Effects of γ-Aminobutyric Acid Type A Receptor Modulation by Flumazenil on Emergence from General Anesthesia

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ABSTRACT

Background: Transitions into conscious states are partially mediated by inactivation of sleep networks and activation of arousal networks. Pharmacologic hastening of emergence from general anesthesia has largely focused on activating subcortical monoaminergic networks, with little attention on antagonizing the γ-aminobutyric acid type A receptor (GABAAR). As the GABAAR mediates the clinical effects of many common general anesthetics, the authors hypothesized that negative GABAAR modulators would hasten emergence, possibly via cortical networks involved in sleep.

Methods: The authors investigated the capacity of the benzodiazepine rescue agent, flumazenil, which had been recently shown to promote wakefulness in hypersomnia patients, to alter emergence. Using an in vivo rodent model and an in vitro GABAAR heterologous expression system, they measured flumazenil’s effects on behavioral, neurophysiologic, and electrophysiologic correlates of emergence from isoflurane anesthesia.

Results: Animals administered intravenous flumazenil (0.4 mg/kg, n = 8) exhibited hastened emergence compared to saline-treated animals (n = 8) at cessation of isoflurane anesthesia. Wake-like electroencephalographic patterns occurred sooner and exhibited more high-frequency electroencephalography power after flumazenil administration (median latency ± median absolute deviation: 290 ± 34 s) compared to saline administration (473 ± 186 s; P = 0.042). Moreover, in flumazenil-treated animals, there was a decreased impact on postanesthesia sleep. In vitro experiments in human embryonic kidney-293T cells demonstrated that flumazenil inhibited isoflurane-mediated GABA current enhancement (n = 34 cells, 88.7 ± 2.42% potentiation at 3 μM). Moreover, flumazenil exhibited weak agonist activity on the GABAAR (n = 10 cells, 10.3 ± 3.96% peak GABA EC20 current at 1 μM).

Conclusions: Flumazenil can modulate emergence from isoflurane anesthesia. The authors highlight the complex role GABAARs play in mediating consciousness and provide mechanistic links between emergence from anesthesia and arousal.

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What We Already Know about This Topic

- γ-Aminobutyric acid type A (GABAAR) receptors are one of the main targets of anesthetic agents
- It is conceivable that negative modulation of GABAAR receptors might accelerate recovery of wakefulness after general anesthesia
- The administration of flumazenil, a competitive antagonist at the benzodiazepine binding site on GABAAR receptors, was evaluated in rodents anesthetized with isoflurane for the effects on behavioral and neurophysiologic markers of emergence as well as postanesthesia sleep patterns

What This Article Tells Us That Is New

- Flumazenil had a modest effect on accelerating emergence from isoflurane anesthesia
- Prolongation of sleep time in the first day after isoflurane anesthesia was significantly reduced by flumazenil
- The data suggest that inhibition of γ-aminobutyric acid type A receptors can not only hasten emergence but can mitigate postanesthesia sleep disturbances

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long-standing clinical use as an emergency treatment for benzodiazepine overdose. Its potential for improving recovery from anesthesia/sedation using benzodiazepines has been examined in clinical studies with mixed results. One study found that patients who were administered flumazenil upon emergence from isoflurane anesthesia had a faster recovery, improved vigilance, and a subjective rating of recovery as more pleasant. Conversely, studies have shown that flumazenil can potentiate the sedative qualities of propofol in patients undergoing minor surgery. Our previous work demonstrated that flumazenil can promote wakefulness in hypersomnic patients. Here, we explore the possibility that the effect of flumazenil on promoting wakefulness translates to the postanesthetized state.

Transitions between conscious and unconscious states (i.e., sleep and anesthesia) involve similar brain networks that mediate arousal. Despite the heavy influence of cortical electroencephalography (EEG) on sleep categorization, the manipulation of subcortical networks for anesthetic emergence has received more research attention. This might be because many of the brain stem nuclei involved in arousal are fairly well characterized and the pharmacology of these exhibits regional anatomic specificity. For example, pharmacologic or electrical stimulation of the ventral tegmental area (rich in dopaminergic neurons) has successfully reversed the effects of general anesthesia. Similarly, excitation of the thalamus through microinfusion of potassium channel antagonists or nicotine resulted in behaviors indicative of arousal. The cholinergic system has also been manipulated to reverse volatile anesthesia globally via intracerebroventricular injection of neostigmine.

We hypothesized that flumazenil can reverse the actions of isoflurane anesthesia on the GABA<sub>R</sub> and expedite emergence. To test these hypotheses, we used a rodent model of anesthesia emergence to test the effect of flumazenil on several physiologic correlates of emergence, as well as a heterologous expression system to test the effect of flumazenil on GABA<sub>R</sub> function. We predicted that animals treated with flumazenil would exhibit hastened behavioral and neurophysiologic markers of emergence from isoflurane anesthesia. At the cellular level, we further predicted that flumazenil would antagonize the actions of isoflurane at the GABA<sub>R</sub>.

**Materials and Methods**

**Experimental Design**

Figure 1 summarizes our experimental paradigm. Briefly, adult male Sprague-Dawley rats (Charles River, USA) recovered from EEG implantation surgery (see EEG/EMG Recording; Surgery section below) for 7 to 10 days before receiving isoflurane anesthesia. Baseline EEG data were collected in activity chambers for 48 h before anesthesia. After 60 min of isoflurane anesthesia, rats were given an intravenous injection of either saline or flumazenil, returned to their activity chamber, and passively observed through emergence from anesthesia. During emergence, we used qualitative behavioral measures and quantitative EEG to characterize time to wakeful behaviors and neurophysiologic effects on brain activity. Animals remained connected to EEG and electromyography (EMG) electrodes for 48 h after anesthesia. During this time, animals continued their normal active/inactive cycle, and sleep periods were analyzed.

A total of 26 animals were used in the study: 14 animals in the saline control group and 12 animals in the flumazenil group. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Emory University (Atlanta, Georgia) and the Atlanta VA Medical Center (Atlanta, Georgia). Throughout the protocol, animals were housed under a 12:12 h light–dark cycle with food and water provided ad libitum. Note that the anesthesia challenge and recovery occurred during the inactive portion of the animal’s circadian cycle.

**EEG/EMG Recording: Surgery**

EEG electrodes were surgically implanted into male rats weighing 370 to 450 g as described previously. Animals were anesthetized with chloral hydrate (400 mg/
kg intraperitoneal) and placed into a stereotactic frame. Four holes were drilled in the skull and two pairs of sterile 0–80 × 3/32 screw electrodes (Plastics One, USA) inserted. With reference to bregma, the first electrode pair was placed anterior-posterior (AP) −1.5 mm, medial-lateral (ML) 3.0 mm and AP −6.3 mm, ML 3.5 mm. These lateral coordinates overlaid the primary forelimb somatosensory cortex as well as the primary visual cortex; thus, this allows recording of cortical areas (referred to as the “cortical” lead). The second electrode pair was placed contralaterally at AP +2.3 mm, ML 1.5 mm and AP −3.6 mm, ML 1.5 mm and overlaid the primary and secondary motor cortices. These medial coordinates allow recording of hippocampal areas (referred to as the “hippocampal” lead). Note that this is not a true hippocampal lead, nor can the electrical activity measured from this lead be explicitly linked to the hippocampus. Neck EMG was recorded with intramuscular electrode pairs inserted into the left and right nuchal muscles. EEG and EMG electrodes were attached to a microconnector (Winchester Electronics, USA), and a dental acrylic compound (Plastics One) was applied to ensure fixation of the assembly to the skull. Once the acrylic skullcap had dried, the free ends of the incision were sutured, leaving the microconnector pins exposed for recurrent, minimally invasive recording.

EEG/EMG Recording: Isoflurane Emergence Protocol and Testing

EEG-outfitted animals were placed in an anesthesia induction chamber precharged with 4% isoflurane in oxygen and maintained for 2 min; during this time, all animals had loss of righting reflex. Animals were then moved to an absorbent, covered heating pad and fitted with a nose cone for anesthesia, a rectal temperature probe, and a pulse oximetry clip. Animals were maintained at 2% isoflurane for 60 min. During steady-state anesthesia delivery, body temperature, respiratory rate, heart rate, oxygen saturation measured by pulse oximetry (SpO₂), and expired gases were measured at 5-min intervals. At 56 min of steady state, animals were fitted with a 24-gauge lateral tail vein catheter, and from 58 to 60 min, animals were randomized to receive an intravenous injection of either 0.9% sterile saline or 0.4 mg/kg flumazenil (West-Ward Pharmaceuticals, USA), a dose that roughly corresponds to 5 mg flumazenil in a 70 kg human (the maximum recommended intravenous dosing for treatment of benzodiazepine overdose).21 Animals were then fitted with an EEG recording cable, and the isoflurane was stopped. Animals were immediately placed in the EEG recording chamber, tethered to the commutator, and visually monitored and timed for eye blink and ambulation as behavioral signs of emergence from anesthesia. Ambulation was defined as a coordinated activity involving the fore and hind limbs in a diagonal crossmatched pattern (not ataxic). Observers were blinded to the animal’s treatment group for all behavioral studies and sleep scoring. The positioning of the EEG recording cable precludes measurement of a righting reflex, so the first signs of ambulation were recorded as a replacement metric along with the ongoing EEG/EMG monitoring. EEG wake was defined empirically from EEG traces as the transition from high-amplitude, low-frequency oscillations to low-amplitude, high-frequency oscillations. Note that ambulation always occurred after EEG wake.

A subset of animals was used for quantifying sleep characteristics (eight in each group). In these animals, EEG and EMG signals were preamplified by Grass model 12A5 amplifiers (Natus Neurology, USA). Signals were then processed for sleep stage scoring via Somnologica Science (Embla, USA); EEG signals were high-pass filtered at 1 Hz and low-pass filtered at 30 Hz, while EMG signals were high-pass filtered at 10 Hz and low-pass filtered at 40 Hz. The amplified and filtered signals were outputted to a analog-to-digital converter (PCI-MIO-16E-4; National Instruments, USA), digitally processed, and viewed on a real-time basis. Data were parsed into 10-s epochs for sleep stage scoring using standard criteria; each epoch was scored by one blinded observer and categorized as wake, non–rapid eye movement sleep (NREM), or rapid eye movement sleep (REM).

To evaluate the changes in sleep behavior, we compared total sleep time, NREM, and REM sleep for 24-h periods before isoflurane anesthesia (baseline) and after isoflurane anesthesia (postanesthesia day 1 [PAD1]). For both periods, analysis began at the beginning of the animal’s active phase (7:00 PM). This time was chosen as it minimized disturbances of the animal and was most convenient for cage maintenance. Note that the PAD1 period began more than 5 h after anesthetic emergence, at which point it is estimated that 95% of isoflurane has been eliminated.22

EEG Spectral Analysis

For a subset of animals (12 in each group), reamplified raw EEG traces from emergence and recovery periods were imported into MATLAB (MathWorks, USA) for spectral analysis using custom functions. Two saline animals were not included as their EEG headcap had fallen off shortly after anesthesia. In order to preserve frequency content throughout alpha, delta, and theta spectral bands, raw EEG traces were low-pass filtered at 40 Hz and high-pass filtered at 0.25 Hz. Spectrogams were computed for individual animals in MATLAB (spectrogram.m) to show the change of spectral EEG properties during anesthesia emergence. Spectrogams were calculated using 10-s windows with a 1-s shift and covered the entire emergence period, beginning at cessation of isoflurane anesthesia to 3 min after EEG wake. Note that spectrograms were used for qualitative analyses only and no statistical analyses were performed on these data.

To quantitatively compare the differences in EEG between flumazenil and saline groups, we calculated power spectral density (PSD) from 20-s EEG episodes during early emergence and late emergence. We defined early emergence as the 20-s epoch beginning 30 s after cessation of isoflurane and late emergence as the 20-s epoch beginning at EEG wake. In 2 out of 24 cases, the late emergence episode was
distorted by artifacts; in these animals, we used the first artifact-free 20-s episode after EEG wake.

PSDs were calculated in MATLAB using the pwelch function with default settings. The presented results are based on the relative power in the 0.5 to 30 Hz range, normalized by division through the total power in this range.

**In Vitro Electrophysiology**

**Cell Culture and Transfection.** Human (wild-type: α1, β2, and γ2) GABA_ARs and green fluorescent protein cDNAs were subcloned into the pCIS2 vector and transfected into human embryonic kidney-293 cells (American Type Culture Collection, USA) using X-tremeGENE 9 transfection reagent (Roche Diagnostics, USA). These cells were maintained in culture on poly-D-lysine-treated glass coverslips supplemented with 5% fetal bovine serum (Hyclone, USA), l-glutamine (0.292 µg/ml), penicillin G sodium (100 U/ml), and streptomycin sulfate (100 mg/ml).

**Recording.** For whole cell patch clamp recording, coverslips were transferred 36 to 72 h after transfection and cDNA removal to a recording chamber and continuously superfused with extracellular solution (145 mM NaCl, 3 mM KH2PO4, 1.5 mM CaCl2, 1 mM MgCl2, 6 mM d-glucose, and 10 mM HEPES–NaOH adjusted to pH 7.4). GABA_A expressing cells were identified by green fluorescent protein fluorescence and voltage clamped at −60 mV using either a Multiclamp 700A amplifier or an Axopatch-1D amplifier (Molecular Devices, USA). The resistance of the patch pipette was 4 to 6 MΩ when filled with intracellular solution (145 mM N-methyl-d-glucamine hydrochloride, 5 mM dipotassium adenosine triphosphate, 1.1 mM EGTA, 2 mM MgCl2, 5 mM HEPES–KOH, and 0.1 mM CaCl2 adjusted to pH 7.2). Responses were low-pass filtered (100 Hz, −3 dB, four-pole Bessel) and digitized with a 1322A interface (Molecular Devices) using pCLAMP and stored for off-line analysis. All experiments were performed at room temperature (21°C to 24°C).

Rapid application of drugs to activate ligand-gated chloride currents was performed as described previously.23 Solutions including GABA and/or modulators were perfused onto the cell using a motor-driven solution exchange device (Rapid Solution Changer RSC-160; Molecular Kinetics, USA). Solutions were exchanged within approximately 50 ms. Laminar flow out of the rapid solution changer head was achieved by driving all solutions at identical flow rates (1.0 ml/min) via a multichannel infusion pump (KD Scientific, USA). The solution changer was driven by protocols in the acquisition program pCLAMP 9.2 (Molecular Devices).

**Pharmacology.** To study the effect of modulators on GABA currents, cells were superfused with extracellular saline before switching solutions or concentrations for 2 s followed by a return to saline for at least 8 s before any subsequent drug application. GABA was diluted in extracellular solution shortly before use. Flumazenil was dissolved in dimethyl sulfoxide, and when used, equivalent amounts of dimethyl sulfoxide were added to the other solutions for control comparisons. Care was taken to remove all air bubbles from the perfusion setup when working with isoflurane. Previous dose–response studies of GABA on evoked current in our laboratory consistently demonstrated that responses evoked by 100 µM GABA or less did not desensitize, and the amplitude of the responses to 100 µM and above typically declined by 10 to 15% in the continued presence of agonist. For flumazenil dose–response studies, flumazenil or GABA doses were individually infused through dedicated syringes to avoid chemical contamination. To study anesthetic-reversal effects of flumazenil, we measured peak currents evoked by the EC20 of GABA (10 µM), by the EC20 of GABA plus 280 µM isoflurane (approximately equivalent to 1 minimum alveolar concentration24–26), or by both plus increasing doses of flumazenil. Flumazenil doses for *in vivo* studies were chosen based on approximate plasma and cerebrospinal fluid concentrations10 adjusted for the higher metabolic rate of rats.21

**Analysis.** To quantify dose–response relationships, peak current amplitudes were extracted from raw data using custom MATLAB functions and organized in Microsoft Excel (USA). To fit a nonlinear dose–response curve and derive standard Hill coefficients, current peaks were fitted to a Hill equation of the form

\[
I = I_{\text{max}} \times \frac{[\text{GABA}]^{nH}}{[\text{GABA}]^{nH} + EC_{20}^{nH}}
\]

where \(I\) is the peak of each current, \(I_{\text{max}}\) is the maximum whole cell current amplitude, \([\text{GABA}]\) is the GABA concentration, \(EC_{20}\) is the GABA concentration eliciting a current equal to half of \(I_{\text{max}}\), and \(nH\) is the Hill coefficient.

To measure flumazenil reversal of isoflurane-induced potentiation of the GABA_A function, isoflurane potentiation was first calculated as the percentage increase in the peak response to the application of 280 µM isoflurane and GABA, relative to the 10 µM GABA response, or

\[
100 \times \left( I_{\text{IsoGABA}} - I_{\text{IsoGABA}} \right) / I_{\text{GABA}}
\]

Reversal by flumazenil was calculated as the difference between this potentiation and the potentiation observed in the presence of an ascending dose of flumazenil (0.1, 1.0, 3.0, 10, or 30 µM), or

\[
100 \times \left( I_{\text{IsoGABA}} - I_{\text{IsoGABA}} \right) / I_{\text{GABA}} - \left( I_{\text{IsoGABA}} - I_{\text{GABA}} \right)
\]

where \(I_{\text{IsoGABA}}\) is the peak current evoked by GABA, \(I_{\text{IsoGABA}}\) is the current evoked by application of both GABA and isoflurane, and \(I_{\text{isoGABA}}\) is the current evoked by application of GABA, isoflurane, and flumazenil.

**Statistical Analysis**

To compare latencies to emergence, we used the Mann–Whitney U test (unpaired data using MATLAB function *ranksum*); to compare differences in baseline and
postanesthesia sleep behavior, we used the Wilcoxon signed-rank test (paired data using MATLAB function `signrank`). Center estimations are presented as median ± median absolute deviation (MAD) for these nonparametric tests. For emergence times and sleep times, we presented measures of effect size together with 95% CIs derived through 10,000-fold bootstrapping in addition to the $P$ values, allowing us to quantify the magnitude of an effect. The effect size of choice was the area under the receiver-operating characteristic curve (AUC) for the independent emergence times and Hedges $g$ for the dependent sleep behavior. The Hedges $g$ is a modification of Cohen $d$ that makes it better applicable to small sample sizes. We consider values of $g$ greater than 0.8 a strong effect and $g$ less than 0.5 a medium effect. It must be noted that these boundaries follow no strict rules, but may help to give an impression regarding the strength of an effect. We only present the $g$ values if the corresponding 95% CIs do not contain 0. Consistent with the statistical comparison of EEG spectra used by other groups, we compared PSDs between saline and flumazenil groups; we calculated the 95% CI using the jackknife method and evaluated significant differences using the MATLAB `two_group_test` function with a significance level set to $P < 0.001$ using methods from the Chronux toolbox. For in vitro electrophysiologic studies, statistical significance for dose–response relationships was assessed using one-way ANOVA with Dunnett post hoc comparisons ($\alpha = 0.05$). Data are presented as mean ± SEM.

**Results**

**Intravenous Flumazenil Hastens EEG and Behavioral Correlates of Emergence from Isoflurane Anesthesia**

Representative EEG/EMG traces during EEG emergence and recovery are shown for a saline-treated animal (fig. 2, A and B) and a flumazenil-treated animal (fig. 2, C and D). Arrows indicate EEG wake (left), eye blink (middle), and ambulation (right). Note that nuchal EMG activity precedes ambulation as the animal is making subtle movements while tonic muscle activity is returning. The corresponding box plots of the emergence times are presented in figure 3. Time to EEG wake was significantly shorter for the flumazenil group (median latency ± MAD, 290 ± 34 s) than for the saline group (473 ± 186 s; $P = 0.042$; AUC: 0.74 [0.55 to 0.93]). A similar trend was noted with other markers of emergence. Flumazenil-treated animals had shorter latencies to eye blink (293 ± 25 s) compared to the saline-treated animals (482 ± 193 s; $P = 0.054$; AUC: 0.73 [0.53 to 0.92]) and shorter latencies to ambulation (flumazenil 348 ± 109 s, saline 646 ± 169 s; $P = 0.117$; AUC: 0.68 [0.45 to 0.89]) that did not achieve statistical significance. The corresponding box plots of the emergence times are presented in figure 3. There were no differences in the latency from EEG wake to ambulation across groups ($P = 0.643$; AUC: 0.44 [0.21 to 0.67]). Although changes in ventilatory parameters are a major contributor to isoflurane elimination and consequently latency to end emergence, we recorded respiratory parameters in pilot studies and did not observe any statistically significant differences among the groups (table 1).

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**Fig. 2.** Electroencephalography (EEG)/electromyography (EMG) recordings of the transition to arousal from anesthesia. (A) Representative waveforms from an animal given saline (SAL) via tail vein catheter at the cessation of isoflurane anesthesia (ISO OFF). EEG from the medial (hippocampal) lead is shown. Arrows designate (from left to right) isoflurane cessation and administration of saline, EEG wake (defined as a transition to lower amplitude, faster oscillatory activity), eye blink, and ambulation. (B) Time-expanded 10-s epoch capturing the transition to a wakeful EEG (before the onset of movement). The gray rectangle in (A) corresponds to this expansion in (B). (C) Representative waveforms from an animal given flumazenil (FLZ) via tail vein catheter at the cessation of isoflurane anesthesia. Arrows designate (from left to right) isoflurane cessation and administration of flumazenil, EEG wake, eye blink, and ambulation. (D) Time-expanded 10-s epoch capturing the transition to a wakeful EEG (before the onset of movement). The gray rectangle in (C) corresponds to this expansion in (D).
Flumazenil administration mitigates increase in sleep times post anesthesia challenge. Although there was no significant difference in baseline total sleep time between the saline and flumazenil groups ($P = 0.383$), the total sleep time for the saline group was significantly increased from 11.1 ± 0.5 h (median ± MAD) at baseline to 12.7 ± 0.2 h on PAD1 ($P = 0.008$). Hedges $g$ was $-1.98 (-3.42$ to $-0.54)$ and indicated a strong effect on sleeping behavior caused by isoflurane. There were no significant differences in sleep time after anesthesia compared to baseline in flumazenil-treated animals ($P = 0.195$). The total sleep time changed from 11.8 ± 1 h to 12.5 ± 0.3 h. Hedges $g$ was $-0.55 (-1.57$ to $0.46)$.

Saline-treated animals exhibited significantly more time spent in NREM sleep compared to flumazenil-treated animals. Figure 6A shows the change in the total sleep time due to isoflurane anesthesia and the contribution of NREM (fig. 6B) and REM (fig. 6C) sleep to the total sleep time. For saline-treated animals, isoflurane had a strong effect on the time spent in NREM sleep (baseline, 9.4 ± 0.3 h; PAD1, 10.8 ± 0.3 h; $P = 0.008$; $g = -1.69 [-4.06$ to $-1.12])$ as well as the time spent in REM sleep (baseline, 1.5 ± 0.3 h; PAD1, 2.0 ± 0.1 h; $P = 0.008$; $g = -0.96 [-2.36$ to $-0.66])$. With flumazenil, these effects disappeared for NREM (baseline, 10.0 ± 0.7 h; PAD1, 10.4 ± 0.3 h; $P = 0.461$; $g = -0.39 [-1.53$ to $0.50]$). The time spent in REM sleep changed from 1.8 ± 0.3 h to 2.0 ± 0.3 h; $P = 0.008$; $g = -0.77 (-1.77$ to $0.06)$.

**Quantitative EEG Reveals Neurophysiologic Effects of Flumazenil Administration**

Similar to raw EEG traces, all animals regardless of group showed a transition of EEG frequency content. Specifically, we observed a shift in EEG from predominance in delta power (0.5 to 4 Hz) during early emergence toward a more uniform distribution of power among higher frequencies during late emergence. Representative spectrograms for an animal in each group are shown in figure 4. In general, both cortical (fig. 4A) and hippocampal (fig. 4B) leads exhibited this shift in frequency profiles from emergence to recovery. Qualitatively, these two examples also demonstrate more beta activity (approximately 14 to 30 Hz) pre- and post-EEG wake and a more modest theta power (4 to 8 Hz) on the spectrogram from the hippocampal lead (fig. 4B) in the flumazenil-treated example.

Figure 5 shows averaged PSDs in both leads for all animals by treatment group. During early emergence, the spectral content is dominated by delta waves in both groups. This frequency distribution becomes more uniform during late emergence, with an increase in beta power. The spectral properties of the corresponding EEG differed between flumazenil and saline groups. During late emergence, flumazenil-treated animals had significantly increased power over a broad band of frequencies within the beta range in the cortical lead (fig. 5B, top). We did not observe such a phenomenon in the hippocampal lead (fig. 5B, bottom).

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**Flumazenil Is a Competitive Antagonist of GABAA Rs in the Absence and Presence of Isoflurane**

The dose–response relationship of the applied GABA concentration with and without coapplied flumazenil was tested using α1β2γ2s GABA A Rs heterologously expressed in human embryonic kidney-293 cells. Electrophysiologic recordings of GABA A R-mediated chloride current amplitudes were measured using whole cell patch clamp recordings. Figure 7A shows representative traces from a single cell of a chloride current evoked by increasing concentrations of GABA in the absence (black trace) and presence of flumazenil (orange trace). These experiments were done pairwise: with each cell, flumazenil decreased the maximal peak GABA current without shifting the dose–response relation, consistent with a competitive rather than noncompetitive antagonist. The summative dose–response curve (fig. 7B) demonstrates that this effect is most consistently seen at higher GABA concentrations (GABA > 30 μM).

Flumazenil also partially reversed the isoflurane enhancement of GABA A R currents. Figure 8A shows representative traces from a single cell of a chloride current evoked by EC20 GABA (black bars) and isoflurane (purple bars). Addition of isoflurane enhanced chloride currents through GABA A Rs. However, in

**Table 1. Flumazenil Does Not Change Respiratory Parameters in a Rodent Model of Emergence**

<table>
<thead>
<tr>
<th>Agent</th>
<th>−10′</th>
<th>−5′</th>
<th>0′</th>
<th>1′</th>
<th>2′</th>
<th>3′</th>
<th>4′</th>
<th>5′</th>
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<tbody>
<tr>
<td>Saline</td>
<td>51.2±5.0</td>
<td>49.7±4.5</td>
<td>48.7±4.7</td>
<td>63.2±8.7</td>
<td>66.8±6.9</td>
<td>72.8±5.2</td>
<td>73.6±8.3</td>
<td>78.0±8.5</td>
</tr>
<tr>
<td>Flumazenil</td>
<td>55.0±3.6</td>
<td>52.6±4.2</td>
<td>52.6±5.1</td>
<td>60.0±6.2</td>
<td>62.8±7.2</td>
<td>69.0±10.5</td>
<td>72.0±10.6</td>
<td>80.0±11.3</td>
</tr>
</tbody>
</table>

Average respiratory rate for animals is represented as mean ± SD (saline: n = 6, flumazenil: n = 5).

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Flumazenil Has Intrinsic Agonist Activity on the GABA$_A$R
Flumazenil modulated GABA$_A$R function in the absence of GABA or any other agonist or receptor modulator. Figure 9A shows that flumazenil elicited chloride currents in a sigmoidal, dose-dependent manner. In comparison to an EC$_{20}$ GABA response elicited from the same cell, the maximal flumazenil evoked current is relatively small, suggesting weak intrinsic activity. Figure 9B represents an agonist dose–response relationship for flumazenil’s effects on measured chloride current, scaled to the within-cell EC$_{20}$ GABA current value. These effects suggest that flumazenil is a weak agonist of the GABA$_A$R when GABA itself is low or nonexistent and competes with GABA at high doses. This competitive antagonist property also allows flumazenil to incompletely reverse the enhancement of the GABA$_A$R by isoflurane even at moderate (EC$_{20}$) doses of GABA.

Discussion
The current work focused on a potential role of flumazenil in altering emergence from anesthesia. Previous work with hypersomnic patients has demonstrated that modulation of GABA signaling via flumazenil improves vigilance and wakefulness in these patients. Our work supports the notion that antagonism of GABAergic signaling can contribute to the reestablishment of waking behavior. While this arousal from anesthesia could in part be due to modulation...
of common subcortical arousal networks involved in sleep, our work cannot exclude modulation of cortical GABAergic synapses. We expected that negative modulation of the GABA_R could mitigate the hypoactive effects of isoflurane and subsequently expedite emergence. In this study, we found that flumazenil had a modest effect on hastening emergence in rats, and this is associated with an increase in high-frequency neurophysiologic activity in the cortex. Flumazenil administration prevented prolongation of the total sleep time in the first postanesthesia day and did not significantly change sleep architecture. Taken together, administration of flumazenil during emergence may prevent an
adverse effect of isoflurane on normal sleep–wake cycles. The results from pharmacologic experiments of flumazenil on the GABA, R suggest that although antagonism of the GABA, R is possible with flumazenil, partial agonism can also be exhibited, consistent with the pharmacologic profile of a competitive antagonist with weak intrinsic agonist activity. These in vitro findings might help explain some of the variability in emergence characteristics previously shown with flumazenil administration in humans.

Flumazenil Hastens Behavioral and Neurophysiologic Markers of Emergence from Anesthesia through Its Interaction with Sleep and Arousal Networks

Our neurophysiologic findings support the notion that flumazenil’s effect on postanesthesia behavior is related to its effects on sleep and arousal networks. Metrics of emergence such as time to ambulation and EEG wake occurred significantly earlier in flumazenil-treated animals. In both saline- and flumazenil-treated animals, EEG wake was readily detected by a transition in the EEG record from predominantly large, slow delta waves to a more uniform distribution of EEG power. This increased EEG power in higher frequency bands (greater than 14 Hz) is indicative of arousal and occurred significantly earlier in animals treated with flumazenil. Human EEG data gathered during recovery from general anesthesia characteristically exhibit an increase in high-frequency power. Our results are also consistent with those of Dahaba et al., who determined that flumazenil changed the frontal EEG of patients undergoing intravenous anesthesia toward a pattern more indicative of awakening. In humans, increased power in higher frequency bands is also more indicative of the lighter stages of sleep (such as N1 and REM), more common in the early morning in preparation for waking.

Although hypoactivity is expected after general anesthesia, flumazenil administration appeared to mitigate this effect for isoflurane. As compared to their baseline, saline-treated animals spent significantly more time asleep in the 24 h after anesthesia (PAD1). Conversely, the percentage of time spent asleep for flumazenil-treated animals was more similar to their baseline. The observed differences in postanesthesia sleep may be explained by the differences observed during emergence from isoflurane anesthesia. For example, flumazenil-treated animals exhibited overall higher beta power during the periemergence period, whereas theta power was higher in the saline group. This shift in EEG power toward higher frequency bands with flumazenil administration has also been

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**Fig. 7.** Flumazenil can act as a competitive antagonist at high γ-aminobutyric acid receptor type A (GABA) concentrations. (A) Representative traces of GABA-evoked chloride currents in the presence and absence of 4 µM flumazenil. Black traces are GABA alone and orange traces are coapplication of GABA and flumazenil. (B) Summative dose–response curves for application of GABA and GABA plus flumazenil to α1β2γ2s receptors (n = 8 cells). Error bars represent SEM. DMSO = dimethyl sulfoxide; DRC = dose–response curve.

**Fig. 8.** Flumazenil can inhibit the enhancement of γ-aminobutyric acid receptor type A (GABA) currents by isoflurane. (A) Representative traces of GABA receptor activation in multiple treatment conditions. Black bars represent coapplication of isoflurane; orange bars represent coapplication of increasing flumazenil concentrations (from left to right: 0.1, 0.3, 1.0, 3.0, 10, and 30 µM). The inset is an overlay of EC20 GABA response (black), EC20 GABA response potentiated by isoflurane (280 µM), and the flumazenil antagonized EC20 GABA response potentiated by isoflurane. (B) Summative dose–response curve for the effect of increasing doses of flumazenil on isoflurane-enhanced EC20 response (n = 34 cells). Error bars represent SEM.
observed in humans. The higher beta frequencies seen with flumazenil at late emergence may represent a more active cortex. Theta EEG frequencies have been associated with mnemonic processing as well as with acute stress. Thus, the increased theta activity seen with saline-treated animals during late emergence may be caused by a more stressful emergence from anesthesia that consequently led to a higher need of sleep and a higher proportion of REM sleep in the 24 h post anesthesia, especially since REM sleep seems to play a major role in dealing with traumatic and stressful experiences. Our results suggest that increased theta power may be an inconsistent marker of emergence; more evidence will be needed to fully understand the role of increased theta power after anesthesia.

Evidence in the sleep literature demonstrates that the predominance of lower frequency bands during awakening is more associated with abnormal arousals during slow-wave sleep. Furthermore, parasomnias such as confusional arousal, night terrors, and sleepwalking are more common during these stages.

The effects of flumazenil on promoting an awake state were not limited to the emergence period, as flumazenil-treated animals did not exhibit significant increases in sleep in the 24 h after anesthesia as compared to their baseline. In contrast, saline-treated animals exhibited a significant increase in sleep (total sleep time, NREM, and REM) in the day after cessation of anesthesia. Whereas propofol anesthesia appears to satisfy some sleep need, accrual of sleep debt (specifically REM) is known to occur with the administration of isoflurane in rodents. Although sleep deprivation was not a part of our study, the animals that received a single administration of flumazenil before emergence did not demonstrate the same change in sleep patterns as the saline-treated animals. These results may highlight the importance of the arousal process on subsequent sleep architecture. Similarly, we observed a lasting effect (greater than 5 h) on vigilance in hypersomnic patients administered a single dose of flumazenil. Of course, the possibility remains that an a priori difference in general waking behaviors (from sleep or anesthesia) could exist among our drug-/vehicle-treated groups. Thus, further experiments characterizing these differences would be warranted.

**A Dual Mechanism of Flumazenil Explains Variability in Clinical Emergence from Anesthesia**

Taken in the context of our previous work with flumazenil in patients with hypersomnia and clinical case reports of flumazenil in reversing emergence delirium, our results suggest that flumazenil has a more complex relationship with GABAₐₐ than pure antagonism. Our results are consistent with clinical studies that suggest that flumazenil can reverse anesthesia even in the absence of benzodiazepines. However, Schwieger et al. found that flumazenil had no effect on the anesthetic requirements of dogs undergoing inhaled anesthesia and other studies show that flumazenil can potentiate the sedative qualities of propofol in patients undergoing minor surgery. Because of its weak agonist activity, flumazenil has been suggested as having utility in treating anxiety from alcohol withdrawal. In contrast to pure GABA antagonists with epileptogenic properties, flumazenil actually has anticonvulsant properties in the absence of chronic benzodiazepine use. These observations fit a mixed or partial agonist action for this drug at the GABAₐₐ. Therefore, it is possible that higher-dose flumazenil administration regimes could augment the unconsciousness produced by general anesthesia via enhancement of GABA signaling. It would be interesting to determine whether similar effects on sleep architecture were observed after a prolongation of anesthetic effects via flumazenil.

Our results suggest that inhibition of GABAₐₐ with flumazenil is effective at hastening wakeful states and perhaps can mitigate postanesthesia sleep disturbances in a rodent model of general anesthesia with inhaled isoflurane. Understanding the role of GABA in transitions to conscious states may help us prevent significant neurologic consequences after administration of general anesthesia, such as postoperative delirium. Similarly, by identifying overlapping neurophysiologic features in the transitions to consciousness, we can better determine how to capitalize on the beneficial effects of sleep in the perioperative period.
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Competing Interests
Dr. Rye wishes to disclose the following relationship: US Patent Application pending (20110028418), “The Use of GABAA Receptor Antagonists for the Treatment of Excessive Sleepiness and Sleep Disorders Associated with Excessive Sleepiness”; Jazz Pharmaceuticals, Palo Alto, California; UCB Pharma, Smyrna, Georgia; and Xenpoort, Inc., Santa Clara, California. The other authors declare no competing interests.

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