled using a one-dimensional diffusion equation.[34] This model was chosen because of the geometry of the brain slices, which were approximately $10 \times 10 \times 0.4$ mm thick. The model is given in dimensionless terms by the equation

$$Y = \sum_{j=1}^{\infty} \left(\frac{(-2(-1)^j)}{[(2j-1)/2]\pi} e^{-[(2j-1)\pi/2]^2\tau} \cos\left[\left(\frac{2j-1}{2} \right) \pi \cdot n \right] \right), \quad (1)$$

where

$$Y = \frac{C_f - C}{C_f} \quad (2)$$

$$n = x/x_o \quad (3)$$

$$\tau = \frac{D \cdot t}{x_o^2}. \quad (4)$$

D is the diffusivity, x_o is half of the slice thickness (200 μm), x is the depth within the slice, C is the concentration in the slice at time t, and C_f is the equilibrium concentration in the slice.

Electrophysiologic Experiments

Brain slices were prepared as described above and, after another hour of recovery in ACSF, were transferred to a recording chamber. Carbogen-saturated ACSF flowed through the recording chamber at a rate of 2.8 ml/min. Experiments were performed at room temperature (22°–24°C) on the stage of an upright microscope (BX50WI; Olympus, Melville, NY) equipped with a 10×, 0.25 NA Olympus objective. Pipettes were fabricated from borosilicate glass (1.7 mm OD, 1.1 mm ID; KG-33, Garner Glass, Claremont, CA) using a two-stage puller (Flaming-Brown model P-87; Sutter Instruments, Novato, CA). Pipette tips were fire polished and had open tip resistances of 2–4 MΩ when filled with ACSF. Bipolar stimulating electrodes were fabricated from tungsten (Microelectrodes Tungsten; World Precision Instruments, Sarasota, FL). The experimental setup for the "conditioned depression" paradigm was as follows: One pair of stimulating electrodes was placed in the alveus to activate recurrent (feedback) inhibition (conditioning pulse), whereas a second pair was placed in stratum radiatum to evoke population responses in CA1 pyramidal neurons by activating Schaffer collateral inputs. The recording electrode was placed in the CA1 layer. Conditioned responses were obtained at interstimulus intervals ranging from 5 to 1,000 ms, in the absence (control) and presence of etomidate (1 μM) and compared with the unconditioned response, which was the population spike evoked without previous alveus stimulus, or for some experiments the response at an interstimulus interval of 2,000 ms. Current pulses (0.1 ms duration) were delivered *via* constant current stimulus isolators (model A365D; World Precision Instruments) at a stimulus rate of 0.05 Hz and were adjusted throughout the course of the experiment such that alveus stimulation (200–800 μA) elicited supramaximal and stratum radiatum stimulation (60–250 μA) elicited half-maximal responses. All recordings were obtained in current clamp mode using an Axopatch 200B patch clamp amplifier (Axon Instruments, Union City, CA) and pClamp 8.0 software (Axon Instruments). Field potentials were amplified (×500), low-pass filtered at 5 kHz using internal amplifier circuitry, sampled at 10 kHz (Digidata 1200; Axon Instruments), and stored on the hard disk of a Pentium-based computer. For data analysis, ClampFit 8.0 (Axon Instruments), Origin 6.1, Microsoft Excel, Prism 4.0 (GraphPad, San Diego, CA), and custom written Matlab 6.5.1 routines were used.

Chemicals and Drugs

All chemicals were obtained from Sigma (St. Louis, MO). Ultrapure water was purified with a Millipore Milli-Q® system (Billerica, MA) and used to prepare all solutions. Isoflurane was purchased from Abbott Laboratories (Abbott Park, IL), and ketamine HCl was from Lloyd Laboratories (Shenandoah, IA). Etomidate as a 0.2% (wt/vol) solution dissolved in propylene glycol (35% vol/vol) was obtained from Bedford Laboratories (Bedford, OH).

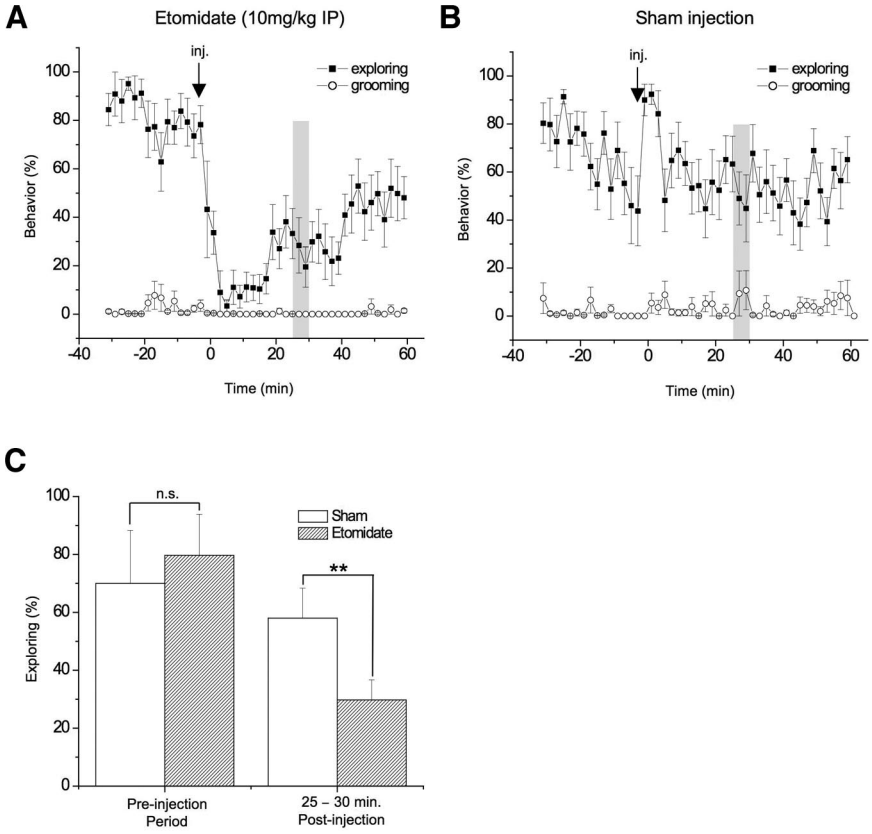

Fig. 1. Time course of the effect of etomidate and sham (saline) injections on locomotor activity. (*A*) Effect of etomidate (10 mg/kg intraperitoneal) on exploratory and grooming behavior. The 25- to 30-min postinjection period (*gray bar*) was chosen as an appropriate time window for further experiments. (*B*) Effect of saline injection on exploratory and grooming behavior in the control (sham-injected) group. (*C*) Levels of exploratory behavior did not differ significantly in etomidate- *versus* sham-injected animals during the 30-min preinjection period ($P > 0.05$, unpaired *t* test), but locomotor activity was significantly reduced during the 25- to 30-min postinjection period (n = 11 for etomidate and n = 10 for sham-injected animals; ** $P < 0.001$, unpaired *t* test). IP = intraperitoneal; n.s. = not significant.

Statistical Analysis

Results are reported as mean ± SD. Unpaired *t* tests were used to assess differences between etomidate- and sham-injected animals in open field activity experiments. One-way analysis of variance followed by Dunnett *post hoc* test or F test as appropriate was used to compare results from intraperitoneally injected *versus* control animals, with a level of $P < 0.05$ considered to be statistically significant.

Results

Time Course of the Effect of Etomidate on Open Field Activity

Mice were allowed to move freely (open field activity) for 30 min before and for 60 min after intraperitoneal injection of etomidate (10 mg/kg) and were observed for "exploring," "immobile," "grooming," or "undefined" behavior. "Exploring" and "immobile" comprised more than 90% of the total behavior, whereas "grooming" varied between 0 and 10% among animals. Because "exploring" and "immobile" showed an inverse relation and "undefined" behavior contributed less than 2% to the total behavior, only changes in "exploring" and "grooming" are shown (fig. 1). Animals showed the highest level of exploration during the first 10 min after their transfer to the recording chamber, after which locomotor activity declined to a steady lower level (figs. 1A and B). Injection of etomidate (10 mg/kg intraperitoneal) induced complete immobility within 3–5 min. Approximately 50–60 min after injection, levels of exploration had returned to approximately 50% (fig. 1A) and as such were indistinguishable from baseline behavior in sham-injected animals. In the latter, injection of saline (sham injection) briefly increased locomotor activity for several minutes before exploratory behavior returned to a steady state level contributing to approximately 50% of total behavior (fig. 1B). Although sham- and etomidate-injected animals did not differ in exploratory behavior during the 30-min preinjection period (70.0 ± 5.8% [n = 11] *vs.* 79.7 ± 4.5% [n = 10]; $P > 0.05$, unpaired *t* test), locomotor activity was significantly reduced in etomidate-injected *versus* control animals 25–30 min after injection (58.0 ± 4.6% [n = 11] *vs.* 29.8 ± 3.1% [n = 10]; $P < 0.001$, unpaired *t* test) (fig. 1C). Therefore, we chose the middle of this slow recovery phase, 25–30 min after injection (gray bar, fig. 1A), as an appropriate time window for further experiments, because we expected brain concentrations to change only slowly during this time period.

Etomidate-induced Learning Impairment

We assessed the effect of a range of doses of etomidate on learning using contextual fear conditioning 25–30 min after injection (fig. 2). In the absence of etomidate (vehicle-injected controls), training in-

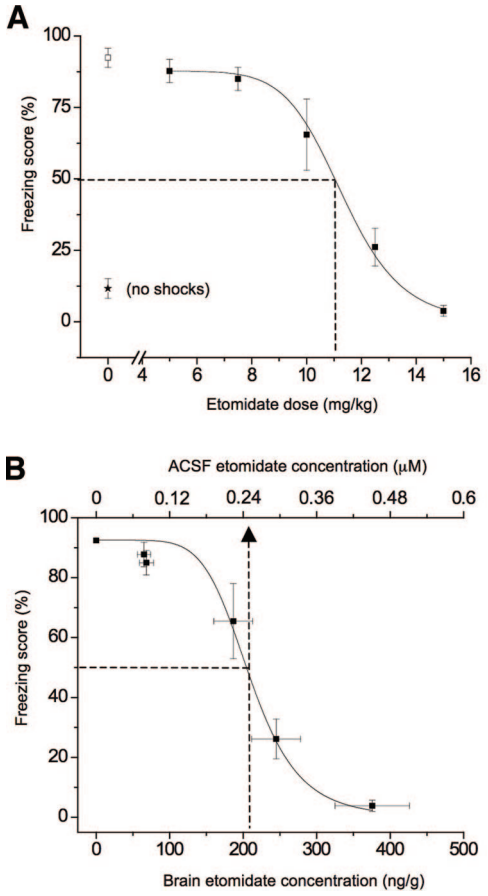

Fig. 2. Effect of etomidate on learning and memory, as assessed by contextual fear conditioning. (*A*) Etomidate administered by intraperitoneal injection 30 min before the training session resulted in a dose-dependent reduction of freezing to context (ED_{50}: 11 ± 0.1 mg). (*B*) Etomidate-induced learning impairment plotted as a function of the brain etomidate concentration (*lower axis*) and corresponding artificial cerebrospinal fluid (ACSF) concentration (*upper axis*).

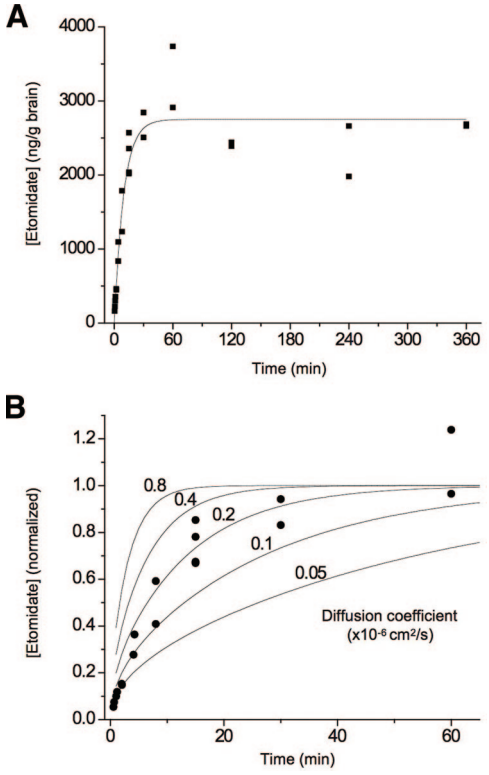

Fig. 3. Partition and diffusion coefficients for etomidate in brain tissue. Brain slices (400 μm) were incubated in artificial cerebrospinal fluid containing 3.27 μM etomidate for durations ranging from 0.67 to 360 min. The total amount in each slice was measured by extraction and high-performance liquid chromatography analysis. Each data point represents the average of duplicate measurements within the same slice. (*A*) The data were fitted by a monoexponential function with a time constant of 9.5 min. The steady state concentration of etomidate, 2,682 ± 155 ng/g brain, yielded a partition coefficient of 3.35 ± 0.18. (*B*) Uptake data obtained during the first 60 min of incubation conformed most closely to a diffusion coefficient of D = 0.2 × 10^{-6} cm^2/s.

duced 92.4 ± 3.3% (n = 8) freezing to context. In comparison, untrained mice froze 11.7 ± 3.2% (n = 13) of the time during examination. Intraperitoneal injection of etomidate (5–15 mg/kg) resulted in a dose-dependent reduction of freezing to context, with an ED_{50} dose of 11 ± 0.1 mg (fig. 2A). Etomidate doses higher than 12.5 mg/kg resulted in behavioral responses that were indistinguishable from untrained animals ($P > 0.05$, one-way analysis of variance). High-performance liquid chromatography analysis of etomidate concentrations in the brain showed that this ED_{50} amnesic dose corresponded to an EC_{50} brain concentration of 208 ± 9 ng/g brain (fig. 2B).

Determination of the EC_{50} Concentration of Etomidate for Amnesia

To establish the aqueous (free) concentration of etomidate corresponding to the brain concentration that impairs learning, we determined the partition coefficient by measuring the ratio of etomidate concentration in ACSF and in 400-μm-thick brain slices exposed to ACSF containing 0.8 mg/l (3.27 μM) etomidate in 35% propylene glycol for up to 360 min. Results from a total of 24 such measurements revealed that the concentration in the brain slice reached a plateau after 30–60 min of equilibration (fig. 3A). In some samples, the concentration slowly declined after 60 min, perhaps because of a change in tissue characteristics (deterioration) during prolonged incubation. The final equilibrium concentration used to determine the partition coefficient was taken as the average concentration from 30 to 360 min. This yielded a value of 2,682 ± 155 ng/g brain (fig. 3A). Taking into account the molecular weight of etomidate (244.3), the density of brain tissue (1.04),[35] and the concentration of etomidate in ACSF (3.27 μM), this concentration corresponds to a brain:ACSF partition coefficient of 3.35. Therefore, the ACSF concentration that corresponds to a 50% reduction in freezing to context *in vivo*, *i.e.*, $EC_{50,amnesia}$, is 0.25 μM (fig. 2B).

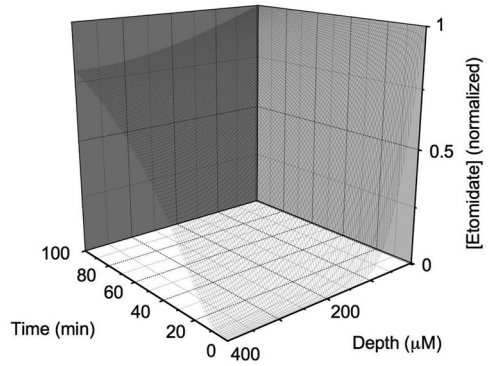

Fig. 4. Model of the concentration profile of etomidate as a function of time and depth in a 400-μm-thick brain slice (top perfused) based on a diffusion coefficient of D = 0.2 × 10^{-6} cm^2/s. The concentration is seen to rise rapidly near the perfused surface of the slice (depth = 0 μm), but the concentration at the deepest sites (400 μm) has reached only approximately 80% of the final equilibrium level after 100 min.

Determination of the Diffusion Coefficient of Etomidate and Its Concentration–Time–Depth Profile in Brain Slices

To determine the diffusion coefficient of etomidate in brain slices, we plotted the uptake data together with the expected average tissue concentrations corresponding to a variety of diffusion coefficients, which we calculated using a one-dimensional model of diffusion into both surfaces of a 400-μm-thick tissue slice (equation 1). The rate of uptake corresponded to a diffusion coefficient of approximately 0.2 × 10^{-6} cm^2/s (fig. 3B). To gauge the expected increase in concentration at different depths through a brain slice under typical recording conditions, we used this one-dimensional diffusion model[34] and the estimated diffusion coefficient of D = 0.2 × 10^{-6} cm^2/s to calculate the concentration of etomidate as a function of time and depth for a 400-μm-thick brain slice with only the top surface perfused (fig. 4). As expected, the concentration increased more rapidly near the surface than at deeper sites within the tissue. At a depth of approximately 100 μm, where electrophysiologic (extracellular) recordings are typically performed, approximately 80% of the final equilibrium concentration is reached only after 60 min of application.

Concentration Profile of Etomidate in a Brain Slice Preparation and Its Correlation with Electrophysiologic Data

To examine whether the concentration profile predicted by the diffusion model is compatible with physiologic data, we applied 1 μM etomidate to 400-μm-thick brain slices for up to 105 min and measured the effect of etomidate on neuronal function using the conditioned depression paradigm (fig. 5A).[21] Etomidate (1 μM) did not alter the unconditioned response (fig. 5B, single response) because the maxima of the population spike amplitudes as assessed by input/output curves in the presence of the anesthetic were not significantly different compared with those ones obtained under control or wash conditions ([etomidate/(control + wash)/2] = 1.05 ± 0.15; P = 0.46, n = 9). In contrast, etomidate prolonged the duration of depression of the conditioned response (fig. 5B, interstimulus interval 10/100/500). This effect was more pronounced the longer the slice was exposed to etomidate (fig. 5C). To compare the onset rate of this physiologic effect with the onset predicted by our diffusion model (fig. 4), we superimposed the normalized effects of etomidate at an interstimulus interval of 100 ms with the expected tissue concentrations (fig. 5D). Solid lines show calculated concentrations at different depths based on the estimated diffusion coefficient for etomidate of 0.2 × 10^{-6} cm^2/s. The onset of physiologic responses corresponded most closely to a recording depth 100 μm below the surface of the slice. Because it is likely that the electrophysiologic signals we recorded were generated not at a single point source at this depth but rather came from tissue sites above and below this location, we also compared this value with the increase in average tissue concentration (integral) in the top half of a 400-μm-thick slice (dashed line). This again revealed a good correspondence between the observed onset rate for etomidate actions and the predicted drug concentration profile.

Discussion

An important consideration in relating results from *in vitro* experiments to behavioral or physiologic consequences *in vivo* is assuring that equivalent drug concentrations are achieved. This requires that the free aqueous concentration present during general anesthesia be determined, commonly by measuring the serum concentration and the extent of plasma protein binding. We took a different approach and measured brain concentration and the brain:ACSF partition coefficient. In principle, under steady state conditions *in vivo*, these two approaches should yield the same values for the free drug concentration. However, under non–steady state conditions, such as following bolus injection of a drug, direct measurements of brain concentrations should more accurately reflect the concentration at the site of action. The aqueous concentration that yields this tissue concentration may be calculated using the partition coefficient. Applying this concentration in aqueous solution should then produce the same tissue concentration *in vitro* as *in vivo*.

In Vitro *Anesthetic Concentrations That Correspond to* in Vivo *Responses*

Because different anesthetic endpoints, such as hypnosis, amnesia, and immobility, are achieved at different anesthetic concentrations, assessing drug effects on pu-

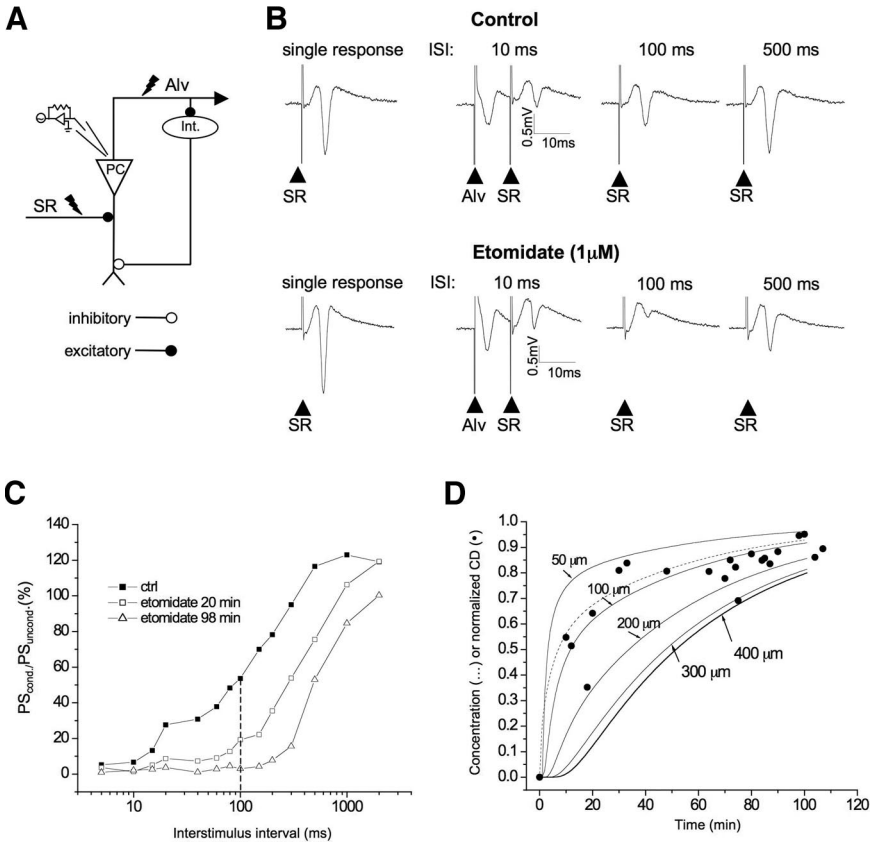

Fig. 5. Physiologic effects of etomidate in the hippocampal brain slice, as assessed by the conditioned depression (CD) paradigm. (*A*) Schematic diagram of the circuit underlying CD, illustrating the positioning of two stimulating electrodes and one recording electrode. (*B*) Averaged extracellular responses of CA1 pyramidal cells after stimulation of the Schaffer collateral pathway in stratum radiatum (SR) or direct antidromic stimulation of CA1 pyramidal neuron axons in the alveus (Alv). Current traces illustrate the depression of the population spike (PS) after a conditioning stimulus. Etomidate (1 μM) profoundly enhanced PS depression resulting from the conditioning stimulus but did not affect the single (unconditioned) response evoked by SR stimulation only. (*C*) Enhancement of CD by etomidate, expressed as PS_{cond}/PS_{uncond} (%) and plotted as a function of interstimulus intervals, continued to increase over an hour. With single-sided perfusion of a 400-μm-thick brain slice, etomidate effects had reached approximately 65% of their maximum possible effects at interstimulus intervals from 100 to 1,000 ms after 20 min. (*D*) The time course of the onset of physiologic responses complies well with the onset predicted by our diffusion model. Expected concentrations at depths ranging from 50 to 400 μm (single-sided perfusion, D = 0.2 × 10^{-6} cm^2/s) were plotted together with the measured effects of etomidate on CD at an interstimulus interval of 100 ms, normalized to the maximum possible effect. The average concentration of etomidate in the upper half of the slice (0–200 μm), plotted as a *dotted line*, is similar to the concentration at 100 μm, a typical recording depth for field potentials. Data were obtained from n = 14 animals. ctrl = control; Int. = interneurons; PC = pyramidal cell.

tative anesthetic targets requires the use of concentrations corresponding to the endpoint of interest. The concentration of etomidate that corresponds to the minimal amnesic dose—whether plasma or *free* aqueous—has not been measured previously. Plasma concentrations have been determined after induction (12–24 μM),[29] and during continuous infusion for maintenance of anesthesia in the clinical setting (5–8 μM).[1,36,37] Also, minimal hypnotic concentrations in humans (1.2 μM)[38] and the concentration that produces loss of righting reflex in rodents (1.6 μM)[39] have been reported. Taking into account 78% plasma protein binding,[1] these plasma concentrations correspond to *free* aqueous concentrations of 2.5–5.0 μM at induction, 1–2 μM for maintenance of "anesthesia," and 0.25–0.35 μM for awakening or recovery of righting reflex. The latter concentration is similar to that which we determined impairs fear conditioning to context. Taking into account the brain:ACSF partition coefficient of 3.35 that we measured, this concentration is also similar to the concentration that impairs water maze learning in mice (0.74 μM in brain tissue/3.35 = 0.22 μM).[40] Based on the observation that the concentration of anesthetics required to suppress learning depends on the specific learning task used, and in general hippocampus-dependent learning is the most susceptible to disruption by drugs,[30] a higher free concentration of etomidate could be required to impair other types of memory. Our measurements of locomotor activity (fig. 1) suggest that recovery of righting reflex may actually occur at a higher concentration than the estimate derived from plasma concentration and protein binding,[1,39] because not only was the righting reflex intact at a drug level that impaired conditioning to context by 50%, but at the same time the mice were spontaneously exploring their environment (albeit at a reduced level).

Etomidate Diffusion into Brain Slices

In addition to applying the appropriate equilibrium drug concentration, a second consideration when performing brain slice experiments is knowing that the concentration at tissue sites of action is near equilibrium. Like many other anesthetics, etomidate is more soluble in lipid than in aqueous environments, and for drugs such as propofol and diazepam, the slowness of diffusion mandates several hours to reach equilibrium.[27] The diffusion coefficient that we measured for etomidate (D = 0.2 × 10^{-6} cm^2/s) is 10 times that of propofol (D = 0.02 × 10^{-6} cm^2/s)[27] but not as great as that of halothane (D = 0.8 × 10^{-6} cm^2/s).[26] Therefore, using a one-dimensional diffusion model, we calculated that when etomidate is applied to only the top surface of a 400-μm-thick

brain slice, approximately 60 min is required before the average concentration in the top half has reached 80% of the final equilibrium value, and concentrations increase even more slowly at greater depths (fig. 4). Our physiologic measurements of the rate of onset of drug action were consistent with these estimates (fig. 5). The implication of this finding is that the low rate of etomidate diffusion should be taken into account when designing and interpreting experiments with brain slices, either by providing a sufficient duration for equilibration or by using thinner preparations, such as organotypic slice cultures, to assure more rapid equilibration.[41] Alternatively, delivery of the drug to both surfaces of a slice instead of single-sided perfusion can have a large impact on drug delivery, because equilibration time is inversely related to the square of the tissue thickness (equation 4). A further implication is that in previous experiments in which etomidate was applied to brain slices for only a brief time, equilibrium levels were unlikely to have been achieved except near the surface of the slice, and drug effects may have been observed at significantly lower concentrations had longer durations of drug application been tested.[25,42–47]

Although its slow diffusion through brain tissue substantially influences the rate of drug equilibration in brain slice experiments, in clinical use, etomidate results in a rapid (30–60 s) onset of hypnosis after a standard induction dose of 0.15–0.3 mg/kg administered intravenously. This is due at least in part to the short distance between the site of delivery *via* the microvasculature and tissue sites of action. In addition, induction doses produce blood levels higher than the EC_{50} equilibrium concentrations, and this also contributes to the rapid onset of clinical effects.

Mechanisms of General Anesthesia

The mechanism by which etomidate produces general anesthesia is thought to be primarily *via* enhancement of the activity of β_2- and/or β_3-containing $GABA_A$Rs.[17,18] The results of recent experiments with genetically modified mice carrying mutations in the β_2 or β_3 subunits provide strong support for this hypothesis, at least with regard to immobility and sedation/hypnosis.[48–50] However, additional presynaptic and postsynaptic actions of etomidate have been described, such as block of different classes of calcium[51] and sodium channels,[52,53] block of nicotinic acetylcholine receptors,[54–56] agonistic effects on α_2-adrenergic receptors,[57] inhibition of brain nitric oxide synthase activity,[58] and inhibition of glutamate release during ischemia.[59] Our current results demonstrating that amnesia is produced at concentrations of approximately 0.25 μM suggest that these other actions are unlikely to play major roles in etomidate-induced amnesia, because they occur only at much higher concentrations ($>$ 10 μM). In addition, our finding that $GABA_A$R-mediated feedback inhibition in the hippocampus is strongly enhanced by 1 μM etomidate (fig. 5) further supports a role for β_2- and/or β_3-containing synaptic receptors in producing behavioral effects.

Population responses are produced by the synchronous firing of many pyramidal cells, reflecting the integration of several different influences. Our finding that 1 μM etomidate did not alter the unconditioned response to Schaffer collateral stimulation (single response, fig. 5B) indicates that under the conditions of our experiments etomidate did not enhance tonic inhibition enough to suppress action potential generation after excitatory synaptic stimulation, although it did substantially enhance phasic (feedback) inhibition. Also, these results provide additional evidence against sodium channels as significant targets at this concentration of etomidate. By contrast, the etomidate concentration that corresponds to the EC_{50} for amnesia (0.25 μM) does fall within the concentration range at which effects of etomidate were reported for expressed $GABA_A$Rs[13] and for other integrative responses that are known to depend on $GABA_A$Rs, including spontaneous action potential firing[49] and θ-oscillations[41] in cortical slice cultures.

In conclusion, the *free* aqueous EC_{50} concentration of etomidate that corresponds to amnesia produced by intraperitoneal administration *in vivo* is 0.25 μM. Diffusion of etomidate through brain tissue is slow enough that after 1 h, the concentration has reached only 80% of the final equilibrium concentration at a typical brain slice recording depth of 100 μm. These factors should be considered when interpreting existing studies and when designing *in vitro* experiments.

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