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Modulation of γ-Aminobutyric Acid Type A Receptor–mediated Spontaneous Inhibitory Postsynaptic Currents in Auditory Cortex by Midazolam and Isoflurane

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Background: **Anesthetic agents that target γ-aminobutyric acid type A ($GABA_A$) receptors modulate cortical auditory evoked responses *in vivo*, but the cellular targets involved are unidentified. Also, for agents with multiple protein targets, the relative contribution of modulation of $GABA_A$ receptors to effects on cortical physiology is unclear. The authors compared effects of the $GABA_A$ receptor–specific drug midazolam with the volatile anesthetic isoflurane on spontaneous inhibitory postsynaptic currents (sIPSCs) in pyramidal cells of auditory cortex.**

Methods: **Whole cell recordings were obtained in murine brain slices at 34°C. $GABA_A$ sIPSCs were isolated by blocking ionotropic glutamate receptors. Effects of midazolam and isoflurane on time course, amplitude, and frequency of sIPSCs were measured.**

Results: **The authors detected no effect of midazolam at 0.01 μM on sIPSCs, whereas midazolam at 0.1 and 1 μM prolonged the decay of sIPSCs by approximately 25 and 70%, respectively. Isoflurane at 0.1, 0.25, and 0.5 mM prolonged sIPSCs by approximately 45, 150, and 240%, respectively. No drug-specific effects were observed on rise time or frequency of sIPSCs. Isoflurane at 0.5 mM caused a significant decrease in sIPSC amplitude.**

Conclusions: **The dose dependence of isoflurane effects on $GABA_A$ sIPSCs in pyramidal cells is consistent with effects on auditory evoked response *in vivo*. By contrast, comparable effects of midazolam on $GABA_A$ sIPSCs arise at concentrations exceeding those currently thought to be achieved *in vivo*, suggesting that the cellular targets of midazolam reside elsewhere in the thalamocortical circuit or that the concentration of midazolam reached in the brain is higher than currently believed.**

THE importance of γ-aminobutyric acid type A ($GABA_A$) receptor–mediated synaptic inhibition for regulating activity in cortical networks is suggested by the profound behavioral effects of benzodiazepines and general anesthetics that target $GABA_A$ receptors in the central nervous system. The effects of these drugs on cognition and perception are ascribed to actions on postsynaptic receptors in which binding or gating properties of $GABA_A$ receptor channels or both are modified to produce inhibitory postsynaptic currents (IPSCs) with prolonged time course and altered peak amplitude, typically resulting in an increased net charge transfer during phasic inhibition.[1–3] Increases in tonic inhibition are also observed,[4] presumably secondary to direct activation of postsynaptic $GABA_A$ receptors or enhanced sensitivity of these receptors to ambient γ-aminobutyric acid. Many general anesthetics also target other proteins in cortical cells, *e.g.*, voltage-gated channels, and therefore, the relative contribution of modulation of $GABA_A$ receptors by these drugs to their effects *in vivo* is unclear. One approach to resolving this issue is to compare the physiologic effects of drugs with multiple protein targets, *e.g.*, volatile anesthetics, with the effects of drugs that are specific for the $GABA_A$ receptor, *e.g.*, benzodiazepines. As a first step toward this end, we investigated the modulation of $GABA_A$ receptor–mediated spontaneous IPSCs by the benzodiazepine midazolam and the volatile anesthetic isoflurane in murine cortical brain slices.

There are no published reports of the effects of midazolam and only one of isoflurane[5] on neocortex at the cellular level. In other areas of the brain, it has been shown that benzodiazepines such as midazolam modulate $GABA_A$ receptor–mediated IPSCs similarly to general anesthetics that target these receptors, *i.e.*, primarily prolonging the time course of IPSCs.[6] A quantitative comparison of the *in vitro* and *in vivo* effects of midazolam and isoflurane requires a comparison of the effects on IPSCs over a range of drug concentrations, as well as estimates of the concentrations of midazolam and isoflurane in cerebrospinal fluid (CSF) after drug applications *in vivo*. We used whole cell voltage clamp recordings to compare modulation of IPSCs in cortical pyramidal cells by three different concentrations of midazolam and isoflurane. In the Discussion, we draw on published reports of drug concentration measurements *in vivo* to relate our data to the effects of midazolam and isoflurane on physiologic responses *in vivo*.

We have chosen the auditory cortex (ACx) as our model neocortical system because cortically mediated auditory-evoked responses are known to be modulated by general anesthetics,[7] whereas the cellular effects of these agents in ACx has not been previously studied. Intraoperative measurement of the midlatency auditory evoked response (MLAER) forms the basis of a promising new technology for monitoring depth of anesthesia.[8] The MLAER is a multiphasic waveform representing the activity of neurons in the primary and secondary ACx in response to transient acoustic stimuli,[9,10] with possible contributions by thalamic and higher-order cortical

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sources.[11] (Primary ACx is functionally defined as that region of the neocortex receiving direct input from the ventral division of the medial geniculate, and secondary ACx is the region immediately adjacent to primary ACx that receives input from both primary ACx and other regions of the medial geniculate.) The MLAER is also observed in nonhuman primates, cats, and rodents, with latencies for the various peaks and troughs of the response similar to those observed in humans.[9,12,13] The effects of midazolam on the MLAER seem to depend on the time course of drug application: Bolus injection followed by continuous infusion of midazolam produces effects on the MLAER that are comparable to those produced by low concentrations of isoflurane,[14,15] whereas bolus injections alone have little effect on the MLAER.[16] For both isoflurane and midazolam, however, the cellular and molecular site mediating the effects of these agents on the MLAER is unknown. Thalamic activation of primary and secondary ACx triggers a cascade of activity in multiple cell types, including excitatory spiny stellate and pyramidal cells of layers IV, V, and VI; pyramidal cells of layers II and III; and several classes of γ-aminobutyric acid–mediated (GABAergic) interneurons in multiple cortical layers.[17–19] This activity cascade is represented in the multiphasic waveform of the MLAER, and therefore, all of these cell types are candidate targets for agents that modulate the MLAER. To gain insight into the relative contribution of modulation of $GABA_A$ receptors on pyramidal cells in the ACx, we measured the effects of isoflurane and midazolam on IPSCs in pyramidal cells of layers IV and V.

Materials and Methods

Slice Preparation

All experimental protocols conformed to American Physiologic Society/National Institutes of Health guidelines and were approved by the University of Wisconsin Research Animal Resources Committee (Madison, Wisconsin). Male and female CBA/J mice (P28-P35) were decapitated during isoflurane anesthesia, and the brain was extracted and immersed in artificial CSF (ACSF; composition: 126 mM NaCl, 26 mM $NaHCO_3$, 1.8 mM KCl, 2.1 mM $CaCl_2$, 1.4 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 10 mM glucose) at 0°–4°C. Slices (500 μm) were obtained from the left hemisphere in a plane 15° off the horizontal plane, as described for auditory thalamocortical slices.[20] Slices were maintained in ACSF saturated with 95% O_2–5% CO_2 at 34°C for 1 h followed by 24°C for 1 h before transfer to the recording chamber, which was perfused at 3 ml/min with ACSF saturated with 95% O_2–5% CO_2 at 34°C.

Layer IV and V pyramidal cells in ACx were visualized and identified based on morphology using a video camera (VE-1000; DAGE MTI, Michigan City, IN) connected to an upright microscope (BX-50WI; Olympus America Inc., Melville, NY) equipped with an infrared band-pass filter (775 ± 75 nm), a long working-distance water-immersion objective (Olympus, 40X, N.A. 0.7), and differential interference contrast optics. Pyramidal cells were identified by the triangular shape of their somata, prominent single apical dendrites, and two smaller caliber basal dendrites. The microscope, recording, and stimulating electrodes were under remote control using an integrated motorized control system (Luigs & Neumann, Ratingen, Germany). Most cells were also labeled with 0.3% biocytin, and morphologic identification was confirmed after processing the tissue using standard histologic techniques.[21]

Patch Clamp Electrophysiology and Recording Protocol

Patch pipettes were fabricated from borosilicate glass (KG-33, 1.7-mm OD, 1.1-mm ID; Garner Glass, Claremont, CA) using a Flaming-Brown two-stage puller (P-87; Sutter Instruments, Novato, CA). Patch pipettes had open-tip resistances of 2–4 MΩ. Whole cell voltage clamp recordings were obtained at 34°C with patch pipettes filled with 100 mM KCl, 40 mM K-gluconate, 10 mM NaCl, 10 mM HEPES, 0.1 mM EGTA, 4 mM MgATP, and 5 mM QX-314 (pH 7.3). Access resistances were less than 20 MΩ and compensated by 20–60%. Cells were held at −60 mV. Spontaneous IPSCs (sIPSCs) were isolated by bath application of 4 mM kynurenic acid to block ionotropic glutamate receptor–mediated currents. All remaining currents were blocked by 20 μM bicuculline (n = 5 cells; data not shown). Kynurenic acid, bicuculline, and all components of the pipette solution and control ACSF were obtained from Sigma-Aldrich (St. Louis, MO).

After forming a gigaohm seal and rupturing the membrane to achieve intracellular access, glutamate receptor antagonists were allowed to equilibrate for 5 min before recording a baseline control period of 15 min. Midazolam was then bath applied for 20–25 min (0.1 and 1 μM) or 35 min (0.01 μM). The midazolam data were taken from the final 5 min of drug application. It was not possible to obtain reliable recovery data from slices exposed to midazolam even when slices were washed for more than 30 min. Midazolam hydrochloride (injectable form, pH approximately 3.0) was obtained from Bedford Labs (Bedford, OH) as a 1-mg/ml solution; to obtain a solution of 1 μM, this stock solution was diluted 2,500-fold.

Isoflurane (Novaplus; Abbott Labs, N. Chicago, IL) was bath applied to slices as follows. Isoflurane was prepared as an aqueous solution from a saturated stock solution (15 mM in ACSF[22]) and diluted to final concentration in ACSF on the day of the experiment. The isoflurane solution was prepared in 500 ml Teflon gas sampling bags (Fisher Scientific International Inc., Hampton, NH; cat. No. 10-923-5) that contained ACSF bubbled with 95% O_2–5% CO_2. Teflon tubing was used between the ACSF

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reservoirs and the recording chamber to minimize loss of the volatile agent before it reached the tissue. Preliminary experiments in which isoflurane concentration was measured in the liquid phase using Ca^{2+}-sensitive electrodes[23] indicated that approximately 15% of the isoflurane was lost before it reached the center of the recording chamber, similar to a previous study using identical techniques and equipment.[24] We calibrated the Ca^{2+}-sensitive electrode by determining the relation between electrode potential and isoflurane concentration in solutions bubbled with isoflurane gas, and in parallel with solutions prepared as for our slices from stock concentrations assuming a saturating concentration of 15 mM.[23] We then measured the electrode potential in the slice chamber while perfusing with different isoflurane concentrations and calculated percent loss according to the measured linear relation between electrode potential and isoflurane concentration. This loss was accounted for in the reported values of the concentration of isoflurane used in these experiments. A drug application protocol identical to that used for the midazolam experiments was used for the isoflurane experiments, *i.e.*, 5 min equilibration with kynurenic acid, 15-min baseline recording period, 20- to 30-min drug recording period. Recovery data were obtained in the isoflurane experiments by recording for more than 30 min after removal of isoflurane from the bathing medium.

We typically observed a significant decrease in mean interevent interval over time, independent of whether midazolam or isoflurane was applied. This likely reflects changes in network activity under our recording conditions over time, unrelated to the application of midazolam or isoflurane, *e.g.*, due to application of glutamate receptor antagonists.[25] Because we were focused on the effects of midazolam and isoflurane on the time course of IPSCs and because similar effects on mean interevent interval were observed independent of drug condition, we do not anticipate that this phenomenon has any effect on our results.

Data were amplified (MultiClamp-700A; Axon Instruments, Union City, CA), low-pass filtered (10 kHz), digitized (20 kHz, DigiData 1322A; Axon Instruments), and recorded using pCLAMP v8.2 (Axon Instruments).

Analysis

Data were analyzed using ClampFit (Axon Instruments) and Origin (OriginLab Corporation, Northampton, MA). Spontaneous IPSCs were analyzed using an automated event detection algorithm.[26] In this algorithm, two windows were moved along the data, a "peak" window and a "baseline" window. At each time point, the data within each window was averaged and the baseline point subtracted from the peak. This yielded a pseudodifferentiated form of the data that was characterized by large, rapid peaks at the onset of $GABA_A$ IPSCs. Threshold-level crossings were identified in this pseudodifferentiated data, with threshold set as $3^*\sigma_{Noise}$, where σ_{Noise} was measured during periods of no visually detectable events and was typically 3–5 pA. Because the baseline value was constantly updated during the analysis, slow changes in baseline had no effect on the accuracy of the algorithm. This algorithm successfully detected more than 99% of sIPSCs. Individual sIPSCs ($n > 100$ in each cell for each condition) were selected for averaging when no other detected events occurred within ±100 ms of the peak. Averaged sIPSCs were then used to measure 10–90% rise times (t_{Rise}). Decay phases were best fit by two exponential components, and decay kinetics were characterized in addition by the weighted sum of the exponential decay components, $\tau_{Dec,Wt} = A_1\tau_1 + A_2\tau_2$, where A_1 and A_2 are the amplitudes of the decay components and τ_1 and τ_2 are their time constants. The time courses of individual, uncontaminated events were also characterized by the time to decay from peak to within 37% of the baseline. Statistical comparisons were performed using the nonparametric Wilcoxon signed rank test with a significance level of 0.05, because 12 of 48 variables tested did not pass the Shapiro-Wilk normality test ($P < 0.05$). Identical results were obtained with the Student *t* test. Data are expressed as mean ± SD.

Results

Properties of Spontaneous IPSCs in Pyramidal Cells

Spontaneous IPSCs were recorded from layer IV and V pyramidal cells in ACx in the presence of ionotropic glutamate receptor antagonists at −60 mV and 34°C and served as an assay of IPSC properties under physiologic conditions (*i.e.*, release from small numbers of release sites due to action potential activity or spontaneous vesicle fusion). Recordings were obtained from 73 pyramidal cells. Most sIPSCs had rapid rise kinetics ($t_{Rise} < 0.6$ ms) and biexponential decay kinetics (see below). A small fraction (typically < 1%) of the sIPSCs in these cells had slower rise times (> 2 ms) and slower decay times (> 15 ms) than the majority of the recorded sIPSCs, presumably reflecting a second population of distal dendritic inhibitory inputs, as observed in the hippocampus.[26] Because of the small number of these events, it was not possible to obtain reliable information on pharmacologic modulation, and we excluded these events from our analyses by restricting individual sIPSC rise times to be less than 1 ms. The remaining sIPSCs on which these data are based occurred at rates of 7.8 ± 6.6 Hz and had rapid rise and decay kinetics ($t_{Rise} = 0.33 \pm 0.087$ ms; $\tau_1 = 3.25 \pm 1.19$ ms, $A_1 = 0.61 \pm 0.12$, $\tau_2 = 13.4 \pm 4.08$ ms).

Effects of Midazolam on IPSCs

Previous studies have shown that the modulation of binding and gating kinetics of $GABA_A$ receptors by ben-

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Table 1. Effects of Midazolam and Isoflurane on Inhibitory Postsynaptic Currents in Auditory Cortex

	Control			Drug			
	Amplitude	t_{Rise}	$\tau_{Dec,wt}$	Amplitude	t_{Rise}	$\tau_{Dec,wt}$	$\tau_{Dec,wt}$ Ratio
ACSF control (n = 13)	50.7 ± 14.1	0.33 ± 0.063	7.52 ± 2.09	48.7 ± 10.9	0.34 ± 0.061	7.70 ± 2.02	0.0271 ± 0.0637
Midazolam + flumazenil (n = 11)	45.7 ± 12.2	0.34 ± 0.11	7.16 ± 1.58	43.1 ± 11.8	0.34 ± 0.11	6.86 ± 2.35	−0.0625 ± 0.134
10 nM midazolam (n = 12)	42.5 ± 12.7	0.36 ± 0.12	6.77 ± 1.90	41.7 ± 9.47	0.38 ± 0.093	6.46 ± 1.91	−0.0291 ± 0.172
100 nM midazolam (n = 6)	57.7 ± 16.4	0.32 ± 0.041	6.73 ± 1.91	59.8 ± 15.1	0.35 ± 0.032	8.42 ± 2.69*	0.244 ± 0.0491
1 mM midazolam (n = 12)	62.4 ± 26.8	0.34 ± 0.076	6.28 ± 1.39	61.1 ± 26.3	0.39 ± 0.065	10.8 ± 3.32*	0.701 ± 0.288
0.1 mM isoflurane (n = 7)	53.1 ± 9.53	0.33 ± 0.068	7.01 ± 1.98	52.9 ± 7.22	0.37 ± 0.075	10.7 ± 2.60*	0.450 ± 0.209
0.25 mM isoflurane (n = 5)	48.1 ± 26.8	0.33 ± 0.084	6.55 ± 1.26	44.2 ± 23.1	0.33 ± 0.076	15.9 ± 4.97*	1.47 ± 0.766
0.5 mM isoflurane (n = 6)	48.1 ± 16.9	0.34 ± 0.074	7.57 ± 1.64	36.5 ± 19.3*	0.38 ± 0.11	30.0 ± 9.91*	2.35 ± 0.640

Effects of drug treatments on mean amplitude (pA), 10–90% rise time (t_{Rise}), and weighted decay time constant ($\tau_{Dec,wt}$; ms) of spontaneous inhibitory postsynaptic currents in pyramidal cells of auditory cortex. The number of cells (n) in each group is indicated in the left column. Data are presented as mean ± SD. The rightmost column shows the fractional change in weighted decay time constant, defined as (Drug − Control)/Control. All statistical comparisons were made within rows, *i.e.*, between the control period and the drug period for that drug condition.

* Significantly different from control period, $P < 0.05$, Wilcoxon signed rank test.

ACSF = artificial cerebrospinal fluid.

zodiazepines is manifested at GABAergic synapses by an increase in the amplitude or the duration of $GABA_A$ receptor–mediated synaptic currents or both.[6,27,28] We observed no consistent effects of midazolam on the mean peak amplitudes of sIPSCs (table 1 and fig. 1). In contrast, bath application of 1 μM midazolam prolonged the decay of spontaneous IPSCs by approximately 70% (table 1 and fig. 1). These effects were observed in every cell tested, indicating that in layer IV and V pyramidal cells in ACx, most $GABA_A$ receptors are sensitive to benzodiazepine agonists. (Distributions of 63% decay times within cells were tested with the Kolmogorov-Smirnoff test [$P < 0.05$].) Application of 0.1 μM midazolam caused a smaller (approximately 24%) increase in decay time course of sIPSCs, whereas 0.01 μM midazolam produced no significant effects on sIPSCs (table 1 and figs. 2 and 3). The latter observation should be interpreted with caution, however, because the value of SD for $\tau_{Dec,Wt}$ (table 1) precludes detecting changes in this parameter smaller than approximately 23% (power analysis with $\alpha = 0.05$, $\beta = 0.8$). However, plotting the decay time as a function of time during the experiment (fig. 2A) strongly suggests that this concentration of midazolam produced no change in decay time course of sIPSCs. Changes in decay time course were not observed in the absence of drug application or when the benzodiazepine antagonist flumazenil (5 μM) was coapplied with 1 μM midazolam (table 1 and fig. 3). Significant decreases in mean interevent interval were observed during bath application of 0.01 and 1 μM midazolam, but

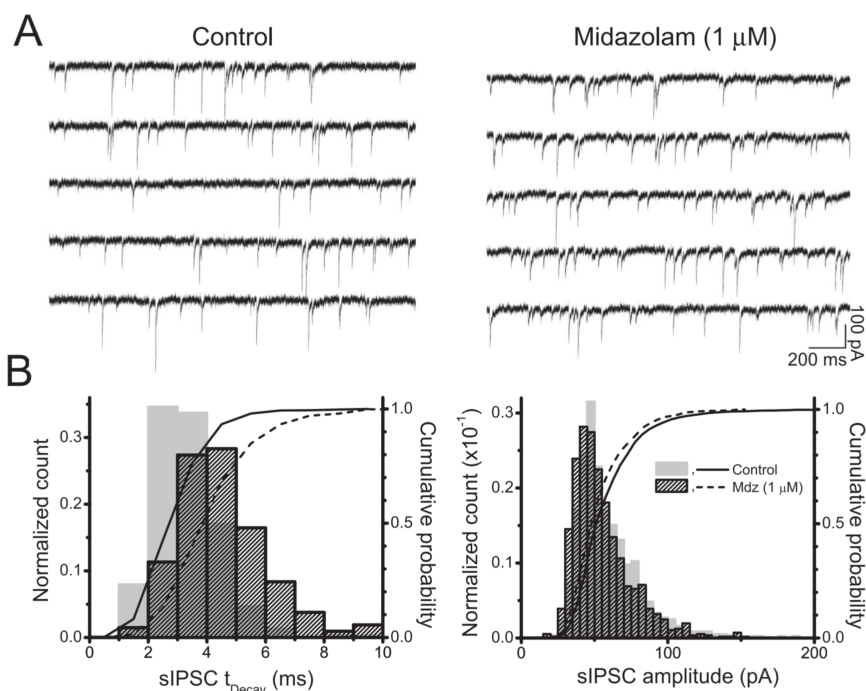

Fig. 1. Properties of spontaneous inhibitory postsynaptic currents (sIPSCs) under control and drug conditions. (*A*) Two epochs comprising 10 s of continuous spontaneous activity recorded under control conditions (*left*) and in the presence of midazolam (1 μM; *right*). The small change in holding current observed in this cell was typically also observed in the absence of drug application. (*B*) Probability distributions (*column plots*) and cumulative probability functions (*line plots*) of 63% decay times (t_{Decay}; *left*), and peak amplitude (*right*) for the same cell as in *A* in control (*gray bars, solid line*) and midazolam (*hatched bars, dashed lines*).

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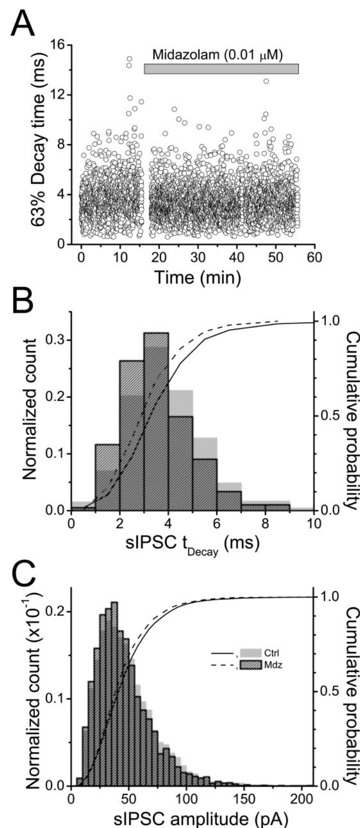

Fig. 2. Midazolam at 0.01 μM has no detectable effect on spontaneous inhibitory postsynaptic currents (sIPSCs). Recording from a cell showing a lack of effect of 0.01 μM midazolam on the decay time course (*A* and *B*) and amplitude (*C*) of sIPSCs. All data are from the same cell. (*A*) Time series plot of 63% decay time measured for approximately 4,500 sIPSCs recorded for 15 min in control solution followed by 40 min in midazolam. (*B*) Probability distribution (*column plots*) and cumulative probability function (*line plots*) of 63% decay times (t_{Decay}) in control (*gray bars, solid line*) and midazolam (*hatched bars, dashed lines*). (*C*) Probability distribution (*column plots*) and cumulative probability function (*line plots*) of spontaneous inhibitory postsynaptic currents peak amplitude in control (*gray bars, solid line*) and midazolam (*hatched bars, dashed lines*).

these effects were likely secondary to nonspecific changes in receptor and network properties over time, because they were also observed under control conditions and in the presence of 5 μM flumazenil (data not shown).

Recent reports suggest that changes in tonic inhibition secondary to either direct activation of $GABA_A$ receptors or increases in the level of or sensitivity to ambient γ-aminobutyric acid may mediate many of the suppressive effects on cortical circuit activity of benzodiazepines and general anesthetics.[29] However, we observed no drug-specific effects on holding current in these experiments. The magnitude of the holding current typically increased by approximately 50 pA over the hour-long course of a recording (*e.g.*, fig. 1), and no significant differences were observed between the magnitude of this change in holding current for control *versus* drug conditions (one-way analysis of variance, $F_{4,47} = 2.523$, $P > 0.05$).

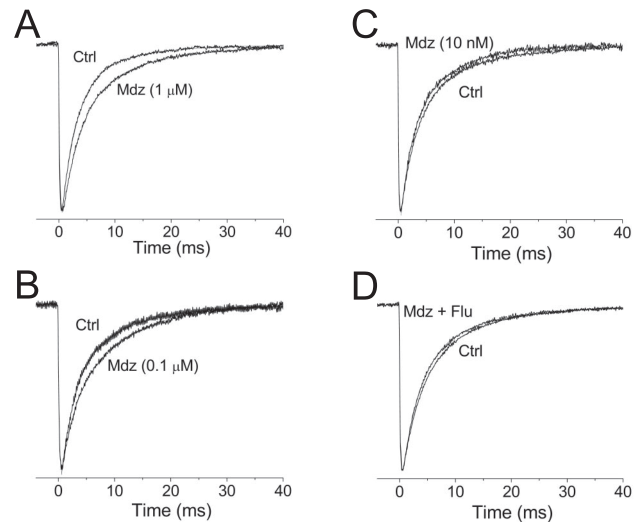

Fig. 3. Modulation of the time course of inhibitory postsynaptic currents by midazolam. Averaged and normalized spontaneous inhibitory postsynaptic currents recorded in four different cells in control conditions and in the presence of 1 μM midazolam (*A*, same cell as in fig. 1), 0.1 μM midazolam (*B*), 0.01 μM midazolam (*C*; same cell as in fig. 2), and 1 μM midazolam plus 5 μM flumazenil (*D*). Ctrl = control; Flu = flumazenil; Mdz = midazolam.

Effects of Isoflurane on IPSCs

Isoflurane is an inhalation anesthetic that in addition to $GABA_A$ receptors is reported to target other protein targets in cortical cells.[3] We tested the effects of isoflurane on sIPSCs in pyramidal cells in ACx to compare with the $GABA_A$ receptor–specific drug midazolam. We chose three concentrations of isoflurane, 0.1 mM (approximately 0.3 minimum alveolar concentration [MAC]), 0.25 mM (approximately 0.85 MAC), and 0.5 mM (approximately 1.7 MAC) to span the range of doses evaluated for their effects on MLAERs in previous studies. At 0.1 mM, isoflurane reversibly prolonged sIPSC decay by approximately 45% (table 1 and fig. 4; data after wash of isoflurane not shown), an effect intermediate between that observed in response to 0.1 and 1 μM midazolam. The decay of sIPSCs was reversibly prolonged by approximately 140 and 230% by 0.25 and 0.5 mM, respectively, effects far greater than those achieved by even the highest concentration of midazolam tested (table 1 and fig. 4; data after wash of isoflurane not shown). No significant changes in holding current were detected (isoflurane *vs.* control, $P > 0.05$, Student *t* test; not shown). Similar to previous reports in the hippocampus and neocortex,[24] the highest dose of isoflurane caused a significant decrease in the mean amplitude of sIPSCs (table 1).

Discussion

The time course and magnitude of $GABA_A$ receptor–mediated inhibition are important for determining the ability of interneurons to regulate integration and spike

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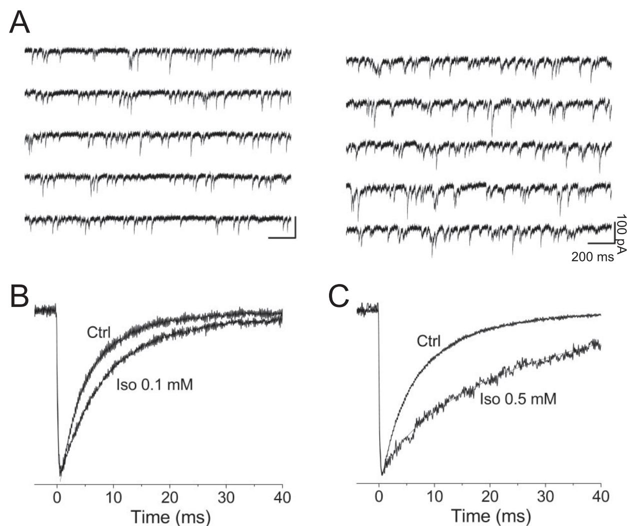

Fig. 4. Effects of isoflurane on spontaneous inhibitory postsynaptic currents. (*A*) Two epochs comprising 10 s of continuous spontaneous activity recorded under control conditions (*left*) and in the presence of isoflurane (0.1 mM; *right*). The small change in holding current observed in this cell was typically also observed in the absence of drug application. (*B* and *C*) Averaged and normalized spontaneous inhibitory postsynaptic currents recorded in 0.1 mM isoflurane (*B*; same cell as in *A*) and 0.5 mM isoflurane (*C*). Ctrl = control; Iso = isoflurane.

initiation in pyramidal cells. There is an ongoing debate in the anesthesia research community about the relative importance of the various protein targets of general anesthetics such as isoflurane for the effects of these agents *in vivo*. We have taken the approach of comparing quantitatively the effects of isoflurane with a receptor-specific agent, midazolam. We have shown that sIPSCs in the primary ACx of mice were prolonged by bath application of both drugs in a dose-dependent manner.

Cell type–specific expression of $GABA_A$ receptors in neocortical circuits may contribute to circuit-specific modulation by anesthetic agents and thus to distinct sites of action and dose dependencies for different behavioral endpoints.[30] We focused on layer IV and V pyramidal cells of ACx because these cells are likely to participate in the MLAER, as they are activated by thalamic excitation both directly and *via* local excitatory interneurons and are inhibited by feedback and feedforward GABAergic inhibition.[31–33] However, the cellular origin of the MLAER is likely to extend to other cell types as well, because this multiphasic evoked response contains components with latencies ranging from 8 to 80 ms and spans multiple cortical areas.[10] In relating the experiments described here to modulation of the MLAER *in vivo*, we focused on effects of the drugs on sIPSCs in concentration ranges spanning those known to modulate the MLAER *in vivo*, the assumption being that if modulation of sIPSCs was observed, this modulation likely contributed to the effects observed on the MLAER, whereas if no modulation of sIPSCs was observed, effects on the MLAER likely were mediated by actions on other cell types in ACx. One challenge in this approach is relating the pharmacology of evoked responses recorded in humans to those recorded in rodents. The MLAER recorded from the scalp in humans consists of a series of peaks and troughs with latencies ranging from approximately 8 ms to approximately 80 ms. In rodents, the latencies of the first two pairs of peaks and troughs, spanning latencies of approximately 6–30 ms,[34] are nearly identical to those recorded in humans,[7] and there is substantial evidence for a cortical basis of these components in both species.[35] Effects of anesthetic agents on the MLAER are also similar in humans and rodents,[7,34,36,37] although the effects of midazolam have not been reported. This similarity in time course, cellular generators, and pharmacology of the MLAER in humans and rodents supports the validity of the correlative approach we have used.

We found that under control conditions, sIPSCs recorded in layer IV and V pyramidal cells in ACx had kinetics comparable to those reported for other primary sensory areas in rat neocortex, with rise times of approximately 0.3–0.4 ms and decay time constants of approximately 5–10 ms.[38,39] Surprisingly, the kinetics of IPSCs recorded in our experiments were substantially slower than those reported for similar experiments performed in rats,[40] presumably reflecting a species difference restricted to ACx.

We observed that midazolam at either 0.1 or 1 μM but not 0.01 μM prolongs the duration of IPSCs in pyramidal cells in ACx, consistent with its effects in other cortical areas. The effects of midazolam on the decay time of IPSCs reported here are similar to those observed in hippocampal pyramidal cells, in which midazolam at 1 μM prolonged τ_{Dec} by 50–75%,[29,41] but no effects were observed in response to 0.01 μM.[29] Much larger effects were reported for concentrations greater than 1 μM in dentate granule cells.[6,42] The effects of midazolam on neocortical pyramidal neurons have not been reported previously, but the benzodiazepine receptor agonist zolpidem at 1 μM produced effects in layer V pyramidal cells of visual cortex similar to those reported here.[28,43]

These effects on IPSCs can be compared with isoflurane, an anesthetic agent that has multiple protein targets in the brain in addition to $GABA_A$ receptors. Here, we found that 0.1 mM isoflurane, corresponding to approximately 0.3 MAC,[44] prolonged IPSCs by approximately 50%, intermediate to the effects of 0.1 and 1 μM midazolam, whereas 0.25 mM and 0.5 mM isoflurane (approximately 0.85 and 1.7 MAC, respectively), prolonged IPSCs to a far greater degree than 1 μM midazolam. These results are similar to a previous study in hippocampus, in which isoflurane prolonged IPSC decays by 75% at approximately 0.15 mM and 150% at approximately 0.3 mM,[24] although larger effects were observed in a separate study in cerebellum.[45]

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The appropriate concentration to use *in vitro* is difficult to determine for a drug such as midazolam, because it is typically given as a bolus injection and complicated kinetic modeling is required to determine approximate CSF concentrations during the period after the injection. In one study, steady plasma concentrations of approximately 200 ng/ml midazolam were obtained by combining a bolus injection of 0.2 mg/kg midazolam and a continuous perfusion of 0.1 mg $\cdot$ kg^{-1} $\cdot$ h^{-1} for 2 h.[15] At steady state, the plasma concentration of free midazolam is the same as the CSF concentration, and therefore, these data can be used to approximate the CSF concentration of midazolam given an estimate of the free unbound fraction of midazolam in plasma, measured at approximately 2% in humans,[46] and the molecular weight of midazolam (362.25 g/M). These data yield an estimated CSF concentration of approximately 0.01 μM midazolam, consistent with what is currently considered the clinically relevant concentration to use *in vitro*[29] but which produces negligible effects on IPSCs and holding current in cells in ACx (table 1 and figs. 2 and 3). This is in contrast to pyramidal cells in hippocampus, in which 0.01 μM midazolam produced a significant increase in holding current although no effects on the time course of IPSCs.[29] Direct measurements of the concentration of midazolam in the brain after bolus or continuous perfusion of benzodiazepines *in vivo* or both will be a significant step toward understanding the mechanism and locus of benzodiazepine effects in cortex.

If these estimates of the concentration of midazolam in CSF are accurate, it has implications for determining the cellular basis for modulation by midazolam of activity in ACx *in vivo*. Investigations of the effects of benzodiazepines on ACx *in vivo* are primarily limited to studies of the modulation of auditory evoked responses in humans and other animals. The effects of midazolam on the MLAER depend on the method of drug application: Bolus injections at doses sufficient for surgical procedures (0.2–0.3 mg/kg) produce only modest effects on latencies and amplitudes of the components of the MLAER.[16,47] However, in both of these studies, recordings of the MLAER were taken only at one or two time points within 10 min of the bolus injection, and therefore, it is possible that delayed effects of midazolam were not detected. In contrast, bolus injections of 0.2 mg/kg followed by continuous perfusions of midazolam (0.1 mg $\cdot$ kg^{-1} $\cdot$ h^{-1} for 2 h) produced decreases in component amplitudes of 35–40%,[15] nearly as large as those reported for isoflurane at approximately 0.3 MAC in humans and rats (45–50%),[14,34] although increases in latencies due to midazolam (5–10%) were smaller than those reported for low doses of isoflurane (20–40%). We and others have observed that this concentration of isoflurane is sufficient to prolong IPSCs in cortical cells by approximately 50–200% (fig. 4).[5,24] If midazolam can produce effects on the MLAER nearly comparable to those of isoflurane without measurable effects on IPSCs in layer IV and V pyramidal cells in ACx, it is likely that the targets of midazolam underlying the observed effects on the MLAER are other cell types in ACx contributing to the MLAER (*e.g.*, supragranular pyramidal cells, spiny stellate cells, GABAergic interneurons), and presumably these cellular targets have $GABA_A$ receptors that are more sensitive to midazolam. Identification of these targets awaits further studies on modulation of IPSCs by midazolam in ACx.

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