An essential role for orexins in emergence from general anesthesia

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The neural mechanisms through which the state of anesthesia arises and dissipates remain unknown. One common belief is that emergence from anesthesia is the inverse process of induction, brought about by elimination of anesthetic drugs from their CNS site(s) of action. Anesthetic-induced unconsciousness may result from specific interactions of anesthetics with the neural circuits regulating sleep and wakefulness. Orexinergic agonists and antagonists have the potential to alter the stability of the anesthetized state. In this report, we refine the role of the endogenous orexin system in impacting emergence from, but not entry into the anesthetized state, and in doing so, we distinguish mechanisms of induction from those of emergence. We demonstrate that isoflurane and sevoflurane, two commonly used general anesthetics, inhibit c-Fos expression in orexinergic but not adjacent melanin-concentrating hormone (MCH) neurons; suggesting that wake-active orexinergic neurons are inhibited by these anesthetics. Genetic ablation of orexinergic neurons, which causes acquired murine narcolepsy, delays emergence from anesthesia, without changing anesthetic induction. Pharmacologic studies with a selective orexin-1 receptor antagonist confirm a specific orexin effect on anesthetic emergence without an associated change in induction. We conclude that there are important differences in the neural substrates mediating induction and emergence. These findings support the concept that emergence depends, in part, on recruitment and stabilization of wake-active regions of brain.

anesthetic hypnosis | arousal | narcolepsy | NREM sleep circuits | volatile anesthetics

While the critical neuroanatomic sites that mediate various anesthetic endpoints remain largely unknown, recent reports suggest that the hypnotic properties of several anesthetics arise via specific interactions with the neural circuits that regulate non-rapid eye movement (NREM) sleep (1). Sleep deprivation potentiates the action of anesthetics (2), whereas administration of the somnogen adenosine decreases anesthetic requirements (3). At hypnotic doses of inhaled anesthetics, there is slowing and synchronization of the electroencephalogram (EEG) as in NREM sleep (4, 5).

Although there are important differences between sleep and the anesthetized state, the similarities have led to speculation that anesthetic- and NREM sleep-induced hypnosis share a common neural substrate (6, 7). We hypothesize that emergence from anesthesia may use endogenous arousal-promoting mechanisms.

Orexinergic neurons play a critical role in promotion and maintenance of wakefulness. Impaired orexinergic signaling leads to narcolepsy (8–10). Orexinergic neurons are distinguished by the presence of two neuropeptides, orexin-A and orexin-B (hypocretin-1 and hypocretin-2), which are processed from a single transcript (11). Both peptides stimulate wakefulness. Moreover, because of their direct monosynaptic projections throughout the forebrain, activation of orexinergic neurons stabilizes wakefulness. Genetic and pharmacologic blockade of orexin-mediated signaling impairs arousal (12–16). Two parallel lines of evidence suggest that orexin signaling can modify the anesthetic state, with orexin agonists decreasing anesthetic duration and an orexin-1 receptor antagonist, SB-334867-A, increasing anesthetic duration (13, 17). Because hypersensitivity to induction of anesthesia and delayed emergence from anesthesia could independently alter anesthetic duration, we sought to provide a direct link of orexin signaling on anesthetic induction and emergence.

Results

The Inhaled Anesthetics Isoflurane and Sevoflurane Inhibit c-Fos Expression in Orexinergic Neurons While Leaving c-Fos Expression in Adjacent Melanin-Concentrating Hormone (MCH) Neurons Unchanged. Wakefulness results in translocation of c-Fos protein to the nucleus in orexinergic neurons. To determine whether volatile anesthetics affect orexinergic neurons, we examined the effects of isoflurane and sevoflurane on c-Fos expression in orexinergic neurons. Adult C57BL/6J mice were exposed to either an oxygen control, or anesthetizing doses of isoflurane (1.25%) in oxygen, or sevoflurane (2.14%) in oxygen for 2 h beginning at lights out, the period of maximal wakefulness, and were sacrificed for immunohistochemical analysis. Isoflurane resulted in a 30% reduction in c-Fos-expressing neurons, consistent with the reduction seen in NREM sleep ($P < 0.05$; Fig. 1). In mice anesthetized with a comparable hypnotic dose of sevoflurane, a 50% reduction in c-Fos-positive nuclei was observed ($P < 0.001$; Fig. 1). The specificity of volatile anesthetic-induced reduction in c-Fos staining in wake-active neurons within perifornical hypothalamus was investigated by counting c-Fos immunoreactive cells in adjacent non-wake-active MCH neurons (18). Exposure to either 2 h of oxygen or 1.25% isoflurane in oxygen during the first 2 h of the dark period did not significantly alter the number of MCH neurons that coexpressed c-Fos ($P = 0.977$; Fig. 1).

Inhibition of Orexinergic Signaling Does Not Alter Induction. We next investigated the functional consequences of impaired orexin signaling on induction and emergence from anesthesia by using transgenic mice that express a cell death gene placed under the control of the prepro-orexin promoter, orexin/ataxin-3 mice, and in their age-matched wild-type siblings. As reported, orexin/ataxin-3 mice acquire murine narcolepsy with onset of symptoms between 4 and 6 weeks of age, in parallel with the selective genetic ablation of orexinergic neurons (19). We hypothesized that orexin/ataxin-3 mice would be hypersensitive to the hypnotic properties of inhaled anesthetics.

Loss of righting reflex was used to determine induction of

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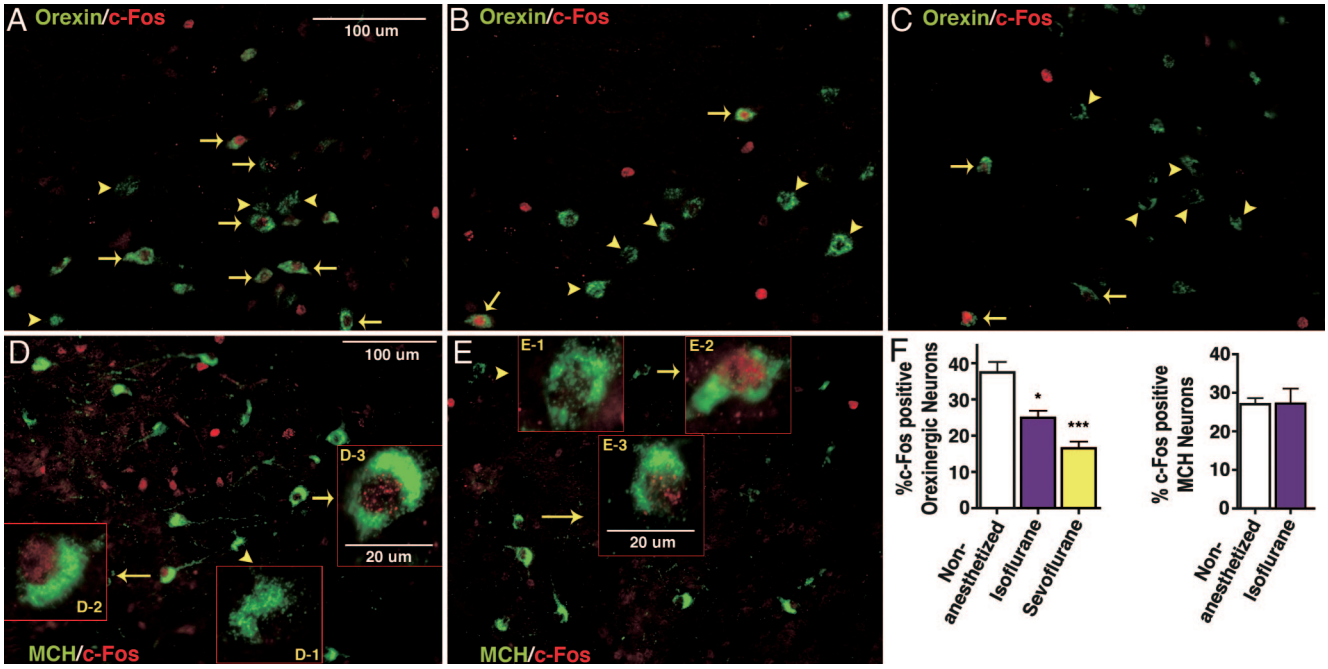

Fig. 1. Specific inactivation of orexinergic neurons in wild-type mice by exposure to anesthetizing doses of isoflurane and sevoflurane. Coronal sections through the perifornical hypothalamus depict c-Fos staining (red nuclei) in orexinergic neurons (green cytoplasm, *A–C*) or in MCH neurons (green cytoplasm, *D* and *E*) prepared from adjacent sections of perifornical hypothalamus. (*A*) Nonanesthetized oxygen control mouse. (*B*) Isoflurane-anesthetized mouse. (*C*) Sevoflurane-anesthetized mouse. (*D*) Nonanesthetized oxygen control mouse. (*E*) Isoflurane-anesthetized mouse. (*F*) Bar graphs summarizing c-Fos expression in both neuronal populations. Arrows depict examples of double-positive neurons, and arrowheads mark MCH or orexinergic neurons that lack c-Fos expression. (Scale bar: *A–E*, 100 μm.) *Insets D-1* and *E-1* show higher magnification of MCH-positive, c-Fos-negative neurons, and *Insets D-2*, *D-3*, *E-2*, and *E-3* show higher-power views of strong or weak c-Fos signals above background, which were all scored as c-Fos positive. (Scale bar: *Insets,* 20 μm.) All bar graphs reveal mean ± SEM counts. Cell counts were analyzed by ANOVA with post hoc Bonferroni correction for multiple comparisons. *, $P < 0.05$; ***, $P < 0.001$; both relative to nonanesthetized oxygen control group.

anesthesia (20). Both orexin/ataxin-3 and wild-type sibling control mice demonstrated equivalent sensitivity. The anesthetic dose [minimum alveolar concentration at which half the mice lose their righting reflex, MAC_{LORR} (ED_{50})] at which half of the orexin/ataxin-3 mice lost their righting reflex was indistinguishable from wild-type sibling controls for both isoflurane and sevoflurane (Table 1 and Fig. 2*A*). Additionally, there was no change in the Hill coefficients for isoflurane or sevoflurane for either group, suggesting that both populations exhibited similar transitions from wakefulness into general anesthesia. In support of the genetic data, by contrary to our hypersensitivity hypothesis, acute blockade of endogenous orexinergic signaling in C57BL/6J mice by using an i.p. injection of the orexin-1R antagonist SB-334867-A, in doses known to block orexin-A-mediated behavior (21, 22), failed to alter the MAC_{LORR} and Hill coefficients for induction (Fig. 2*B* and Table 1).

To exclude altered pharmacokinetics and pharmacodynamics, we exposed C57BL/6J mice treated with vehicle or two different doses of the orexin-1R antagonist to 1.25% isoflurane and found no difference in time to induction of anesthesia (Fig. 2*C*). Four minutes after inhaling isoflurane, when vehicle and 20 mg/kg SB-334867-A-treated mice had lost their righting reflexes, brain concentrations of isoflurane were indistinguishable ($n = 9$ mice per group, $P = 0.76$).

Inhibition of Orexinergic Signaling Delays Emergence. Although genetic and pharmacologic treatments that impair orexin signaling failed to alter induction of anesthesia, they induced dramatic differences in emergence from anesthesia. Orexin/ataxin-3 mice showed markedly delayed emergence from anesthesia (50% more time to emerge), for both isoflurane and sevoflurane ($F_{1,72} = 18.17$, $P < 0.001$) (Fig. 3*A*). To determine whether delayed emergence was unique to the genetic ablation of orexin neurons or applied to other disruptions of orexin signaling, we again used the selective orexin-1 receptor antagonist, SB-334867-A. SB-334867-A produced a dose-dependent delay in emergence of wild type C57BL/6J mice exposed to isoflurane, and significantly delayed emergence at 20 mg/kg i.p. ($F_{2,45} = 8.80$, $P < 0.001$) (Fig. 3*B*). To exclude a

Table 1. Genetic and pharmacologic blockade of endogenous orexin signaling fails to alter induction of isoflurane or sevoflurane anesthesia

	Isoflurane		Sevoflurane		Isoflurane	
	Orexin/ataxin-3	Wild-type siblings	Orexin/ataxin-3	Wild-type siblings	Orexin-1R antagonist (20 mg/kg SB334867A)	Vehicle
MAC_{LORR}, %	0.81	0.79	1.25	1.25	0.90	0.89
MAC_{LORR} 95% C.I., %	0.78–0.84	0.76–0.82	1.20–1.31	1.21–1.31	0.89–0.92	0.88–0.90
Hill slope	13.0	13.2	12.0	13.9	28.4	29.4
Hill slope 95% C.I.	7.5–18.4	6.9–19.5	5.4–18.5	6.0–21.7	19.6–37.1	20.2–38.6

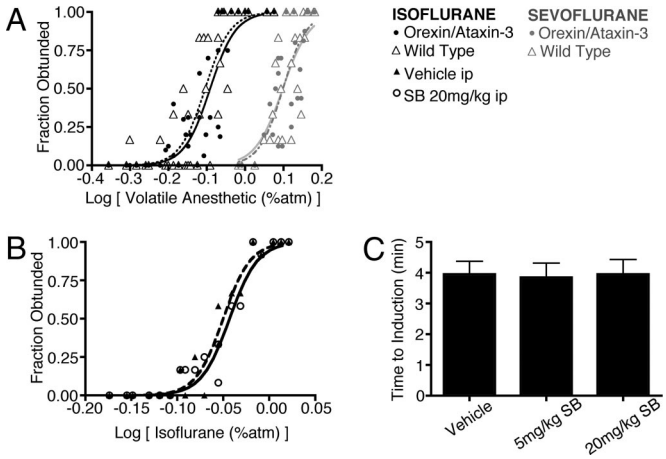

Fig. 2. Dose–response curves demonstrate equivalent sensitivity to induction of anesthesia despite genetic or pharmacologic impairment of orexin signaling. *y* axis depicts the fraction of mice that have lost their righting reflex as a function of the $\log_{10}$ of increasing concentrations of isoflurane or sevoflurane. (*A*) The ED_{50}, also known as the minimum alveolar concentration at which half the mice lose their righting reflex (MAC_{LORR}), and Hill slopes are equivalent for wild-type mice (open triangles) and orexin/ataxin-3 mice (filled circles) after stepwise increases in isoflurane (black symbols) or sevoflurane (gray symbols). Best-fit sigmoidal dose–response curves are shown for wild-type mice (dashed lines) and for orexin/ataxin-3 mice (solid lines). (*B*) Equivalent isoflurane dose–response for mice treated with SB-334867-A (open circles) or vehicle (black triangles). Best-fit sigmoidal dose–response curves are shown for vehicle-treated (dashed lines) and for SB-334867-A-treated mice (solid lines). (*C*) Pharmacodynamic assessment demonstrating identical onset of isoflurane anesthesia in mice treated with vehicle or increasing doses of SB-334867-A.

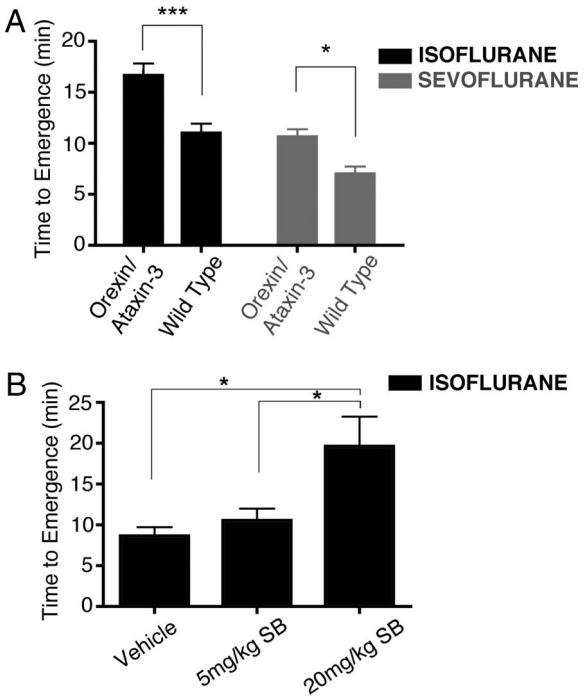

Fig. 3. Genetic and pharmacologic inhibition of orexin signaling delays emergence from anesthesia. Emergence from anesthesia was determined by the time elapsed from discontinuation of an anesthetic until the return of the righting reflex. (*A*) Relative to wild-type sibling controls, emergence times for orexin/ataxin-3 mice are significantly longer for isoflurane (shown in black) and for sevoflurane (shown in gray). Two-way ANOVA followed by Bonferroni testing. (*B*) Emergence times for wild-type C57BL/6J mice (in minutes) after a 2-h exposure to 1.25% isoflurane and i.p. injections of vehicle, 5 mg/kg SB-334867-A, or 20 mg/kg SB-334867-A. ANOVA followed by Bonferroni corrections. All data are expressed as mean ± SEM. ***, $P < 0.001$; *, $P < 0.05$.

pharmacodynamic explanation for the delay in emergence seen with inhibition of orexin signaling, we directly measured brain levels of anesthetic drugs. HPLC extracts prepared from brains of mice killed during emergence at the time when control mice had regained their righting reflex (isoflurane levels, 0.61 ± 0.06 μmol/g brain), yet 20 mg/kg SB-334867-A-treated mice remained anesthetized (isoflurane levels, 0.61 ± 0.04 μmol/g brain), revealed no differences ($n = 6$ mice per group).

Delay in Emergence Is Not an Anesthetic Exacerbation of Cataplexy. To exclude the possibility that delayed emergence in the orexin/ataxin-3 mice is cataplexy, we instrumented orexin/ataxin-3 mice and wild-type sibling controls with EEG and electromyelogram (EMG) leads and monitored EEG along with nuchal muscle tone during induction, maintenance, and emergence from isoflurane anesthesia. Cataplexy is defined by sudden loss of EMG activity with a fast-frequency desynchronized EEG. In contrast, wakefulness is characterized by the presence of increased EMG activity and fast-frequency desynchronized EEG. As typified by Fig. 4, wakefulness returned to euthermic wild-type controls 9.8 ± 2.2 min after discontinuation of 2 h of 1.30% isoflurane. Conversely, in age-matched, euthermic narcoleptic orexin/ataxin-3 mice, the electrographically defined wakefulness did not return until 19.2 ± 2.9 min after terminating the same anesthetic ($P = 0.015$ by Student's t test). Cataplectic episodes were not observed during induction, maintenance, or emergence from anesthesia. Moreover, during the first 2 h after isoflurane exposure was terminated, we failed to find any evidence of cataplexy. Thus, EEG/EMG analysis confirms that orexin/ataxin-3 mice do not exhibit a state resembling awake paralysis. Because mice will not enter a state of natural sleep in a supine position, and because mice turned from prone to supine awaken immediately, righting reflex studies together with EEG/EMG recordings demonstrate that orexin impairment asymmetrically delays emergence.

Discussion

With this series of studies, we demonstrate that orexinergic signaling modifies emergence from the inhaled volatile anesthetics, isoflurane and sevoflurane, without affecting induction. This work supports the concept that emergence is not a mere reflection of induction. Rather, emergence involves, at least in part, distinct neural substrates. We show that exposure to anesthetizing doses of isoflurane and sevoflurane inhibits c-Fos activation in orexinergic neurons. This inhibition is not global, because c-Fos levels in adjacent perifornical MCH neurons remain unchanged. The percentage of c-Fos-activated orexin-

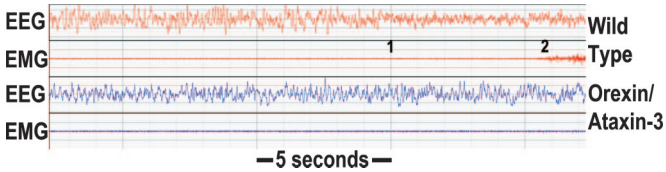

Fig. 4. Emergence by EEG and EMG criteria in wild-type mice while orexin/ataxin-3 siblings remain anesthetized. At 9.8 ± 2.2 min after discontinuation of the isoflurane, wild-type mice exhibit EEG and EMG evidence of emergence from anesthesia as depicted by the top two tracings that show the abrupt return of low-amplitude, fast-frequency EEG (tracing 1), along with concurrent EMG tone (tracing 2). Emergence occurs with a similar appearance in orexin/ataxin-3 mice but is significantly delayed at 19.2 ± 2.9 min.

ergic neurons in our unanesthetized control mice is in agreement with levels determined for wakefulness in mice confirmed by EEG measures, and the percentage of c-Fos-positive, orexinergic neurons in isoflurane or sevoflurane anesthetized mice concur with that found in mice confirmed to be in NREM sleep by EEG (23). As with behavioral state transitions from natural sleep, our righting reflex and EEG studies confirm that the endogenous orexin system promotes wakefulness, facilitating emergence from anesthesia.

Whether the inhibition of orexinergic neurons is caused by a direct action of volatile anesthetics on orexinergic neurons themselves or on their afferents remains unknown. One possible scenario is that volatile anesthetics induce anesthesia through potentiation of inhibitory GABAergic projections from the ventrolateral preoptic nucleus to multiple wake-active neural groups (24, 25). Emergence requires stabilization of wakefulness, and thus, emergence would depend to a larger extent on orexinergic signaling than on induction. Alternatively, a direct inhibitory action of anesthetics on any of the wake-active groups such as the locus coeruleus, tuberomammillary nucleus, dorsal raphe, or ventral periaquiductal gray would be predicted to destabilize wakefulness and bias this network toward hypnosis. A direct inhibitory action of isoflurane and sevoflurane on G protein-coupled orexinergic receptors would inhibit local interneurons that are known to receive excitatory orexinergic innervation and send reciprocal excitatory projections back to the orexinergic neurons (24, 26). This action would dampen the positive feedback, reduce wake-promoting/stabilizing electrochemical gain, and thus impair orexinergic neuron coordination of arousal. Support for this latter mechanism of volatile anesthetic-induced inhibition of orexin-1 receptor signaling has been reported (27).

If inhibition of orexinergic neurons were essential to induction of general anesthesia, then mice whose orexinergic system was destroyed by genetic ablation should exhibit pronounced hypersensitivity to the hypnotic properties of volatile anesthetics. We selected orexin/ataxin-3 mice for study to reduce the likelihood of developmental confounders. These mice are born and mature with intact orexinergic neurons and subsequently acquire narcolepsy postnatally, as the ataxin neurotoxin accumulates (19). This mouse model more closely mimics human narcolepsy, which is also acquired (28), and contrasts with prepro-orexin knockout mice in whom orexin signaling is impaired from the beginning of embryogenesis. To our surprise, narcoleptic orexin/ataxin-3 mice were not hypersensitive to the hypnotic properties of either isoflurane or sevoflurane.

However, orexin/ataxin-3 narcoleptic mice and orexin-1 receptor-antagonist-treated mice exhibited delayed emergence from both commonly used inhaled anesthetics. This observation replicates the anesthetic phenotype seen in a subset of human narcoleptic patients—some of whom take hours to emerge from standard inhaled anesthetics rather than several minutes as predicted (29, 30). The delayed emergence does not represent altered pharmacokinetics, as evidenced by our tissue isoflurane concentration measurements. Because i.v. or i.p. bolus administration of anesthetics never reaches steady state, separation of emergence from induction and maintenance of anesthesia requires continuous delivery of anesthetics. Hence, a distinct role of orexin signaling on emergence from anesthesia has not previously been recognized. A role for orexin signaling in hastening emergence from anesthesia also provides a systems neuroscience framework for the observation that modafinil, a drug capable of stimulating orexinergic and histaminergic neurons (31), administered to postoperative human patients enhances subjective measures of arousal and wakefulness in the recovery room (32). At the same time, our work highlights the potential for using orexinergic agents to modulate anesthetic emergence, as clinically indicated.

In one of the first articles to recognize a role for orexins in modulating anesthetic action, Higuchi and colleagues demonstrated that intracerebroventricular delivery of orexin-A induced signs of EEG arousal in rats during an inhaled anesthetic. They showed that isoflurane-induced burst suppression could be attenuated by central delivery of orexin-A at doses that did not affect heart rate or mean blood pressure. However, by itself, orexin-A could not fully reverse the state of general anesthesia produced by 1.0 or 1.5 MAC of isoflurane (17). Orexins appear to partially antagonize anesthetic-induced hypnosis by activation of the neural substrates required for arousal. Hence, orexins become the second class of partial antagonists of inhaled anesthetics joining central-acting acetylcholinesterase inhibitors such as physostigmine (33). In light of the stimulatory effects of carbachol, a cholinergic agonist, on orexinergic neurons (24), it is likely that acetylcholinesterase inhibitors may promote wakefulness, in part, by direct activation of orexinergic neurons. Such a mechanism is consistent with orexin-mediated acceleration of emergence.

In summary, we identify a specific neural group essential for prompt emergence from general anesthesia. Inhibition of orexinergic signaling by using genetic or pharmacologic tools does not alter induction of anesthesia, supporting the concept that wake-promoting and -stabilizing orexinergic neurons uniquely impact exit from but not entry into the anesthetized state. This fact dictates that the neural substrates governing transitions into and out of the anesthetized state need not be identical, that induction and emergence from anesthesia are not mirror opposite processes, and that other currently unrecognized neuronal groups may play a critical role in mediating behavioral state transitions like general anesthesia or sleep in which consciousness is transiently yet reversibly impaired.

Materials and Methods

Animals. Studies were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania and were in accordance with National Institutes of Health guidelines. Narcoleptic orexin-ataxin-3 mice were generated by placing a truncated ataxin-3 cDNA under the control of the prepro-orexin promoter (19). Twenty-six transgenic mice, aged 66 $\pm$ 5 days, and 13 wild-type sibling controls, aged 61 $\pm$ 9 days, of both genders were used in righting reflex behavioral assays. For EEG and EMG recordings, six orexin/ataxin-3 mice, aged 150 $\pm$ 8 days, and six wild-type sibling controls, aged 149 $\pm$ 6 days, were used. All studied orexin/ataxin-3 mice had been backcrossed to a 129SvEv strain for 10 or 11 generations. Mice in pharmacologic and immunohistochemical studies were wild-type C57BL/6J males aged 8–14 weeks (Jackson Laboratories).

Immunohistochemistry and Cell Counting. To determine whether isoflurane and sevoflurane reduce c-Fos expression in orexinergic and adjacent MCH neurons, C57BL/6J mice were placed in the loss of righting chambers and exposed either to 1.25% isoflurane dissolved in 100% oxygen (isoflurane group), 2.14% sevoflurane dissolved in 100% oxygen (sevoflurane group), or 100% oxygen (wake control group) in the 2 h just after lights out (ZT12–ZT14) during their period of maximal activity and wakefulness (23). All mice were immediately killed by cervical dislocation followed by rapid brain harvest, postfixation in 4% paraformaldehyde, and paraffin embedding. Sections were cut at 10 μm and stained for c-Fos (rabbit polyclonal antibody PC05, Calbiochem, at 1:1,000) and either prepro-Orexin (mouse monoclonal antibody MAB763, R&D Systems, at 1:10,000) or MCH (chicken polyclonal antibody, AB5857, Chemicon, at 1:4000) according to standard protocols (34). Fluorescent secondary antibodies were an Alexa 594-labeled goat anti-rabbit (Molecular Probes, at 1:200) for c-Fos, or an Alexa 488 goat anti-mouse or anti-chicken (Molecular Probes, at 1:200) for detection of orexin and MCH neurons. Three to six sections centered at the perifornical hypothalamus were counted per brain. Counting was performed by one blinded and confirmed by a second blinded experimenter.

Loss and Return of Righting Reflex. Induction and emergence from isoflurane or sevoflurane were defined behaviorally as the respective loss and return

of the righting reflex and were evaluated as follows. Mice were placed in cylindrical gas-tight, controlled-environment chambers arrayed in parallel (35). After 90 min of habituation with 100% oxygen each day on two successive days, anesthesia was induced with a Dräger model 19.1 isoflurane or sevoflurane vaporizer by using 8–12 stepwise incremental increases in the concentration of anesthetic gas dissolved in 100% oxygen. Anesthetic gas concentrations were determined in triplicate during the last 2 min at each step (35). Initial volatile anesthetic concentrations were 0.44% isoflurane or 0.96% sevoflurane. After 15 min at each concentration to allow for equilibration of the mouse with the anesthetic vapors, the concentration of volatile anesthetic was increased by 6 ± 3% of the preceding value. Peak volatile anesthetic concentrations were 1.19% isoflurane or 1.52% sevoflurane. At the end of each 15-min interval, the cylindrical chambers were rotated 180°. A mouse was considered to have lost the righting reflex if it did not to turn itself prone onto all four limbs within 2 min. After the last mouse lost its righting reflex, volatile anesthetic concentration was increased one additional time before measurements of emergence time, which was defined as the duration that elapsed until each mouse regained its righting reflex by turning prone onto all four feet. Mouse temperature was maintained between 36.6 ± 0.6°C by submerging the controlled environment chambers in a 37°C water bath. In an effort to minimize both the number of mice used and the number of anesthetic exposures, induction and emergence from anesthesia in orexin/ataxin-3 mice and sibling controls were performed during the same experiment.

Pharmacologic Inhibition of Orexin Signaling and Righting Reflex Studies. To determine the effect of the orexin-1R antagonist, SB-334867-A (Tocris Bioscience), on induction of anesthesia, 24 C57BL/6J mice were evaluated for both the concentration at which the righting reflex was lost as well as a separate experiment to measure the latency to loss of righting. Thirty minutes before determining isoflurane sensitivity, one-third of the mice received an i.p. injection of vehicle (10% encapsin and 2% DMSO in sterile water), and the other two-thirds received 5 mg/kg or 20 mg/kg of freshly prepared SB-334867-A dissolved in vehicle (21) and administered i.p. at 20 ml/kg. All three groups of mice were exposed to 1.25% isoflurane. Righting reflex was checked every 15 s. To determine the dose–response curve for loss of righting, stepwise increases in isoflurane concentration were performed every 15 min as described above. To eliminate the questions about the duration of action of SB-334867-A, emergence from isoflurane anesthesia in wild-type C57BL/6J mice was studied as described below rather than after the typical 3 h required to generate induction dose–response curves. C57BL/6J mice were anesthetized in controlled-environment chambers (35) with 1.25% isoflurane for 90 min. Individual mice were rapidly removed, injected with an i.p. bolus of either vehicle, 5 mg/kg, or SB-334867-A, 20 mg/kg, doses known to reverse orexin-A-mediated behaviors (21, 22). All i.p. volumes were delivered at 20 ml/kg over 20–30 s. Immediately after injection, mice were returned to the controlled-environment chambers where they continued to breathe 1.25% isoflurane for an additional 30 min before anesthetic gases were discontinued and emergence $time_{RR}$ was recorded as described above.

EEG/EMG Recordings. To obtain an electroencephalographic measure of anesthetic state, mice were anesthetized with 1.5% isoflurane (15–40 min exposure) and chronically implanted for continuous monitoring of EEG/EMG as described in ref. 8. Animals were housed in a 12-h light/dark cycle and allowed to habituate to recording conditions for 7–14 days. Each mouse was recorded for at least 24 h before anesthetic challenge to verify integrity of EEG and EMG waveforms, beginning at lights-on at 8:00 a.m. Food and water were replenished at 8:00 a.m. The mice were not otherwise disturbed. Ten male and two female transgenic and matched wild-type mice were recorded concurrently. EEG/EMG signals were amplified by using a Grass Model 78 (Grass Instruments) and filtered (EEG, 0.3–100 Hz; EMG, 30–300 Hz) before being digitized at a sampling rate of 250 Hz, and displayed on a polygraph system. To determine whether narcoleptic orexin/ataxin-3 mice exhibited cataplexy during emergence from anesthesia, all mice were exposed to 1.30% isoflurane for 2 h. During this anesthetic and subsequent emergence from anesthesia mice were warmed by submerging the recording chambers in a 37°C water bath. EEG/EMG waveforms were recorded and then were visually scored into 20-s epochs of wakefulness according to standard criteria (8, 19), defined as appearance of increased muscle tone with low-amplitude, fast-frequency EEG. The instant at which wakefulness returned after discontinuation of the anesthetic was noted and averaged for all orexin/ataxin-3 and sibling control mice and is reported as the mean ± standard error.

HPLC Measurement of Brain Isoflurane Concentration. To determine whether impaired orexin signaling alters brain anesthetic concentration, standard solutions of isoflurane in methanol were freshly prepared and injected into a Shimadzu HPLC instrument (Shimadzu) equipped with a C_{18} solid-phase extraction column (J. T. Baker) and both UV and refractive index detectors to generate isoflurane calibration curves. The concentration of isoflurane in mice was determined both at induction and emergence by killing wild-type C57BL/6J mice injected with either vehicle or 20 mg/kg of SB-334867-A i.p. The brain concentration at induction was measured 4 min after exposing both groups of mice to 1.25% isoflurane when both groups had lost their righting reflexes (Fig. 2*C*). The isoflurane concentration was also measured in a separate set of mice 10 min after discontinuing the isoflurane exposure at a time when control mice exposed to 1.25% isoflurane for 2 h had regained their righting reflex, yet 20 mg/kg-treated mice remained anesthetized (Fig. 3*B*). All mice were killed by cervical dislocation. Harvested brains were snap-frozen in liquid nitrogen and stored at −80°C pending analysis. Brains were homogenized in 0.02 M phosphate buffer (pH 4.6) and then spiked with 0.5 μl of halothane in 4 ml of homogenate as an internal control. The homogenate was centrifuged at 30,000 × *g* for 30 min at 4°C, and the supernatant loaded onto the C_{18} cartridges. After eluting with a mixture of methanol and 2-propanol (vol/vol 2:1) with 0.1% trifluoroacetic acid, aliquots were immediately injected into the HPLC. The easily resolved isoflurane peaks (refractive index) were corrected for recovery yield by using the spiked halothane peaks (UV detection), and normalized by grams of brain tissue.

Statistics. To obtain MAC_{LORR} and Hill slope constants, the log of volatile anesthetic gas concentration versus the fraction of the population having lost the righting reflex plots were generated and fit with a nonlinear dose–response curve with a variable slope by using Prism 5.0 (GraphPad Software). To minimize the number of animals used in the study and to confirm the reproducibility of the data generated, a population of mice was exposed to the same anesthetic twice with 24 h between anesthetic exposures. MAC_{LORR} and Hill slope constants are reported as means of two independent trials with 95% confidence limits. Emergence $time_{RR}$ data generated immediately after each anesthetic exposure are reported as a mean ± standard error. Cell counts and emergence $time_{RR}$ were analyzed with one- or two-way ANOVA with post hoc Bonferroni multiple comparison testing where appropriate. Time to return of first EEG/EMG activity consistent with wakefulness was defined as emergence $time_{EEG}$ and was analyzed between wild-type and orexin/ataxin-3 mice by using a *t* test.

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