

Effects on Synaptic Inhibition in the Hippocampus Do Not Underlie the Amnestic and Convulsive Properties of the Nonimmobilizer 1,2-Dichlorohexafluorocyclobutane

Misha Perouansky, M.D., Robert A. Pearce, M.D., Ph.D.†*

Background: **Although it does not suppress movement in response to noxious stimuli, the nonimmobilizer 1,2-dichlorohexafluorocyclobutane (F6, also known as 2N) does cause amnesia and seizures. These occur at 0.48 and 1.3 times, respectively, the concentrations that are predicted from its lipid solubility to cause immobility. The molecular and cellular basis of these effects is not known. The ionotropic γ-aminobutyric acid type A ($GABA_A$) receptor is modulated strongly by anesthetics, and it plays an important role in many seizure models. Also, the hippocampus is a structure central to the formation of memory and is susceptible to seizure generation. The authors therefore investigated the effect of F6 on $GABA_A$ receptor–mediated inhibition in hippocampal neurons.**

Methods: **Transverse hippocampal slices were prepared from young (12- to 21-day-old) Sprague-Dawley rats. Inhibitory postsynaptic currents were recorded from hippocampal CA1 pyramidal cells in the presence of ionotropic glutamate receptor antagonists. F6 was applied with the bath solution. The concentration of F6 achieved during the experiment at the location of synaptic inhibition was derived using a diffusion model.**

Results: **At tissue concentrations of up to 75 μM (approximately 5 × predicted minimal alveolar concentration), F6 had no discernible effect on either the amplitude or the kinetics of GABA-mediated synaptic currents. Isoflurane, by contrast, prolonged the decay time constant of these currents at 100 μM (approximately 0.3 × minimal alveolar concentration).**

Conclusions: **At concentrations that bracket the *in vivo* amnestic and seizure-inducing range, F6 has no discernible effect on fast synaptic $GABA_A$ receptors in hippocampal CA1 pyramidal neurons. Synaptic $GABA_A$ receptors sharply discriminate between volatile anesthetics and a prototype nonimmobilizer. Similar *in vivo* effects of anesthetics and nonimmobilizers may be mediated by different cellular mechanisms.**

ANESTHESIA is a complex drug-induced state that comprises multiple elements, *e.g.*, immobility, amnesia, and hypnosis.[1] Correspondingly, anesthetic drugs, particularly the volatile anesthetics, affect multiple molecular and cellular processes. Separating relevant effects from irrelevant ones and linking effects on the receptor level to desirable and undesirable manifestations *in vivo* are fundamental aims of anesthesia-related research.[2]

One approach to these aims is to compare the effects of anesthetics to those of drugs that have anesthetic-like physicochemical properties but do not produce the full spectrum of anesthetic actions *in vivo*. These compounds were initially termed *nonanesthetics*[3]; subsequently, the term was changed to *nonimmobilizers* after it was discovered that some agents cause amnesia but do not prevent movement in response to noxious stimuli.[4] The volatile compound 1,2-dichlorohexafluorocyclobutane (designated *F6* or *2N* in the literature) is an extensively studied prototype nonimmobilizer. Like anesthetics, it produces amnesia at a concentration of approximately one third the predicted minimal alveolar concentration (MAC) at which, according to its lipid solubility, it should cause immobility to painful stimulus in 50% of subjects if it behaved as a true anesthetic (predicted MAC or MAC_{pred}).[3,4] At concentrations above MAC_{pred}, it induces convulsions,[5] a property that to some degree is shared by some anesthetic ethers, *e.g.*, enflurane.[6]

A number of ligand-gated neuronal ion channels are affected to some degree by anesthetics. Of these, the ionotropic γ-aminobutyric acid type A ($GABA_A$) receptor is thought to play a pivotal role in general anesthetic action. In particular, $GABA_A$ receptor modulation may contribute to the amnestic as well as the proconvulsive properties of volatile anesthetics.[7,8] We decided therefore to investigate in detail the effects of F6 on $GABA_A$ receptor–mediated synaptic activity. We used whole cell patch clamp recordings from rat hippocampal CA1 pyramidal neurons. Volatile anesthetics were shown previously to exert multiple effects in this preparation. Specifically, at concentrations above 150 μM (0.5 × MAC), isoflurane prolonged the decay of fast spontaneous and miniature γ-aminobutyric acid–mediated (GABAergic) inhibitory postsynaptic currents (sIPSCs and mIPSCs, respectively), and at concentrations above 600 μM (2 × MAC), it reduced IPSC amplitude. At equivalent MAC fractions, enflurane had similar effects on inhibitory postsynaptic current (IPSC) decay, but it reduced IPSC amplitude more profoundly. These effects on decay and amplitude were proposed to underlie the amnestic and proconvulsive properties, respectively.[8] We hypothesized that similar effects would be observed at behaviorally equivalent concentrations of F6.

* Associate Professor, † Professor of Anesthesiology.

Received from the Department of Anesthesiology, University of Wisconsin, Madison, Wisconsin. Submitted for publication August 28, 2003. Accepted for publication December 7, 2003. Supported by grant No. GM 55719 from the National Institutes of Health, Bethesda, Maryland (to Dr. Pearce), and by the Department of Anesthesiology, University of Wisconsin-Madison, Madison, Wisconsin. Presented in part at the Annual Meeting of the American Society of Anesthesiologists, Orlando, Florida, October 12–16, 2002; the Annual Meetings of the Society for Neuroscience, New Orleans, Louisiana, November 4–9, 2000, and San Diego, California, November 10–15, 2001; and the 12th Neuropharmacology Conference, Orlando, Florida, October 31–November 2, 2002.

Address correspondence to Dr. Perouansky: Department of Anesthesiology, Room 43 Bardeen Labs, University of Wisconsin Medical School, 1300 University Avenue, Madison, Wisconsin 53792-3272. Address electronic mail to: mperouansky@wiscmail.wisc.edu.

Materials and Methods

All experiments were conducted according to the guidelines in the *Guide for the Care and Use of Laboratory Animals*[9] and were approved by the University of Wisconsin Animal Care and Use Committee (Madison, Wisconsin).

Slice Preparation

Juvenile male Sprague-Dawley rats (aged 14–24 days) were decapitated during isoflurane anesthesia, and the brain was quickly removed and immersed in cold (4°C) artificial cerebrospinal fluid (ACSF) saturated with 95% O_2–5% CO_2 (carbogen gas). A block of tissue containing the hippocampus was removed and glued to a tissue tray using cyanoacrylate glue. Tissue slices 400 μm thick were prepared using a vibrating microtome (Leica VT1000; Bannockburn, IL), incubated at 32°C for 1 h, and then kept in carbogen-saturated ACSF at room temperature until use.

Patch Clamp Electrophysiology

Cells in the stratum pyramidale of CA1 were visualized using a video camera (Hamamatsu C2400; Hamamatsu Corp., Bridgewater, NJ) connected to an upright microscope (Zeiss Axioskop; Thornwood, NJ) equipped with an infrared bandpass filter (Chroma D775/220; Brattleboro, VT), a long working-distance water-immersion objective (Zeiss Achroplan 40×, 0.75 numerical aperture), and differential interference contrast optics (DIC, or Nomarski). Whole cell recordings were obtained at room temperature (22°–24°C) using a Multiclamp 700A patch clamp amplifier and pClamp software (Axon Instruments, Foster City, CA). Data were filtered at 5 kHz, sampled at 10 kHz (Digidata 1200; Axon Instruments), and stored on a Pentium-based computer. Patch pipettes were fabricated from borosilicate glass (KG-33; Garner Glass, Claremont, CA; 1.7 mm and 1.1 mm OD and ID, respectively) using a Flaming-Brown two-stage puller (model P-87; Sutter instruments, Novato, CA), fire polished, and coated with Sylgard (Dow-Corning, Midland, MI) to reduce electrode capacitance when necessary. Tight-seal whole cell recordings were obtained using standard techniques. Patch pipettes had open-tip resistances of 2–4 MΩ when filled with the recording solution of the following composition: 140 mM CsCl, 10 mM NaCl, 10 mM HEPES, 10 mM BAPTA, 2 mM MgATP, and 5 mM lidocaine *N*-methyl bromide (QX-314), pH 7.3. Access resistances were typically 10–20 MΩ and were compensated by 60–80%. The membrane potential was voltage clamped at −60 mV. $GABA_A$ receptor–mediated IPSCs were isolated by bath application of 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 40 μM D,L-2-amino-5-phosphonovaleric acid to block glutamate receptors, and by the inclusion of CsCl and QX-314 in the patch pipette to block regenerative Na^+ and $GABA_B$-mediated currents. Tetrodotoxin (1 μM) was used to isolate non–action potential-dependent, *i.e.*, miniature, IPSCs. IPSCs recorded in the absence of tetrodotoxin were considered to be a combination of both action potential dependent and independent IPSCs and were termed *spontaneous IPSCs*. The remaining currents were exclusively $GABA_A$ receptor mediated because they were completely blocked by bath application of 10 μM bicuculline (data not shown). D,L-2-amino-5-phosphonovaleric acid, CNQX, tetrodotoxin, and bicuculline were prepared as 50- to 100-fold stock solutions and applied using syringe pumps (model 55-1111; Harvard Apparatus, Natick, MA) set to flow at 1 or 2% of the ACSF flow rate to achieve the desired bath concentrations.

Application of Volatile Agents

Experiments were conducted using ACSF of the following composition: 127 mM NaCl, 1.21 mM KH_2PO_4, 1.87 mM KCl, 26 mM $NaHCO_3$, 2.17 mM $CaCl_2$, 1.44 mM $MgSO_4$, and 10 mM glucose, saturated with carbogen gas, pH 7.4. Solutions of artificial cerebrospinal fluid containing isoflurane and F6 were prepared in Chemware Teflon FEP gas sampling bags (North Safety Products, Cranston, RI) fitted with three-way stopcocks. F6 solutions were prepared by filling Teflon bags partially with ACSF that had been preequilibrated with carbogen gas and adding to the headspace appropriate quantities of F6-saturated carbogen and pure carbogen to achieve the desired aqueous concentration of F6, as calculated using a saline/gas partition coefficient for F6 of 0.026.[10] Isoflurane solutions were prepared similarly except that 10 mM isoflurane in ACSF (which is below the saturating concentration of 15 mM at room temperature)[11] was added to the Teflon bag. For electrophysiologic experiments, solutions were equilibrated by shaking on a shaker table for at least half an hour and were then allowed to stand for at least 1 h. F6-saturated carbogen stock was prepared in a separate Teflon bag by first evacuating the bag then adding carbogen, water, and an excess of liquid F6 and shaking for 1 h to equilibrate. Control ACSF solutions were prepared similarly in Teflon bags. Concentrations of F6 and isoflurane in ACSF from the Teflon bags and from the brain slice chamber were measured by gas chromatography to determine the fraction of loss and the concentrations that were actually applied to slices. Volatile agents were applied for 20 to 30 min. The data used for analysis were collected within the last 3 min of drug exposure and after at least 17 min of washing.

For F6, which in contrast to isoflurane does not reach an equilibrium within this time frame, the effective concentration around the cell soma was determined by taking into account the loss of F6 from bag to chamber, the distance of the cell from the slice surface, and the duration of F6 application, using a diffusion model as detailed previously (fig. 1).[10] These are the concentra-

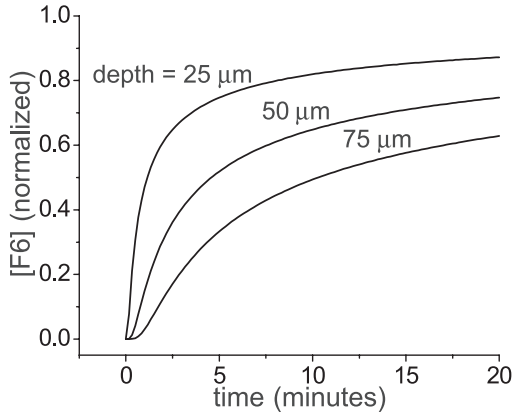

Fig. 1. Determination of "effective" tissue F6 concentrations. The F6 concentrations achieved at various depths are shown as a function of exposure time for a 400μm-thick slice exposed to drug on only one surface.[10] After 15 min of perfusion with F6 containing solution, the concentration of F6 near the soma of a cell lying 25 μm below the exposed surface has reached approximately 80% of the final equilibrium concentration. At the same time, a cell located at a depth of 75 μm will be exposed to less than 60% of the final concentration. The difference is accentuated for shorter exposure times.

tions of F6 referred to in the text. For example, if the measured concentration of F6 in the Teflon bag was 81 μM, the concentration in the bath would be 48.6 μM (40% loss of F6 on the way to and within the slice chamber). After 17 min of drug application, the concentration around a cell soma located 30 μM below the slice surface was estimated by the diffusion model to be 40 μM.

Aqueous Phase Drug Concentration by Gas Chromatography

Aqueous samples (2 ml) were collected from Teflon bags or the brain slice perfusion chamber using a gastight glass syringe (Hamilton Co., Reno, NV) fitted with a Teflon stopcock (Hamilton Co.). Samples were transferred to 3.7-ml glass vials capped with mininert valves (Alltech, Nicholasville, KY). Aqueous and gas phases within the vial were equilibrated by shaking for 1 h. Drug concentrations in the vial headspace were determined by gas chromatography using gas phase calibration standards for F6 or isoflurane. Concentrations in aqueous samples ($C_{aq,\ sample}$) were calculated based on saline/gas partition coefficients ($\lambda_{saline/gas}$) and the relative volumes of aqueous and gas phases within the vial ($V_{gas,vial}$ and $V_{aq,vial}$), according to the equation

$$C_{aq,sample} = C_{gas,vial}(V_{gas,vial}/V_{aq,vial} + \lambda_{saline/gas}). \quad (1)$$

Gas phase concentrations ($C_{gas,\ vial}$) were measured using a Varian 3700 gas chromatograph (Varian Inc., Walnut Creek, CA) with a flame ionization detector. Separation was achieved by on-column injection into a 1.83 m $\times$ 3.2 mm stainless steel column packed with 80/100 Poropak Q.

Data Analysis

Data were analyzed on a Pentium-based personal computer using Clampfit (Axon Instruments), Origin (MicroCal, Northampton, MA), and Instat (GraphPad Software, San Diego, CA). Data were first filtered off-line at 2 kHz. Events were then analyzed using an automated event–detection algorithm as described previously.[8] All events detected by the program were visually inspected, and the peak amplitude and 10–90% rise time measurements were adjusted if necessary. To characterize the decay kinetics of fast IPSCs, a subset of events also was selected for exponential curve fitting. Events were selected only if no other event occurred within 250 ms of the peak. IPSC decays were typically best described by two exponential components. To quantify the overall decay time, we computed the weighted time constant $\tau_w = (A_1\tau_1 + A_2\tau_2)/(A_1 + A_2)$, where τ is the time constant of decay and A is the amplitude.

For statistical comparison of interevent intervals (IEIs), data were first tested for normality using the Kolmogorov-Smirnov test. The largest groups of data (mIPSCs, n = 23, and sIPSCs, n = 7, control conditions) did pass the test of normality. Therefore, it was assumed that subsets of these data would also be normally distributed, so *t* tests were performed to compare group means.

Results

These data are based on recordings from 30 cells located in the stratum pyramidale of CA1. The majority of these cells are likely to be pyramidal cells.[12] However, because we did not conduct histologic analysis, a small number of inhibitory cells may also be included. Under control conditions (without F6) and with isoflurane application, the recordings were usually stable for prolonged periods (greater than 60 min). Exposure to F6, by contrast, seemed to affect the recording conditions, as described in detail in the section Effect of F6 on IPSC Amplitude.

We hypothesized that F6 would either increase (at lower concentrations) and/or decrease inhibition (particularly at higher concentrations) thereby providing cellular substrates compatible with its amnestic and seizure-inducing effects *in vivo*. We therefore examined characteristics of inhibition at the population and the individual IPSC levels that would, either together or in isolation, translate into changes in overall inhibition.

Effect of F6 on IPSC Decay

A prominent effect of anesthetic agents on synaptic $GABA_A$ responses is a slowing of the time constant of decay. Therefore, we investigated the effect of F6 on the decay of sIPSCs and mIPSCs. Decay was usually best fitted with two exponentials, but we report here the weighted time constant τ_w to simplify comparisons. Un-

der control conditions, the τ_w of spontaneous events ranged from 15.7 to 21.9 ms (mean, 19.6 ± 2.8 ms; n = 7) and did not differ significantly from the τ_w of miniature events, which ranged from 10.4 to 24.0 ms (mean, 18.9 ± 3.4 ms; n = 18; $P > 0.05$, two-tailed *t* test). These values are similar to those previously reported in this same preparation.[8] We examined the effect of F6 on the decay of mIPSCs and sIPSCs in 17 cells at concentrations ranging from 15 to 75 μM (approximately 1 to 5 × MAC_{pred}). An example of the (lack of) effect is illustrated in figure 2A. Averaged mIPSCs are shown from a single cell before, during, and after washout of 36 μM F6 (> 2 × MAC_{pred}). Unlike the prominent effect produced by volatile anesthetics, no apparent effect of F6 on decay was observed in this cell. Neither the τ_w of sIPSCS (n = 4) nor the τ_w of mIPSCs (n = 13) were affected by F6 at any of the concentrations tested.

To test whether our experimental conditions and analytical techniques were suitable for detecting minor alterations in IPSC kinetics, we also tested the effect of a low concentration of isoflurane (fig. 2B). Under experimental conditions that were identical to those under which F6 was tested, isoflurane 100 μM (0.3 × MAC) slowed the decay of the averaged IPSC by almost 50% (from 15 ± 4 to 22 ± 8 ms; n = 3; $P < 0.05$, paired *t* test). A comparison of effects of isoflurane and F6 over a range of concentrations is shown in figure 2C. It is apparent that isoflurane significantly slowed mIPSC decay in a dose-dependent manner, but that F6 at concentrations up to 5 × MAC_{pred} had no effect on either mIPSCs or sIPSCs.

Effect of F6 on IPSC Amplitude

Isoflurane, enflurane, and halothane all exert dual effects on IPSCs: In addition to prolonging the decay, they also reduce the peak amplitude of the currents.[8] Both effects are dose dependent, and although the effect on amplitude requires higher concentrations than the effect on decay, it is apparent at sub-MAC concentrations. Because it is more pronounced for enflurane than for isoflurane,[8] it has been suggested that the differential effects of isoflurane and enflurane on decay kinetics *versus* amplitudes of mIPSCs (more block of the amplitude for the same degree of prolongation with enflurane) could underlie the proconvulsive properties of enflurane. F6 reliably induces seizures at concentrations slightly above 1 MAC_{pred} *in vivo*, and we found no effect on the decay kinetics of either mIPSCs or sIPSCs (fig. 2); therefore, we examined whether it alters mIPSC amplitude.

An example of one such experiment is illustrated in figure 3A. In the presence of 1 μM tetrodotoxin, synaptic currents were measured before, during, and after the application of 40 μM F6. No gross effects on either the amplitude or the frequency of mIPSCs are apparent. Figure 3B shows the results of a quantitative analysis of mIPSCs recorded from the cell shown in figure 3A. A minor shift of the distribution of mIPSC amplitudes toward smaller values is apparent from both the amplitude histograms (inset) and the cumulative probability histogram. However, the shift was not reversed on washout of F6. In all, we tested the effect of 15 to 62 μM F6 on mIPSCs in 13 cells. In nine of these neurons, the recordings remained stable throughout the 20-min F6 applica-

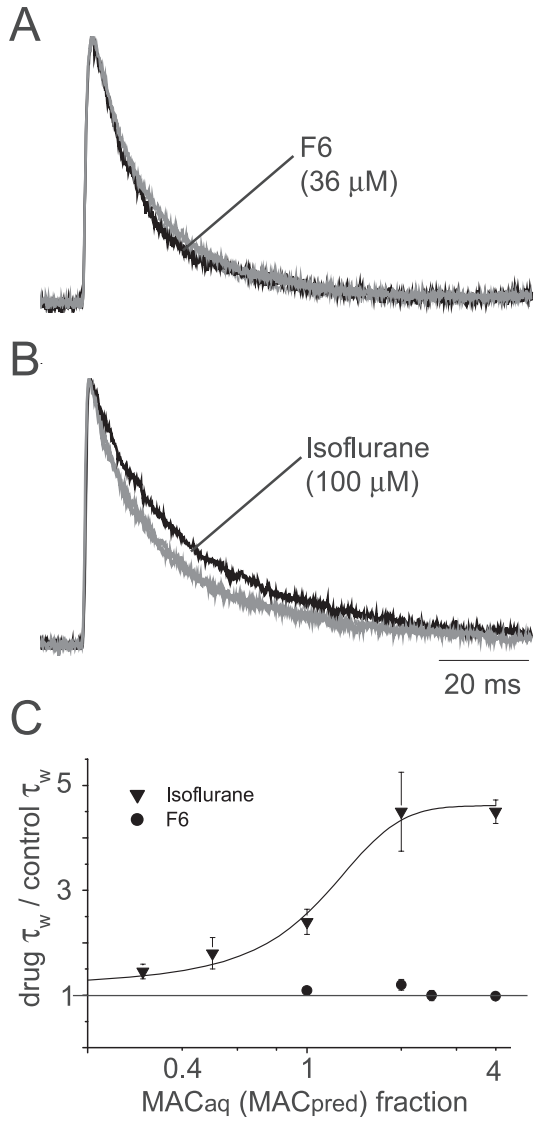

Fig. 2. Synaptic $GABA_A$ receptors discriminate between anesthetic and nonimmobilizer. (*A*) 36 μM F6 (> 2 × MAC_{pred}, *black trace*) had no effect on the decay of $GABA_A$ mIPSCs. (*B*) By contrast, 100 μM isoflurane (0.3 × MAC, *black trace*) slowed the decay by approximately 30% (from 20 ms to 26 ms). (*C*) Summary of the effects of F6 and isoflurane on the decay time constants of $GABA_A$ IPSCs. Isoflurane slowed IPSCs in a dose-dependent manner up to the highest concentrations tested, whereas F6 at similar MAC_{pred} concentrations had no effect. (Data for F6 based on recordings from 4 and 13 cells for sIPSCs and mIPSCs, respectively. Isoflurane data at 0.3 × MAC from 3 cells, other isoflurane concentrations taken from reference 8. Hill coefficient is 3.8 for isoflurane data.) $GABA_A$ = γ-aminobutyric acid type A; IPSC = inhibitory postsynaptic current; MAC_{pred} = predicted minimal alveolar concentration; mIPSC = miniature inhibitory postsynaptic current; sIPSC = spontaneous inhibitory postsynaptic current.

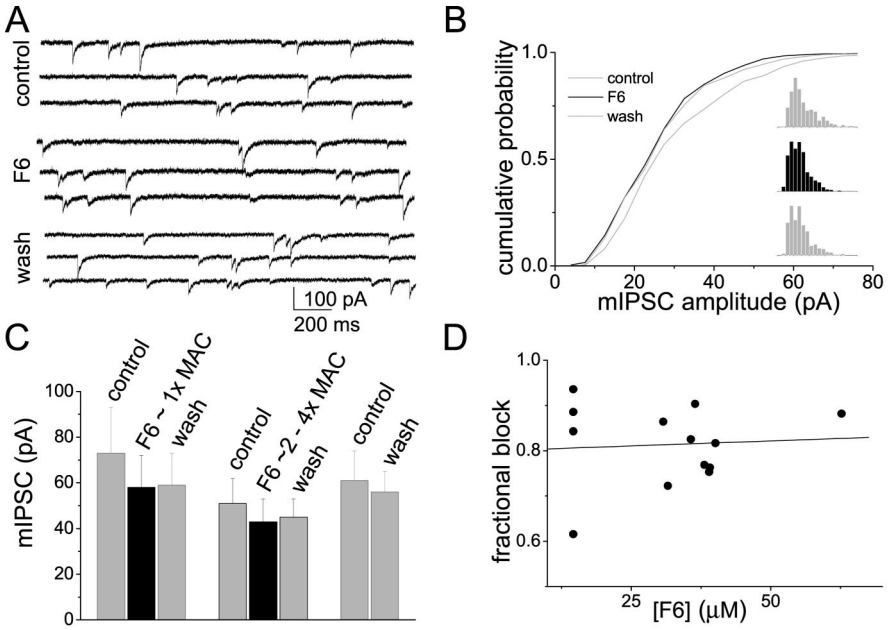

Fig. 3. Effect of F6 on IPSC amplitude is not dose dependent. (*A*) mIPSCs recorded from a hippocampal pyramidal cell before (*top three traces*) during (*middle three traces*) and after wash-out (*bottom three traces*) of 40 μM F6 show no obvious effect on the amplitude of discrete events. (*B*) Cumulative probability of mIPSC amplitudes before the start of F6 application, at the end of a 20-min-long F6 application and after a 17-min wash-out period. The minor shift toward smaller amplitudes is not reversed upon wash-out. Insets show the amplitude histograms for the three periods analyzed (450–500 events in each period, same cell as in (*A*)). (*C*) Mean amplitudes of mIPSCs exposed to F6 of approximately 1 × MAC_{pred} (n = 4) and 2–4 × MAC_{pred} (n = 5) during control, F6, and wash show a trend toward smaller amplitudes after F6 exposure that is only partially reversible (note that only cells with an adequate wash period were included). mIPSCs from five control cells recorded over a similar time period are shown to the right. Note the small decrease in amplitude (run-down). (*D*) Fractional block (mean amplitude in F6/mean amplitude of control) *versus* F6 concentration for 13 neurons exposed to F6 (regardless of whether a wash was obtained). The magnitude of the block did not increase with F6 concentration. IPSC = inhibitory postsynaptic current; mIPSC = miniature inhibitory postsynaptic current.

tion and 20-min washout, and the results from these cells are summarized in figure 3C. We divided the cells into two groups, those exposed to a concentration of approximately 1 × MAC_{pred} and those exposed to approximately twice MAC_{pred} of F6 or higher (n = 4 and n = 5, respectively; see fig. 3, legend). The mean IPSC amplitude was reduced by 20% (from 73 ± 20 pA to 58 ± 14 pA at 1 × MAC_{pred}) and 16% (from 51 ± 11 pA to 43 ± 10 pA at ≥ 2 × MAC_{pred}), and this effect was only partially reversed by washout. To test for changes in IPSC amplitude that may occur with prolonged recordings independently of any drug exposure (so-called run-down), we recorded from five "control" cells for a similar duration but without exposure to F6. In these cells, we observed a mean reduction of the IPSC amplitude of 8% (from 61 ± 13 pA to 56 ± 9 pA). Therefore, approximately half of the change in amplitude under F6 could be attributed to run-down and half could be attributed to effects of the drug. Statistical analysis yielded a significant reduction in IPSC amplitude for the cells in the group exposed to higher F6 concentrations ($P < 0.001$, one-tailed paired *t* test).

It would be tempting to speculate that this (admittedly minor) effect on IPSC amplitude might underlie the proconvulsive effects of F6. However, we do not believe this to be the case for the following reasons. First, we tried to establish a dose–effect relation between [F6] and magnitude of block. The results are shown in figure 3D. We plotted the fractional block by F6 ($I_{F6}/I_{control}$, where $I_{control}$ and I_{F6} represent the mean amplitudes of 400–500 consecutive IPSCs before and during F6 application, respectively). It is evident that the magnitude of the block by F6 did not correlate with changes in F6 concentration. Second, during perfusion with F6-containing solution, we almost invariably observed an increase in access resistance (R_S) between the recording electrode and the cell interior. In a sample of cells where the change could be analyzed, we found that, under F6, the normalized R_S increased to 1.36 (± 0.38; n = 9) times the value under control conditions. This change was independent of the F6 concentration applied. We also noted that, simultaneously, there was a slight increase in the 10–90% rise time of the IPSCs. Figure 4A illustrates these findings in a time series plot for three cells that were exposed to 30–40 μM F6. For each individual cell, the data are averaged over 5-s intervals and normalized to the mean values obtained over the time period before application of F6. The figure illustrates that, shortly after exposure to F6, there is a reduction of mean IPSC amplitude that mirrors the increase in the mean rise time. Figure 4B shows a plot of mean normalized rise times *versus* amplitudes and illustrates the correlation between these two parameters. Our interpretation of these findings is that, for unknown reasons possibly related to its physicochemical properties (extremely low aqueous and moderate lipid solubility), F6 caused an increase of the access resistance to the cell interior. The increased rise time and decreased amplitudes thus are likely to reflect a change in the recording conditions rather than physiologic effects on $GABA_A$ receptors. Similar effects were observed in recordings of sIPSCs: Exposure to F6 (25 and 75 μM; n = 4) led to an increase in R_S, but in this case, the trend toward smaller IPSC amplitude did not reach statistical significance ($P >$

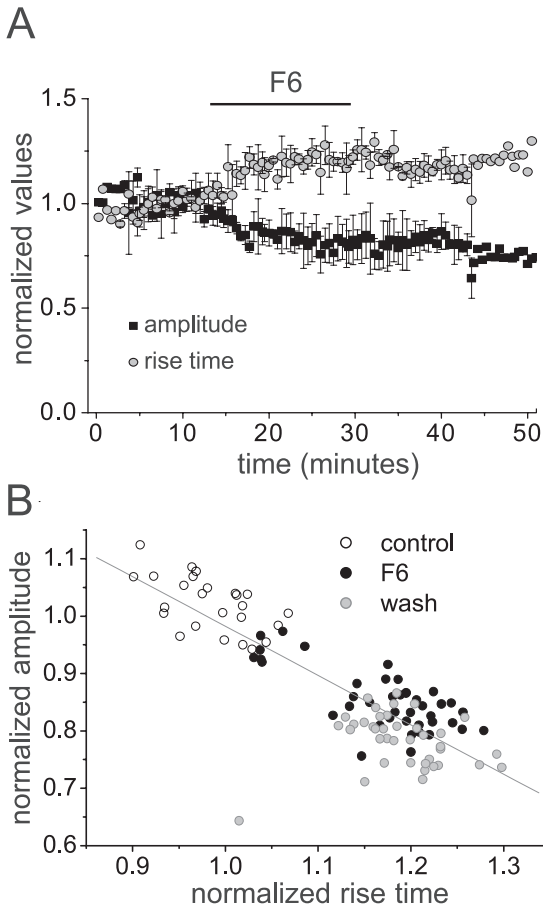

Fig. 4. F6 affects inhibitory postsynaptic current (IPSC) amplitude indirectly. (*A*) A time series plot of averaged normalized IPSC amplitudes and 10–90% rise times from three experiments. Upon perfusion with F6 containing solution, the IPSC rise time increased and there was a concomitant decrease in amplitude. (*B*) IPSC amplitudes varied with changes in rise time. Normalized IPSC amplitudes are plotted against normalized rise times, data from the cells shown in (*A*). Note the linear correlation of the two parameters (correlation coefficient = 0.84). For these cells, the concentration of F6 had reached 30–40 μM after 15 min of application.

0.05, two-tailed paired *t* test). Such systematic changes in R_S and rise time were not seen either in control recordings (*i.e.*, during prolonged recordings without application of any volatile agent: normalized R_S 0.97 [± 0.036; n = 4] times the control value) or in recordings where isoflurane was used (normalized isoflurane R_S = 1.07 [± 0.2; n = 6] times control R_S). This lack of F6-mediated block of native GABA$_A$ receptors is in general agreement with the finding by Mihic *et al.*,[13] who did not observe any effect of F6 in a similar concentration range on responses of expressed $\alpha_1\beta_1\gamma_{2s}$ receptors to application of 5 μM GABA.

Effect of F6 on sIPSC and mIPSC Frequency

Changes in the frequency of mIPSCs reflect effects on the release probability, whereas the rate of sIPSCs is also affected by changes in the excitability of presynaptic interneurons. We measured the rate of sIPSCs and mIPSCs as the IEI. The IEIs of sIPSCs and mIPSCs ranged from 92 to 565 ms (mean, 274 ± 168 ms; n = 7) and from 144 to 675 ms (mean, 335 ± 175 ms; n = 23), respectively. The difference was not significant ($P > 0.05$, two-tailed *t* test), indicating that there was a relatively low rate of spontaneous action potentials in the interneuron population innervating the pyramidal cells under our recording conditions. We studied the effect of

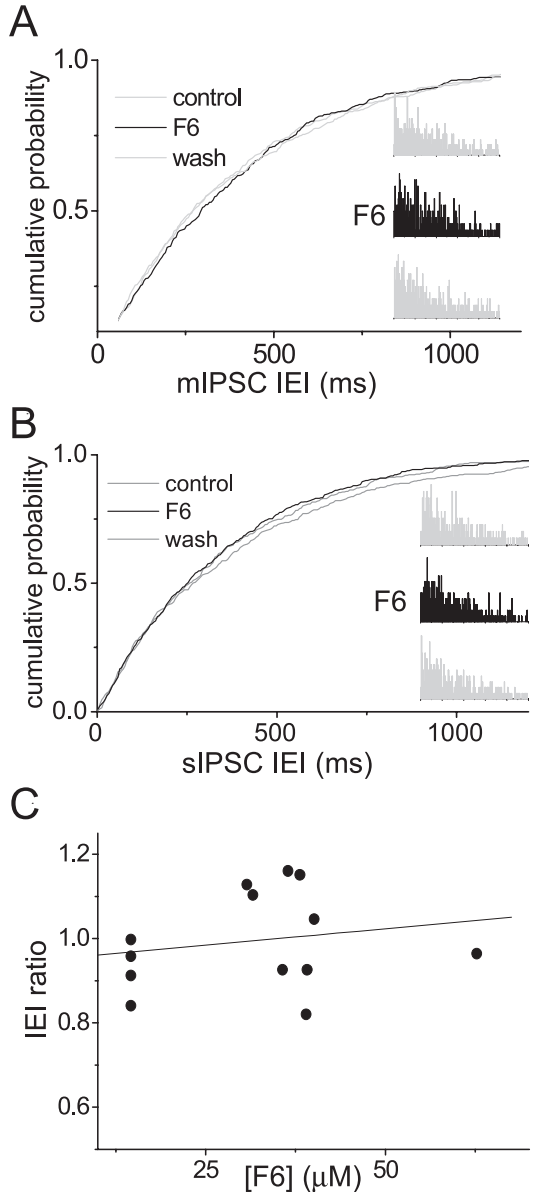

Fig. 5. F6 has no effect on IPSC frequency. (*A*) Cumulative probability and amplitude histograms (insets) of mIPSCs. There was no systematic change with F6 40 μM. (*B*) Cumulative probability and amplitude histograms (insets) of sIPSCs. As for mIPSCs, there was no change in the cumulative probabilities and amplitude distributions in the presence of 25 μM F6. (*C*) The ratio of the IEI in F6 divided by the IEI under control conditions ($IEI_{F6}/IEI_{control}$) is plotted against the concentration of F6 for all mIPSC experiments. IEI = interevent interval; IPSC = inhibitory postsynaptic current; mIPSC = miniature inhibitory postsynaptic current; sIPSC = spontaneous inhibitory postsynaptic current.

F6 on the frequency of both mIPSCs (fig. 5A) and sIPSCs (fig. 5B). Exposure to F6 (25–75 μM; n = 4) did not change the IEI of sIPSCs (313 ± 149 ms; $P > 0.05$, two-tailed paired t test). Likewise, F6 (15–63 μM; n = 13) had no effect on the frequency of mIPSCs (334 ± 179 ms; $P > 0.05$, two-tailed paired t test; fig. 5). Low isoflurane concentrations (40–100 μM) also had no effect on IEI for either sIPSCs (339 ± 237 ms and 332 ± 139 ms for control and test, respectively; n = 3; two-tailed paired t test) or mIPSCs (426 ± 158 ms and 428 ± 171 ms for control and test, respectively; n = 5; two-tailed paired t test), consistent with previous results.[8]

Discussion

We presented data demonstrating the lack of effect of the nonimmobilizer F6, at a wide range of concentrations, on $GABA_A$ receptor–mediated synaptic transmission. These results are relevant to ongoing investigations of anesthetic mechanisms for the following reasons: They illustrate that the $GABA_A$ receptor, which is widely accepted to play a crucial role in the mechanisms of anesthesia, discriminates between anesthetics and nonanesthetics across a wide range of concentrations; they suggest that suppression of fast synaptic inhibition is not the mechanism of seizure induction by F6. Furthermore, in combination with other recent findings,[14] they raise the possibility that the clinical effects that anesthetics and nonanesthetics have in common may be mediated *via* different mechanisms, and therefore that the strategy of using nonimmobilizers in the search for anesthetic targets has important limitations. Each of these issues is discussed in the following sections.

Synaptic $GABA_A$ Receptors Discriminate

In striking contrast to all halogenated volatile and numerous intravenous anesthetics, F6 had no effect on the decay time course of IPSCs at any of the concentrations tested (figs. 2A and C). By contrast, the widely used volatile anesthetic isoflurane slowed IPSCs significantly at concentrations as low as 100 μM (0.3 MAC; figs. 2B and C), a concentration that impairs hippocampal-dependent learning (fear conditioning to context) but not hippocampal–independent learning (fear conditioning to tone).[15] A slowing of IPSC decay has at least two functional consequences: On one hand, it increases the negative charge transfer across the neuronal membrane, thereby hyperpolarizing the cell and moving it away from the firing threshold for action potential generation. On the other hand, it also prolongs the time interval that conductances shunting the membrane are active, thus reducing the membrane resistance and thereby diminishing the efficiency of excitatory synaptic inputs. Both of these effects, the hyperpolarizing and the shunting effect, diminish the excitability of the neuronal membrane.

Effects of isoflurane on synaptic inhibition are likely to at least contribute to, and perhaps even be sufficient to account for, its suppression of synaptic plasticity. In a recent study, it was found that the ability of isoflurane (0.2–0.3 mM, approximately 1 MAC) to block the induction of homosynaptic long-term potentiation in hippocampal CA1 pyramidal cells was prevented by the $GABA_A$ receptor antagonist picrotoxin,[7] indicating that long-term potentiation suppression was therefore probably mediated by anesthetic modulation of the $GABA_A$ receptor. Because synaptic plasticity has been proposed as an *in vitro* model of memory formation,[16] the authors concluded that the effect of isoflurane on GABAergic inhibition is compatible with and may be sufficient to account for its amnestic effects *in vivo*. Recent reports indicate that isoflurane may block synaptic plasticity at even lower amnestic (*i.e.*, 0.3 MAC) concentrations.[17] Therefore, because synaptic $GABA_A$ receptors in the hippocampus are insensitive to F6, they cannot be instrumental in the mechanism by which F6 produces amnesia.

The Amnestic Paradox

Nonimmobilizers, known as nonanesthetics before the discovery that they could produce some effects from the anesthetic spectrum, were introduced into experimental paradigms as a tool to discriminate between relevant and irrelevant targets of anesthetic, particularly volatile anesthetic, action.[3] The rational behind this approach is that nonimmobilizers do not produce the full spectrum of effects *in vivo* that anesthetics do. For example, F6 does not produce immobility at or even well above MAC_{pred}.[3] Therefore, a cellular process that is similarly affected by F6 and an anesthetic cannot underlie immobility—as demonstrated for the nicotinic acetylcholine receptor.[18,19] By contrast, isoflurane and F6 both impair learning at concentrations of approximately 0.3 × MAC and MAC_{pred}.[4] Therefore, following the above argument, a process similarly affected by both drugs could contribute to the impairment of memory formation.

Our findings suggest that if isoflurane and F6 share the same mechanisms for amnesia, it is not *via* actions on classic fast synaptic $GABA_A$ receptors. However, it is conceivable that other types of $GABA_A$ receptors could play a role in the suppression of hippocampal plasticity by isoflurane. A separate class of slow dendritic inhibitory synapses exists in these cells,[20,21] as do extrasynaptic receptors that are a likely source of tonic inhibitory current.[22,23] These receptors differ in their physiologic and pharmacologic properties, and they could be susceptible to modulation by F6. These possibilities must be investigated before definitive conclusions about the interaction of this prototype nonimmobilizer with GABAergic inhibition are drawn. Alternatively, it is possible that isoflurane and F6 induce amnesia *via* interference with separate pathways. In this case, the usefulness

of nonimmobilizers in the search for mechanisms of amnesia is further limited. The latter possibility is supported by the results of a recent *in vivo* study in which learning impairment caused by F6 and flurothyl were antagonized by the concomitant administration of isoflurane.[14]

Mechanisms of Amnesia and Seizure Generation Remain Unresolved

Previous studies *in vivo* demonstrated that there are numerous physiologic processes that are not affected by F6 but that are affected by volatile anesthetics.[24–26] However, F6 does induce amnesia at concentrations below 1 MAC_{pred} and convulsions above 1 MAC_{pred}.[4,5] In the current study, using the hippocampal slice preparation, we failed to detect any effects at the cellular level that could underlie either the amnestic or the convulsive properties of F6. It has been recognized that, because of its extremely low aqueous solubility and its volatility, it can be difficult to achieve targeted concentrations in experiments where F6 is delivered *via* the aqueous superfusate,[19] particularly if it must diffuse significant distances into biologic tissue—the typical conditions of experiments on brain slices. We circumvented the uncertainties posed by diffusion by measuring drug concentrations in the bath as well as the brain tissue diffusion coefficient of F6,[10] thus allowing us to characterize its concentration profile under our experimental conditions. We were therefore able to determine with reasonable accuracy the effective concentrations in the tissue surrounding the structures of interest—in this case, inhibitory synapses that are located primarily on the somata of CA1 pyramidal neurons.[27] Other parameters of GABAergic synaptic inhibition, such as the frequency of spontaneous events and postsynaptic current amplitude, are modulated by anesthetics in a less consistent manner. These parameters also remained unaffected by F6, with the caveat that under our experimental conditions, because of the low frequency of spontaneous relative to action potential–independent activity, subtle effects on action potential–dependent $GABA_A$ release may have been missed. Such effects are not necessarily likely—F6 at concentrations up to 50 μM had no measurable effect Na^+ channel–mediated release of the excitatory neurotransmitter glutamate from cortical synaptosomes and had only modest effects on voltage-gated Na^+ currents in dorsal root ganglion cells.[28]

If F6 does not cause amnesia or convulsions *via* $GABA_A$ receptor modulation, what might be the targets that lead to these effects? Numerous possibilities have been suggested for volatile anesthetics, and each of these could also (or instead) be a target for F6. Studies of recombinant nicotinic acetylcholine receptors expressed in *Xenopus* oocytes showed that F6 inhibits rat and human nicotinic acetylcholine receptors at relevant concentrations.[18,19] Because F6 shares this property with isoflurane and neuronal-type nicotinic acetylcholine receptors are implicated in certain forms of memory,[29] these receptors are one plausible target. However, if as suggested above, anesthetics and nonimmobilizers cause similar behavioral effects *via* independent or even antagonistic actions, identification of common molecular targets may prove to be less instructive than anticipated.

The authors thank Michael Laster, D.V.M. (Assistant Researcher), and Edmond Eger II, M.D. (Professor, Department of Anesthesia, University of California, San Francisco, California), for providing gas phase calibration standards for isoflurane and 1,2-dichlorohexafluorocyclobutane, and Mark Perkins, B.S. (Research Specialist, Department of Anesthesiology, University of Wisconsin Medical School, Madison, Wisconsin), for technical support.

References

1. Eger EI II, Koblin DD, Harris RA, Kendig JJ, Pohorille, Halsey MJ, Trudell JR: Hypothesis: Inhaled anesthetics produce immobility and amnesia by different mechanisms at different sites. Anesth Analg 1997; 84:915–8
2. Raines DE, Miller KW: On the importance of volatile agents devoid of anesthetic action. Anesth Analg 1994; 79:1031–3
3. Koblin DD, Chortkoff BS, Laster MJ, Eger EI II, Halsey MJ, Ionescu P: Polyhalogenated and perfluorinated compounds that disobey the Meyer-Overton hypothesis. Anesth Analg 1994; 79:1043–8
4. Kandel L, Chortkoff BS, Sonner J, Laster MJ, Eger EI II: Nonanesthetics can suppress learning. Anesth Analg 1996; 82:321–6
5. Eger EI, Koblin DD, Sonner J, Gong D, Laster MJ, Ionescu P, Halsey MJ, Hudlicky T: Nonimmobilizers and transitional compounds may produce convulsions by two mechanisms. Anesth Analg 1999; 88:884–92
6. Joas TA, Stevens WC, Eger EI: Electroencephalographic seizure activity in dogs during anaesthesia. Br J Anaesth 1971; 43:739–45
7. Simon W, Hapfelmeier G, Kochs E, Zieglgansberger W, Rammes G: Isoflurane blocks synaptic plasticity in the mouse hippocampus. ANESTHESIOLOGY 2001; 94:1058–65
8. Banks MI, Pearce RA: Dual actions of volatile anesthetics on $GABA_A$ IPSCs: Dissociation of blocking and prolonging effects. ANESTHESIOLOGY 1999; 90:120–34
9. Institute of Laboratory Animal Resources: Guide for the Care and Use of Laboratory Animals, 7th edition. Washington, D.C., National Academy Press, 1996
10. Chesney MA, Perouansky M, Pearce RA: Differential uptake of volatile agents into brain tissue *in vitro*: Measurement and application of a diffusion model to determine concentration profiles in brain slices. ANESTHESIOLOGY 2003; 99:122–30
11. Franks NP, Lieb WR: Stereospecific effects of inhalational general anesthetic optical isomers on nerve ion channels. Science 1991; 254:427–30
12. Freund TF, Buzsaki G: Interneurons of the hippocampus. Hippocampus 1996; 6:347–470
13. Mihic SJ, McQuilkin SJ, Eger EI II, Ionescu P, Harris RA: Potentiation of gamma-aminobutyric acid type a receptor-mediated chloride currents by novel halogenated compounds correlates with their abilities to induce general anesthesia. Mol Pharmacol 1994; 46:851–7
14. Eger EI, Xing Y, Pearce R, Shafer S, Laster MJ, Zhang Y, Fanselow MS, Sonner JM: Isoflurane antagonizes the capacity of flurothyl or 1,2-dichlorohexafluorocyclobutane to impair fear conditioning to context and tone. Anesth Analg 2003; 96:1010–8
15. Dutton RC, Maurer AJ, Sonner JM, Fanselow MS, Laster MJ, Eger EI II: The concentration of isoflurane required to suppress learning depends on the type of learning. ANESTHESIOLOGY 2001; 94:514–9
16. Bliss TV, Collingridge GL: A synaptic model of memory: Long-term potentiation in the hippocampus. Nature 1993; 361:31–9
17. Cheung KH, MacIver MB: Isoflurane-induced depression of synaptic LTP does not involve post-NMDA receptor mediated mechanisms. ANESTHESIOLOGY 2002; 96:A15
18. Raines DE, Claycomb RJ, Forman SA: Nonhalogenated anesthetic alkanes and perhalogenated nonimmobilizing alkanes inhibit alpha(4)beta(2) neuronal nicotinic acetylcholine receptors [published erratum appears in Anesth Analg 2002; 95:869]. Anesth Analg 2002; 95:573–7
19. Borghese CM, Harris RA: Anesthetic-induced immobility: Neuronal nicotinic acetylcholine receptors are no longer in the picture. Anesth Analg 2002; 95:509–11

20. Pearce RA: Physiological evidence for two distinct $GABA_A$ responses in rat hippocampus. Neuron 1993; 10:189–200

21. Banks MI, Li TB, Pearce RA: The synaptic basis of GABAA, slow. J Neurosci 1998; 18:1305–17

22. Bai D, Zhu G, Pennefather P, Jackson MF, MacDonald JF, Orser BA: Distinct functional and pharmacological properties of tonic and quantal inhibitory postsynaptic currents mediated by γ-aminobutyric $acid_A$ receptors in hippocampal neurons. Mol Pharmacol 2001; 59:814–24

23. Semyanov A, Walker MC, Kullmann DM: GABA uptake regulates cortical excitability via cell type-specific tonic inhibition. Nat Neurosci 2003; 6:484–90

24. Maurer AJ, Sessler DI, Eger EI II, Sonner JM: The nonimmobilizer 1,2-dichlorohexafluorocyclobutane does not affect thermoregulation in the rat. Anesth Analg 2000; 91:1013–6

25. Sonner J, Li J, Eger EI: Desflurane and nitrous oxide, but not nonimmobilizers, affect nociceptive responses. Anesth Analg 1998; 86:629–34

26. Steffey EP, Laster MJ, Ionescu P, Eger EI, Emerson N: Ventilatory effects of the nonimmobilizer 1,2-dichlorohexafluorocyclobutane (2N) in swine. Anesth Analg 1998; 86:173–8

27. Soltesz I, Smetters DK, Mody I: Tonic inhibition originates from synapses close to the soma. Neuron 1995; 14:1273–83

28. Ratnakumari L, Vysotskaya TN, Duch DS, Hemmings HC Jr: Differential effects of anesthetic and nonanesthetic cyclobutanes on neuronal voltage-gated sodium channels. ANESTHESIOLOGY 2000; 92:529–41

29. Changeux JP, Bertrand D, Corringer PJ, Dehaene S, Edelstein S, Lena C, Le Novere N, Marubio L, Picciotto M, Zoli M: Brain nicotinic receptors: Structure and regulation, role in learning and reinforcement. Brain Res Brain Res Rev 1998; 26:198–216