Distinctive Recruitment of Endogenous Sleep-promoting Neurons by Volatile Anesthetics and a Nonimmobilizer

Bo Han, M.D., Ph.D., Hilary S. McCarren, Ph.D., Dan O'Neill, M.D., Max B. Kelz, M.D., Ph.D.

Background: Numerous studies demonstrate that anesthetic-induced unconsciousness is accompanied by activation of hypothalamic sleep-promoting neurons, which occurs through both pre- and postsynaptic mechanisms. However, the correlation between drug exposure, neuronal activation, and onset of hypnosis remains incompletely understood. Moreover, the degree to which anesthetics activate both endogenous populations of γ-aminobutyric acid (GABA)ergic sleep-promoting neurons within the ventrolateral preoptic (VLPO) and median preoptic nuclei remains unknown.

Methods: Mice were exposed to oxygen, hypnotic doses of isoflurane or halothane, or 1,2-dichlorohexafluorocyclobutane (F6), a nonimmobilizer. Hypothalamic brain slices prepared from anesthetic-naive mice were also exposed to oxygen, volatile anesthetics, or F6 *ex vivo*, both in the presence and absence of tetrodotoxin. Double-label immunohistochemistry was performed to quantify the number of c-Fos–immunoreactive nuclei in the GABAergic subpopulation of neurons in the VLPO and the median preoptic areas to test the hypothesis that volatile anesthetics, but not nonimmobilizers, activate sleep-promoting neurons in both nuclei.

Results: *In vivo* exposure to isoflurane and halothane doubled the fraction of active, c-Fos-expressing GABAergic neurons in the VLPO, whereas F6 failed to affect VLPO c-Fos expression. Both in the presence and absence of tetrodotoxin, isoflurane dose-dependently increased c-Fos expression in GABAergic neurons *ex vivo*, whereas F6 failed to alter expression. In GABAergic neurons of the median preoptic area, c-Fos expression increased with isoflurane and F6, but not with halothane exposure.

Conclusions: Anesthetic unconsciousness is not accompanied by global activation of all putative sleep-promoting neurons. However, within the VLPO hypnotic doses of volatile anesthetics, but not nonimmobilizers, activate putative sleep-promoting neurons, correlating with the appearance of the hypnotic state. **(Anesthesiology 2014; 121:999-1009)**

THE preoptic area is home to heterogenous cell populations that regulate a variety of homeostatic functions. Since von Economo first demonstrated that lesions to this area produce insomnia,[1] the preoptic area's specific role in arousal state regulation has been repeatedly demonstrated. Localized clusters of neurons within the ventrolateral preoptic (VLPO) nucleus and the median preoptic (MnPO) nucleus increase their expression of the immediate early gene, *c-fos*, during sleep.[2,3] The suggestion that these c-Fos–expressing VLPO and MnPO neurons increase their neuronal activity during sleep has been validated using *in vivo* electrophysiologic unit recordings.[4,5] Moreover, these sleep-active neurons in the VLPO and the MnPO are γ-aminobutyric acid (GABA)ergic and exchange mutual inhibitory projections with a number of wake-active neuronal nuclei to regulate sleep and arousal.[6–8] Finally, lesion or pharmacological modulation of VLPO or MnPO activity alters induction and maintenance of sleep, confirming their sleep-promoting function.[9–15]

Many changes in brain activity observed during sleep are similar to those observed during anesthetic-induced hypnosis.[16,17] The active roles of the VLPO and the MnPO in the genesis of sleep coupled with their inhibitory connectivity to arousal-promoting structures have singled these nuclei out as potential neuronal targets of general anesthetics.

What We Already Know about This Topic

- Anesthetic-induced unconsciousness is accompanied by activation of neurons in hypothalamic sleep-promoting regions.
- The extent to which the same neurons are involved in both sleep and anesthesia is not clear.
- Neuronal activation was evaluated after anesthetic exposure both in live mice and in *ex vivo* brain tissue for two nuclei intimately involved in sleep regulation: the ventrolateral preoptic and the median preoptic areas.

What This Article Tells Us That Is New

- Within the ventrolateral preoptic area, volatile anesthetics increased activation of γ-aminobutyric acidergic neurons. In the median preoptic area, neuronal activation was independent of hypnosis.
- Anesthetics do not activate all neurons that regulate sleep, nor do they universally recruit all sleep-promoting neural nuclei.

The first two authors contributed equally to this work.

Submitted for publication December 4, 2013. Accepted for publication June 30, 2014. From the Department of Anesthesiology and Critical Care (B.H.), Department of Pharmacology (H.S.M.), University of Pennsylvania, Perelman School of Medicine, Philadelphia, Pennsylvania; Department of Anesthesiology, Weill Cornell Medical College, New York, New York (D.O.); and Department of Anesthesiology and Critical Care, Center for Sleep and Circadian Neurobiology, Institute for Translational Medicine and Therapeutics, University of Pennsylvania, Perelman School of Medicine, Philadelphia, Pennsylvania (M.B.K.).

Indeed, exposure to suprahypnotic doses of a variety of general anesthetics including barbiturates, propofol, dexmedetomidine, chloral hydrate, isoflurane, and halothane all induces c-Fos expression in putative sleep-promoting VLPO neurons.[18–22] Although activation of the VLPO could be indirect, arising *via* disinhibition, the volatile anesthetic isoflurane directly depolarizes the subset of VLPO neurons that are electrophysiologically indistinguishable from those involved in the genesis and maintenance of natural sleep.[22] Evidence regarding the MnPO's role in anesthetic-induced hypnosis is less clear. To date, only a single study has evaluated the effects of anesthetics on the MnPO. It failed to find c-Fos induction in the MnPO of rats after exposure to pentobarbital, chloral hydrate, or ethanol.[19] However, it did not address the functional heterogeneity of neurons in the MnPO, in which subsets of neurons also help to regulate blood pressure, body temperature, and endocrine signaling, and might therefore have missed a selective activation confined to a subset of neurons that did not increase the overall number of c-Fos–expressing neurons.

In the current study, we quantify VLPO and MnPO activation, as measured by c-Fos expression, in response to the volatile general anesthetics isoflurane and halothane. As the sleep-promoting subsets of hypothalamic neurons in the VLPO and MnPO are known to be GABAergic,[23] we hypothesized that anesthetic-induced increases in c-Fos immunoreactivity would be significant in these glutamic acid decarboxylase (GAD)–immunoreactive populations. Moreover, we further test the hypothesis that induction of c-Fos would only occur with drugs capable of eliciting hypnosis. Nonimmobilizers are compounds predicted to be anesthetics based on their structure and lipid solubility, but violate the Meyer–Overton hypothesis of anesthetic action.[24,25] These drugs are biologically active, elicit amnesia, and can cause seizures, but critically, they fail to produce unconsciousness, antinociception, or muscle atonia.[25–28] Consequently, members of the nonimmobilizer family form powerful tools that have been used to probe the molecular targets of anesthetic action[29–33] and have the potential to probe neuronal targets of anesthetic action as well. We hypothesized that administration of the nonimmobilizer 1,2-dichlorohexafluorocyclobutane (F6) should not induce c-Fos expression in sleep-promoting GABAergic neurons of the VLPO and MnPO.

Materials and Methods

Animals

All studies were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. Wild-type C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) aged 3 to 6 months were used for all experiments. All animals were housed in a group environment (three to five mice per cage), given access to food and water *ad libitum*, and 2 weeks before experiments were acclimatized to a reverse 12-h light–dark cycle with lights on at 7 PM (zeitgeber time 0). The reversed light cycle allowed experiments to be performed during the animals' active period, when confounding induction of c-Fos due to physiological sleep would be minimized.

In Vivo *Anesthetic Drug Exposure and Tissue Collection*

Mice were exposed to isoflurane, halothane, F6, and 100% oxygen in custom-made controlled environmental chambers while euthermia was maintained as described previously.[34,35] In brief, each mouse was placed in a cylindrical gas-tight chamber submerged in a 37°C water bath. Body temperatures were maintained at 37.1° ± 0.5°C throughout the exposure. Inhaled exposure occurred for 2 h with 1.2% isoflurane (administered *via* a Drager model 19.1 isoflurane vaporizer; Drager Medical Inc., Telford, PA), 1.0% halothane (administered *via* a Drager model 19.1 halothane vaporizer), 3.2% F6 (administered *via* a Drager model 19.1 enflurane vaporizer as previously described),[28] or no drug in 100% oxygen at a flow rate of 200 ml/min per chamber. Anesthetic doses correspond to the EC_{99} for loss of righting reflex in mice.[35] F6 was administered at the highest dose that did not induce seizures. *In vivo* experiments were conducted between zeitgeber time 14 and zeitgeber time 17 (9 AM to 12 PM). After exposure, mice were rapidly overdosed with isoflurane and transcardially perfused with saline followed by 4% paraformaldehyde. Brains were removed and postfixed for 1 to 3 h at 4°C. After cryoprotection in 30% sucrose for 2 days at 4°C, tissue was flash-frozen in liquid nitrogen. Tissue was then sectioned at 40 μm on a 2800 Frigocut N cryostat (Reichert-Jung, Depew, NY) and stored free-floating in antifreeze solution (1.75 M sucrose, 8 M polyvinylpyrrolidone, 15.4 mM sodium azide, and 10.7 M ethylene glycol in 0.1 M sodium phosphate buffer pH 7.2) at −20°C until staining.[36] For VLPO experiments, n = 10 for each group. For MnPO experiment, n = 10 for isoflurane, n = 9 for F6, n = 13 for halothane, and n = 12 for oxygen. Sample sizes were chosen based on previous experience with c-Fos immunoreactivity in the VLPO.[22]

Ex Vivo *Anesthetic Drug Exposure*

Hypothalamic brain slices from anesthetic-naive mice were used for all *ex vivo* studies and were prepared as described previously.[22] Mice were sacrificed by cervical dislocation followed by decapitation. The brain was placed in ice-cold dissecting solution (consisting of 219 mM sucrose, 3.0 mM KCl, 1.25 mM NaH_2PO_4, 1.0 mM $MgSO_4$, 2.0 mM $CaCl_2$, 10 mM glucose, and 26 mM $NaHCO_3$) bubbled with 95% O_2–5% CO_2. Coronal sections cut at 200 μm spanning from bregma +0.26 mm through to −0.10 mm for the VLPO were collected using a motorized vibratome (World Precision Instruments, Sarasota, FL) and transferred to a holding chamber containing artificial cerebrospinal fluid (130 mM NaCl, 3.0 mM KCl, 1.25 mM NaH_2PO_4, 1.0 mM $MgSO_4$, 2.0 mM $CaCl_2$, 10 mM glucose, and 26 mM $NaHCO_3$)

bubbled with 95% O_2–5% CO_2. The temperature of chambers was maintained at 34°C by a water bath. The slices were rested and fully equilibrated in the artificial cerebrospinal fluid for 1 h and then were incubated for another 2 h in the artificial cerebrospinal fluid with or without exposure to isoflurane (0.3, 0.6, 0.9, or 1.2%) bubbled from the vaporizer into the artificial cerebrospinal fluid, or F6 (24 μM).[37,38] Sample sizes were as follows: n = 29 for oxygen, n = 7 for F6, n = 4 for 0.3%, n = 7 for 0.6%, n = 9 for 0.9%, and n = 10 for 1.2% isoflurane. F6 concentration was confirmed to be 29 ± 2.65 μM in a subset of experiments (n = 3) using high-performance liquid chromatography system Gold (Beckman Coulter Inc., Danvers, MA) consisting of a variable wavelength ultraviolet detector operated at 202 nm, a Type U6K injector valve, and a Vydac C_{18} 4.6 × 250 mm column.[39] All high-performance liquid chromatography F6 measurements were carried out at room temperature with a flow rate of 1 ml/min and a mobile-phase mixture of 57% acetonitrile and 0.02 M NaH_2PO_4 buffer containing 0.01 M sodium lauryl sulphate. Under these conditions, our standard curve yielded an r = 0.998. All *ex vivo* slice experiments were conducted between zeitgeber time 14 and zeitgeber time 18. Due to these time constraints, only four to six sections were simultaneously processed on a given day with a single treatment group always compared with an oxygen control. In a subset of experiments, hypothalamic slices were treated with the addition of tetrodotoxin (1 μM) at the beginning of the 2 h before drug exposure. For VLPO experiments, n = 3 in both the tetrodotoxin and tetrodotoxin plus isoflurane groups. Only one slice was harvested from each animal for studies in the VLPO. *Ex vivo* studies in the MnPO were prepared identically to those in the VLPO with the exception that 200-μm coronal sections spanned bregma +0.62 through +0.26 and consequently two slices were harvested from each animal. The number of slices used for each experiment was as follows: n = 15 for tetrodotoxin alone, n = 6 for tetrodotoxin plus 1.2% isoflurane, and n = 8 for tetrodotoxin plus F6.

Immunofluorescent Staining

For the *in vivo* studies, residual antifreeze solution was rinsed off from the slices with three phosphate-buffered saline (1X) washes before staining. Brain sections were incubated for 15 min in 0.3% H_2O_2, blocked in 1% blocking reagent (Oregon green 488 tyramine kit; Life Technologies, Grand Island, NY) for 60 min, and incubated overnight at room temperature with mouse anti-GAD67 antibody (MAB5406, 1:1,000; EMD Millipore, Billerica, MA) diluted in 1% blocking reagent. Sections were rinsed and incubated overnight at room temperature with a biotinylated secondary antibody (MP-7402; Vector Labs, Burlingame, CA) and then placed in Tyramide Signal Amplification solution (1:100, Oregon green 488 tyramine kit; Life Technologies) for 30 min. The sections were permeabilized and blocked for 60 min with 4% normal goat serum in phosphate-buffered saline containing 0.4% triton, and then incubated overnight at room temperature with rabbit anti-c-Fos antibody (PC38, 1:10,000; Calbiochem, San Diego, CA) diluted in the blocking solution. The c-Fos antibody was detected using anti-rabbit secondary antibody conjugated with an Alexa 594 fluorescent dye (A11037, 1:200; Life Technologies). All slides were sealed for visualization with Leica TCS SP5 confocal microscope (Leica Microsystems Inc., Buffalo Grove, IL) equipped with a motorized stage and tile scanning mode. Sections that were incubated without primary or secondary antibodies were used as controls and found to be devoid of signals. For *ex vivo* studies, after drug exposure, the 200-μm brain sections were fixed for 30 min at room temperature with 4% paraformaldehyde in phosphate-buffered saline and were processed for immunofluorescence.

Quantitative Analysis of Immunofluorescence-stained Slices

The immunofluorescence-labeled cells were counted manually using ImageJ (National Institutes of Health, Bethesda, MD) with regions of interest defined by standardized counting windows based on the mouse brain atlas.[40] Tissue from *in vivo* experiments was categorized according to the coronal section of the atlas that it most closely resembled (0.12-mm increments from +0.62 mm to −0.10 mm). VLPO cell counts were performed on sections spanning from +0.26 mm to −0.10 mm relative to bregma using a 400 × 250 μm box positioned 300 μm lateral to midline.[22] The same box was used on *ex vivo* tissue. Bilateral counts from a single slice containing the VLPO were averaged. MnPO sections spanned from bregma +0.62 mm to +0.26 mm. The counting windows (width × height) applied for the MnPO were 250 × 350 μm with the ventral border 120 μm (bregma +0.62 mm) or 200 μm (bregma +0.50 mm) dorsal to the third ventricle, 250 × 500 μm with the ventral border 50 μm dorsal to the third ventricle (bregma +0.38 mm), and 250 × 1,000 μm with the ventral border 100 μm (bregma +0.26 mm) dorsal to the third ventricle.[40] Cells in each box were scored as immunoreactive for c-Fos, GAD, or both.

Statistical Analysis

GraphPad Prism (version 4.0; GraphPad Software Inc., San Diego, CA) was used for all statistical analyses. A one-way ANOVA of GAD-immunoreactive cell number as a function of rostral-caudal VLPO position showed no significant effect on the number of GAD-immunoreactive neurons. Hence, one-way ANOVAs for c-Fos–immunoreactive cell number and for percentage of c-Fos–immunoreactive GABAergic cells as a function of drug treatment were run with counts having been averaged across all VLPO slices. Due to the precedent within the literature for dividing the MnPO into rostral and caudal divisions,[2,4,41] a one-way ANOVA was run to verify that rostral-caudal position did not affect the number of GAD-immunoreactive neurons despite the varying size of our counting window. Thereafter, a two-way ANOVA was

performed that showed neither significant effect on expression of c-Fos in GABAergic MnPO neurons as a function of rostral/caudal position nor an interaction between position and treatment. Consequently, data are presented with the collapsed counts irrespective of MnPO rostral-caudal position. One-way ANOVAs for c-Fos–immunoreactive cell number and for percentage of c-Fos–immunoreactive GABAergic cells as a function of drug treatment were run with counts having been averaged across all MnPO slices. The same analyses were done for both the *in vivo* and *ex vivo* experiments. For *ex vivo* tetrodotoxin experiments, a two-tailed *t* test was used to compare data from slices treated with tetrodotoxin alone to those treated with isoflurane + tetrodotoxin in the VLPO, whereas a one-way ANOVA was used to compare the three tetrodotoxin-treated groups in the MnPO. All results are reported as means ± standard error. A *P* value of less than 0.05 was considered statistically significant.

Results

As expected, there was no significant effect of inhaled drug treatment on the number of GAD-immunoreactive neurons in the VLPO ($F_{3,36}$ = 0.87, P = 0.47). Surprisingly, inhaled drug treatment did not increase the total number of c-Fos–expressing neurons in the VLPO ($F_{3,36}$ = 0.17, P = 0.92). However, as shown in figures 1 and 2, inhaled drug treatment did significantly change the expression of c-Fos in the GABAergic neurons in the VLPO ($F_{3,36}$ = 8.18, P = 0.0003) and decreased c-Fos in the non-GABAergic neurons (data not shown). *Post hoc* Bonferroni testing confirmed that isoflurane and halothane significantly increased the number of c-Fos–immunoreactive GABAergic neurons *in vivo*, roughly doubling the fraction seen both in nonanesthetized, oxygen-exposed controls as well as in mice exposed to the nonimmobilizer, F6. Importantly, c-Fos expression in GABAergic neurons was statistically indistinguishable between control and F6-exposed mice (fig. 2). The percentage of c-Fos–expressing GABAergic neurons was also statistically indistinguishable between halothane- and isoflurane-treated mice.

Consistent with the *in vivo* findings, ANOVA testing revealed a significant effect of drug treatment on c-Fos immunoreactivity specifically in the GABAergic VLPO neurons of hypothalamic slices exposed to drugs *ex vivo* ($F_{5,60}$ = 5.98, P < 0.0001). Compared with oxygen exposure alone, 0.9% isoflurane (P < 0.01) and 1.2% isoflurane (P < 0.01) roughly doubled the number of GABAergic neurons expressing c-Fos (fig. 3). This increase was not observed at lower, subhypnotic isoflurane concentrations (0.3 and 0.6%). Similar to the *in vivo* findings, *ex vivo* exposure to F6 also failed to induce c-Fos expression in GABAergic neurons within the VLPO (fig. 3). Total c-Fos and GAD cell counts did not differ across conditions ($F_{5,60}$ = 2.27, P = 0.06 and $F_{5,60}$ = 2.06, P = 0.08, respectively, data not shown).

As both putative sleep-active and nonsleep active VLPO neurons are known to express GAD but only the former population is depolarized by isoflurane,[22,42] we incubated hypothalamic slices with tetrodotoxin to impair trans-synaptic neurotransmission. In agreement with an anesthetic-induced postsynaptic activation, subsequent *ex vivo* exposure to isoflurane still retained its efficacy and more than doubled the fraction of GABAergic neurons expressing c-Fos as compared with tetrodotoxin-treated anesthetic-naive slices (t = 3.74, P = 0.02; fig. 3E). However, compared with tetrodotoxin treatment alone, isoflurane treatment in the presence of tetrodotoxin did not alter the number of single labeled c-Fos–immunoreactive or GAD-immunoreactive neurons (t = 0.22, P = 0.83 and t = 0.03, P = 0.98, respectively).

Contrary to our initial hypothesis, results in the MnPO do not directly parallel those in the VLPO. One-way

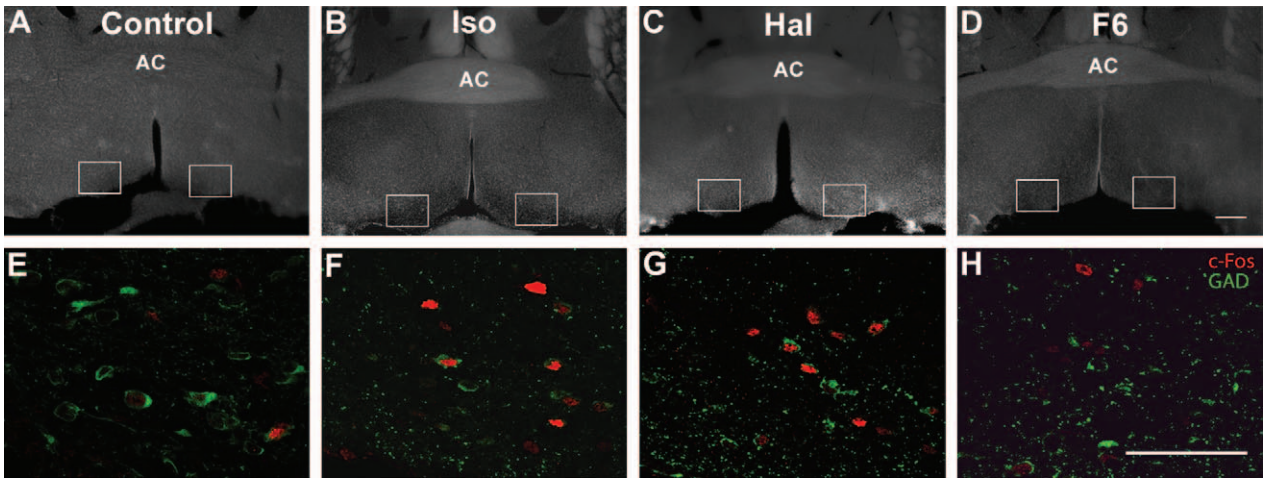

Fig. 1. Representative images showing immunofluorescence-labeled neurons in the ventrolateral preoptic area of mice after a 2-h *in vivo* drug exposure. *White boxes* in *A–D* indicate the region of interest, shown at higher magnification in *E–H* for mice exposed to (*E*) 100% oxygen, (*F*) 1.2% isoflurane in oxygen, (*G*) 1.0% halothane in oxygen, and (*H*) 3.2% F6 in oxygen. c-Fos staining, red nuclei; glutamic acid decarboxylase (GAD) staining, green cytoplasm. AC denotes the anterior commissure. *Scale bar* in *D* is 100 μm and applies to *A–D*; *scale bar* in *H* is 60 μm and applies to *E–H*.

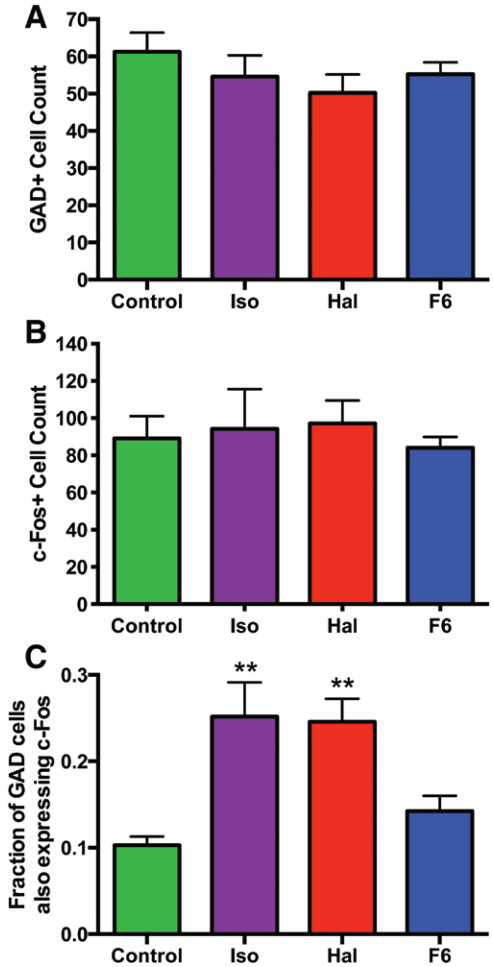

Fig. 2. Quantification of (*A*) glutamic acid decarboxylase (GAD)–labeled neurons, (*B*) c-Fos–labeled neurons, and (*C*) the fraction of GAD-labeled neurons that also express c-Fos in the ventrolateral preoptic area (VLPO) after a 2-h *in vivo* anesthetic or oxygen exposure. Control, 100% oxygen (n = 10); isoflurane, 1.2% in oxygen (n = 10); halothane, 1.0% in oxygen (n = 10); F6, 3.2% in oxygen (n = 10). All bar graphs show mean + standard error. Data analyzed by one-way ANOVA with *post hoc* Bonferroni correction for multiple testing. $^{**}P < 0.01$.

ANOVA revealed that inhaled drug treatment did not change the number of GAD-immunoreactive neurons ($F_{3,40} = 0.18$, $P = 0.91$) but did significantly increase both the total number of c-Fos–immunoreactive neurons ($F_{3,40} = 8.74$, $P = 0.0001$) as well as the percentage of c-Fos–immunoreactive GABAergic neurons ($F_{3,40} = 7.30$, $P = 0.0005$). Surprisingly, these increases were due to a significant induction of c-Fos expression after both isoflurane and F6 treatment, but not after treatment with halothane (figs. 4 and 5). Interestingly, *in vivo* induction of c-Fos by isoflurane and F6 was significant not only in the GABAergic neurons but also occurred in non-GABAergic MnPO neurons that lack GAD immunoreactivity (fig. 4), with the highest levels of c-Fos expression occurring after an identical F6 exposure that failed to alter c-Fos in the VLPO.

To determine whether putative activation of MnPO neurons by isoflurane might also be direct as shown for the VLPO (fig. 3), we conducted additional *ex vivo* studies and exposed anesthetic-naive slices containing the MnPO to isoflurane, F6, or to an oxygen control in the presence of tetrodotoxin. As shown in figure 6, and in contrast with the mechanism of activation in the VLPO, a one-way ANOVA demonstrated that c-Fos induction in GABAergic neurons by isoflurane or F6 in the presence of tetrodotoxin did not differ from tetrodotoxin exposure alone ($P > 0.05$ for each comparison), although the overall ANOVA was significant ($F_{2,26} = 3.51$, $P = 0.04$) due to a difference in the fraction of GAD neurons also expressing c-Fos in the tetrodotoxin plus isoflurane and tetrodotoxin plus F6 groups. The total number of GAD-immunoreactive neurons did not differ between groups ($F_{2,26} = 0.25$, $P = 0.78$), and the total number of c-Fos–immunoreactive neurons only differed between the tetrodotoxin plus isoflurane and tetrodotoxin plus F6 groups ($F_{2,26} = 6.36$, $P = 0.006$ overall, $P > 0.05$ for comparisons with tetrodotoxin-alone group).

Discussion

The exact mechanisms underlying general anesthetic-induced unconsciousness remain hotly debated. Studies during the past decade in animals as well as humans have increasingly focused on the disruption of cortical information transfer that occurs with hypnotic doses of anesthetics.[43–49] Although cortical changes likely represent a final common neuronal mechanism resulting in loss of consciousness, similar modulation of connectivity is known to occur with the onset of natural sleep.[50] This should remind us that changes in subcortical activity, which underlie our nightly sojourn into sleep, are sufficient to directly alter the function of the cerebral cortex.[51,52] General anesthesia and natural sleep are not identical states, yet they do share similar tra its.[17,44,50,53,54] Studies across phyla as distinct as invertebrates and mammals have demonstrated that anesthetic drugs are capable of activating endogenous sleep-promoting neural systems.[18–22,55,56] Hence, we believe that exploring the commonalities and recognizing the key differences in the genesis of these states will be critical to advance our understanding both of anesthetic mechanisms and sleep neurobiology.

In the current study, we demonstrate in GABAergic neurons of the VLPO that volatile anesthetics increase the percentage of c-Fos-expressing neurons by 100% during both *in vivo* and *ex vivo* exposures. Moreover, we show that a similar doubling of c-Fos expression persists despite pretreatment of slices with tetrodotoxin in concentrations that disrupt action potential propagation. This enduring induction of c-Fos occurs as isoflurane closes a background potassium conductance leading to a depolarization of sleep-promoting GABAergic neurons.[22] Because the expression of GAD, the enzyme responsible for the biosynthesis of GABA, is not confined solely to the sleep-promoting neurons in the preoptic area, the preserved relative increase in

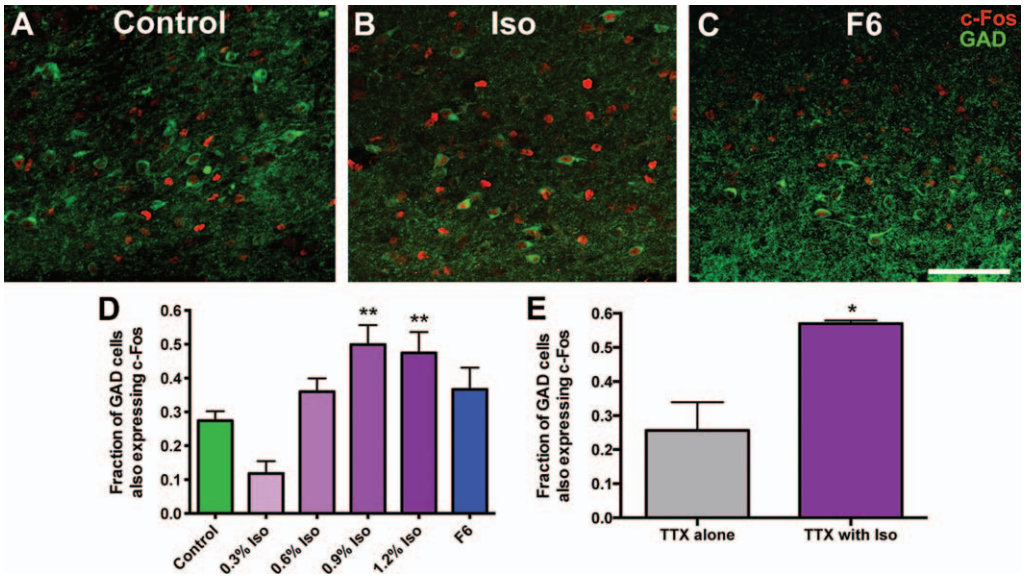

Fig. 3. Representative immunofluorescent images of the ventrolateral preoptic area (VLPO) after a 2-h *ex vivo* exposure to (*A*) oxygenated artificial cerebrospinal fluid (control), (*B*) 1.2% isoflurane in oxygenated artificial cerebrospinal fluid, and (*C*) 24 μM F6 in oxygenated artificial cerebrospinal fluid. *Scale bar* in *C* is 60 μm and applies to *A–C*. c-Fos staining, red nuclei; glutamic acid decarboxylase (GAD) staining, green cytoplasm. (*D*) Shows the fraction of GAD-immunoreactive cells that also label for c-Fos with exposure to oxygen (n = 29), F6 (n = 7), and increasing doses of isoflurane (n = 4 for 0.3%, n = 7 for 0.6%, n = 9 for 0.9%, and n = 10 for 1.2%). (*E*) The fraction of GAD-immunoreactive cells that coexpress c-Fos is also shown for slices that were exposed *ex vivo* to tetrodotoxin (TTX, n = 3) alone or with 1.2% isoflurane (TTX with Iso, n = 3). All bar graphs show mean + standard error. Data in *D* analyzed by one-way ANOVA with *post hoc* Bonferroni correction for multiple testing. Data in *E* analyzed by *t* test. $^{*}P < 0.05$; $^{**}P < 0.01$.

the number of c-Fos–expressing GABAergic neurons in the presence of tetrodotoxin also corroborates that activation is occurring in the sleep-promoting cohort of VLPO neurons. As critically, in the VLPO we demonstrate that c-Fos induction occurs specifically with exposure to doses of anesthetics that elicit unconsciousness and not with inhalation of low doses of isoflurane or with an amnestic dose of the nonimmobilizer, F6.[28,57] Hypothalamic slice exposures occurred at 34°C. Correcting for the 3°C change in temperature would raise the apparent potency of each nominal isoflurane dose bubbled onto the slices by 21% (*i.e.*, the 0.30% exposure would correct to a 0.37% exposure),[58] although one limitation of our study is that the exact aqueous concentrations of anesthetics were not measured. Although we did administer other doses of F6 *in vivo* (from 3.5 to 4.2%), approaching the predicted MAC-immobility concentration in mice, these higher doses of F6 induced seizures in all mice studied. This placed an easily-recognized upper concentration limit on our *in vivo* dose–response studies.[59,60] To overcome this potential limitation, higher doses of F6 corresponding to 29 μM were delivered *ex vivo*.

Although the finding of increased c-Fos immunoreactivity in GABAergic VLPO neurons is congruent with volatile anesthetic-induced activation of sleep-promoting VLPO neurons suggested by previous single label c-Fos expression studies *in vivo* and by electrophysiological recordings in hypothalamic slices,[18–22] important discrepancies exist. Previous work showed an increase in the absolute number of c-Fos–immunoreactive cells in the VLPO after *in vivo* isoflurane and halothane exposure, and a significant induction of c-Fos in VLPO after exposure to a subhypnotic dose of isoflurane[22] not reproduced herein. This may have been due to differences in the light–dark cycle. Although both sets of experiments were performed during the rodents' active phase, mice in this study were adapted to a reverse light cycle. In addition, the c-Fos antibody and method of detection presented here differ from the brightfield staining in previous articles. The polyclonal c-Fos antibody used in the current study did target the same amino acids of the c-Fos protein, but labeled more total neurons under similar experimental conditions to that reported in our previous study,[22] despite the lower sensitivity of immunofluorescent detection.

The VLPO and MnPO form two of the best-studied and closely linked components of the brain's endogenous sleep-promoting network. Neurons within the VLPO are hypothesized to play a major role in the initiation of sleep[61–63] whereas those in the MnPO may stabilize the state of sleep and track homeostatic drive to sleep.[64–66] The presented results suggest that these two preoptic area nuclei likely serve distinct roles in the response to general anesthetics. For isoflurane, GABAergic neurons in both nuclei are activated *in vivo*. *Ex vivo*, induction of c-Fos in the VLPO by isoflurane persists in the presence of tetrodotoxin, whereas isoflurane fails to induce MnPO activation when synaptic transmission is disrupted. This suggests that the MnPO may be recruited indirectly by isoflurane either through

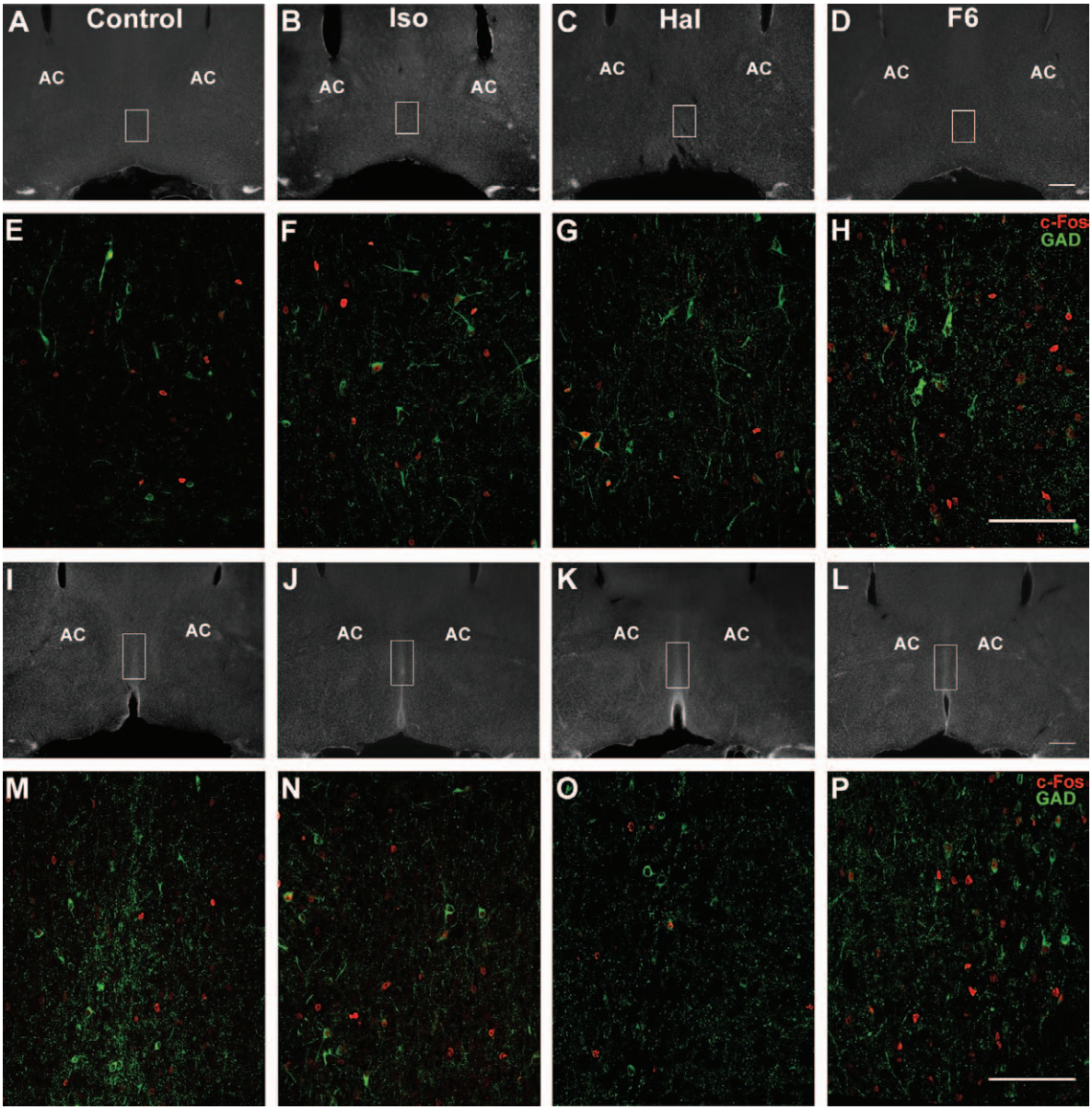

Fig. 4. Representative images showing immunofluorescence-labeled neurons in the median preoptic area (MnPO) of mice after a 2-h *in vivo* drug exposure. *White boxes* in *A–D* indicate the rostral region of interest, shown at higher magnification in *E–H* for mice exposed to (*E*) 100% oxygen, (*F*) 1.2% isoflurane in oxygen, (*G*) 1.0% halothane in oxygen, and (*H*) 3.2% F6 in oxygen. Similarly, *I–L* show a *white box* around the caudal region of interest, which is further magnified in *M–P* for mice treated with (*M*) 100% oxygen, (*N*) 1.2% isoflurane in oxygen, (*O*) 1.0% halothane in oxygen, and (*P*) 3.2% F6 in oxygen. c-Fos staining, red nuclei; glutamic acid decarboxylase (GAD) staining, green cytoplasm. AC marks the anterior commissure. *Scale bars* in *D* and *L* are 100 μm and apply to *A–D* and *I–L*; *scale bars* in *H* and *P* are 60 μm and apply to *E–H* and *M–P*.

disinhibition and/or VLPO-mediated secondary activation. In the case of isoflurane, the ensuing, coordinated inhibitory connections arising from the VLPO and MnPO and projecting to many wake-promoting systems should facilitate a hypnotic state transition when exogenous drugs, such as anesthetics, or endogenous somnogens directly activate or indirectly disinhibit these sleep-promoting neurons.[6] Halothane's neuronal mechanism of action seems to lack this dual recruitment of both sleep-promoting VLPO and MnPO populations. Although it is possible that halothane's failure to significantly induce c-Fos in the GABAergic MnPO represents a false-negative result, substantial evidence confirms that halothane and isoflurane have unique protein targets and mechanisms.[67–69] Halothane has also been shown to cause less depression of the cortical electroencephalogram compared with isoflurane,[70,71] which may be caused in part by halothane's persistent activation of the wake-active locus coeruleus and orexinergic neurons.[72] Consequently, halothane's failure to stimulate GABAergic MnPO neurons is not entirely surprising.

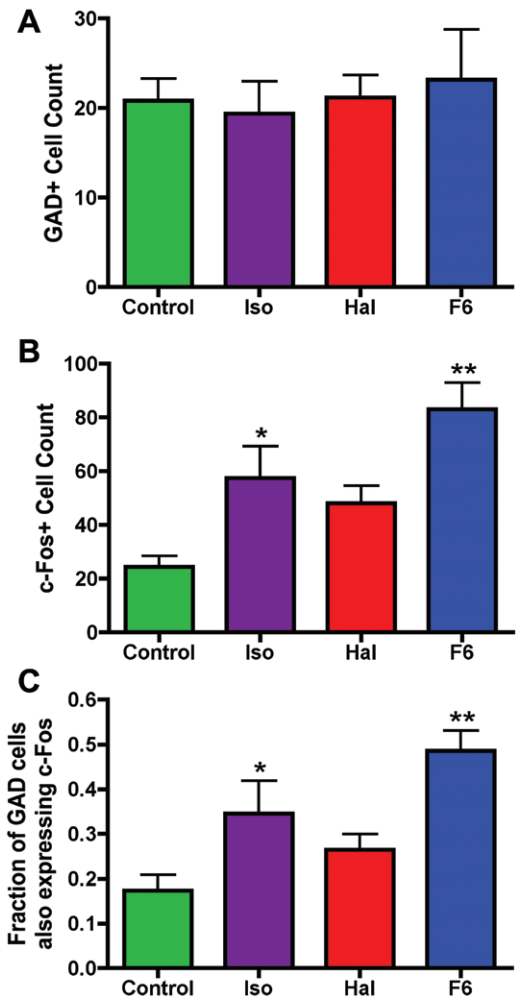

Fig. 5. Quantification of (*A*) glutamic acid decarboxylase (GAD)–labeled neurons, (*B*) c-Fos–labeled neurons, and (*C*) the fraction of GAD-labeled neurons that also express c-Fos in the median preoptic area (MnPO) after a 2-h *in vivo* anesthetic or oxygen exposure. Control, 100% oxygen (n = 12); isoflurane, 1.2% (n = 10); halothane, 1.0% (n = 13); F6, 3.2% (n = 9). All bar graphs show mean + standard error. Data analyzed by one-way ANOVA with *post hoc* Bonferroni correction for multiple testing. *$P < 0.05$; ** $P < 0.01$.

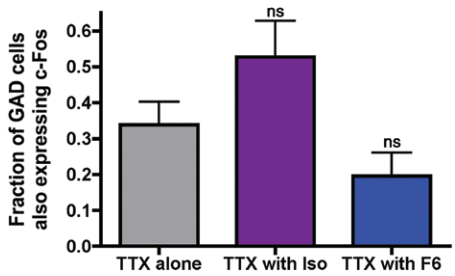

Fig. 6. Quantification of the fraction of glutamic acid decarboxylase (GAD)–labeled neurons that also express c-Fos in the median preoptic area (MnPO) after a 2-h *ex vivo* exposure in oxygenated artificial cerebrospinal fluid to tetrodotoxin (TTX, n = 15) alone or in combination with 1.2% isoflurane (TTX with Iso, n = 6) or F6 (TTX and F6, n = 8). All bar graphs show mean + standard error. Data analyzed by one-way ANOVA with *post hoc* Bonferroni correction for multiple testing. No significant difference (ns) was observed between TTX alone and TTX with Iso or TTX with F6.

Most unexpected, however, are our results with F6 in the MnPO. They demonstrate that isolated activation of GABAergic neurons in the MnPO without the VLPO is not sufficient to elicit anesthetic hypnosis. The ability of F6 and isoflurane to increase c-Fos expression in the MnPO *in vivo* suggests that the MnPO may participate in a nonhypnotic behavioral or physiological effect common to both of these drugs that halothane lacks. F6 and isoflurane could theoretically cause a systemic disruption in some homeostatic function regulated by the MnPO. This theory is consistent with the finding that neither F6 nor isoflurane act directly on GABAergic MnPO neurons to induce c-Fos. The MnPO is known to be sensitive to changes in temperature, cellular osmolarity, blood pressure, and neuroendocrine balance.[66] In our *in vivo* study, mice were kept euthermic in our controlled environmental chambers. Moreover, F6 has been shown to have no effect on thermoregulation in rodents.[73] Pilot trunk blood measurements of serum osmolarity failed to show differences after 2-h exposures between isoflurane-treated (321 ± 9 mOsm/l, n = 3) and oxygen-treated controls (327 ± 5 mOsm/l, n = 4). Based on this lack of a difference and upon the discovery that the osmole-sensitive MnPO neurons are a distinct subset from the sleep-promoting GABAergic ones,[74] serum osmolarity in F6 or halothane-treated mice was not evaluated. Gvilia *et al.*[65] first suggested that the GABAergic neurons in the MnPO might increase their firing rates and c-Fos expression not simply in proportion to the amount of time spent asleep, but rather that these neurons might be responsive to the increasing pressure to sleep, or homeostatic drive for sleep that accrues with increasing time spent in the wake state. In particular, rapid eye movement sleep pressure drives c-Fos activation in GAD-immunoreactive neurons[64] and produces the highest firing rates in MnPO neurons.[4] However, it should be noted that the MnPO's specific function with respect to sleep has recently been called into question.[75] The homeostatic drive for rapid eye movement sleep accrues during isoflurane, sevoflurane, and halothane anesthesia, whereas under halothane a homeostatic drive for nonrapid eye movement sleep also increases.[76] Although the effects of exposure to a nonimmobilizer on subsequent sleep remain unknown, even if sleep pressure were to accumulate during F6 exposure by interfering with the genesis of endogenous sleep states, this might reconcile c-Fos induction in MnPO by isoflurane and F6, but would not explain the halothane result.

Herein, we have shown selective c-Fos induction in the putative GABAergic, sleep-promoting cells of the VLPO by general anesthetics. This supports the notion that the same subset of cells may participate in sleep and

anesthetic-induced unconsciousness. We have previously demonstrated that isoflurane acts directly on the putative sleep-promoting cells of the VLPO to cause an increase in firing rate and membrane potential in *ex vivo* slices.[22] Studies in Drosophila have shown that mutations in single genes can lead to large changes in sleep as well as crucial alterations in anesthetic sensitivity.[55,77] Moreover, increased synaptic activity specifically in sleep-related Drosophila neurons alters responsiveness to isoflurane, unveiling a common neural pathway for sleep and anesthetic sensitivity.[56] However, circuits and genes affecting sleep do not exclusively map onto those regulating anesthetic responsiveness, as demonstrated by our results with halothane in the MnPO. A number of genetic mutations in Drosophila have been shown to reduce sleep without having any effect on isoflurane sensitivity.[55] Different anesthetics also undoubtedly act on distinct molecular and anatomical components of sleep circuitry to varying degrees.[55,72,78–80] From a functional standpoint, the volatile anesthetics distinguish themselves from natural sleep by their accumulation of rapid eye movement sleep debt and inability to relieve preexisting rapid eye movement debt during exposure.[81–83] Although sleep- and anesthetic-induced unconsciousness are clearly distinct states, our findings are consistent with a role for an endogenous sleep-promoting GABAergic neuronal population in the VLPO in genesis of volatile general anesthetic hypnosis but fail to confirm an invariant contribution of MnPO neuronal populations.

Acknowledgments

The authors thank Jason Moore, Ph.D. (Department of Anesthesiology and Critical Care, University of Pennsylvania, Perelman School of Medicine, Philadelphia, Pennsylvania), for his assistance with ImageJ software (National Institutes of Health, Bethesda, Maryland) programming and critical readings of this article and Qing Cheng Meng, Ph.D. (Department of Anesthesiology and Critical Care, University of Pennsylvania, Perelman School of Medicine), for his assistance with high-performance liquid chromatography experiments.

This work was supported by GM088156 and by HL007953 from the National Institutes of Health, Bethesda, Maryland, and by the Department of Anesthesiology and Critical Care, University of Pennsylvania, Philadelphia, Pennsylvania.

Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Kelz: Department of Anesthesiology and Critical Care, University of Pennsylvania, Perelman School of Medicine, 3620 Hamilton Walk, John Morgan Building Room 334, Philadelphia, Pennsylvania 19104. kelzma@uphs.upenn.edu. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

References

1. von Economo C: Sleep as a problem of localization. J Nerv Ment Dis 1930; 71:249–59
2. Gong H, Szymusiak R, King J, Steininger T, McGinty D: Sleep-related c-Fos protein expression in the preoptic hypothalamus: Effects of ambient warming. Am J Physiol Regul Integr Comp Physiol 2000; 279:R2079–88
3. Sherin JE, Shiromani PJ, McCarley RW, Saper CB: Activation of ventrolateral preoptic neurons during sleep. Science 1996; 271:216–9
4. Suntsova N, Szymusiak R, Alam MN, Guzman-Marin R, McGinty D: Sleep-waking discharge patterns of median preoptic nucleus neurons in rats. J Physiol 2002; 543(Pt 2):665–77
5. Szymusiak R, Alam N, Steininger TL, McGinty D: Sleep-waking discharge patterns of ventrolateral preoptic/anterior hypothalamic neurons in rats. Brain Res 1998; 803:178–88
6. Saper CB, Chou TC, Scammell TE: The sleep switch: Hypothalamic control of sleep and wakefulness. Trends Neurosci 2001; 24:726–31
7. McGinty D, Szymusiak R: Brain structures and mechanisms involved in the generation of NREM sleep: Focus on the preoptic hypothalamus. Sleep Med Rev 2001; 5:323–42
8. Lu J, Sherman D, Devor M, Saper CB: A putative flip-flop switch for control of REM sleep. Nature 2006; 441:589–94
9. Benedetto L, Chase MH, Torterolo P: GABAergic processes within the median preoptic nucleus promote NREM sleep. Behav Brain Res 2012; 232:60–5
10. Liu YW, Li J, Ye JH: Histamine regulates activities of neurons in the ventrolateral preoptic nucleus. J Physiol 2010; 588(Pt 21):4103–16
11. Lortkipanidze N, Chidjavadze E, Oniani N, Darchia N, Gvilia I: Sleep-waking behavior following a lesion in the median preoptic nucleus in the rat. Georgian Med News 2009:81–4
12. Zhang J, Yin D, Wu F, Zhang G, Jiang C, Li Z, Wang L, Wang K: Microinjection of adenosine into the hypothalamic ventrolateral preoptic area enhances wakefulness *via* the A1 receptor in rats. Neurochem Res 2013; 38:1616–23
13. Lu J, Greco MA, Shiromani P, Saper CB: Effect of lesions of the ventrolateral preoptic nucleus on NREM and REM sleep. J Neurosci 2000; 20:3830–42
14. McGinty D, Gong H, Suntsova N, Alam MN, Methippara M, Guzman-Marin R, Szymusiak R: Sleep-promoting functions of the hypothalamic median preoptic nucleus: Inhibition of arousal systems. Arch Ital Biol 2004; 142:501–9
15. Sallanon M, Denoyer M, Kitahama K, Aubert C, Gay N, Jouvet M: Long-lasting insomnia induced by preoptic neuron lesions and its transient reversal by muscimol injection into the posterior hypothalamus in the cat. Neuroscience 1989; 32:669–83
16. Franks NP: General anaesthesia: From molecular targets to neuronal pathways of sleep and arousal. Nat Rev Neurosci 2008; 9:370–86
17. Lydic R, Baghdoyan HA: Sleep, anesthesiology, and the neurobiology of arousal state control. ANESTHESIOLOGY 2005; 103:1268–95
18. Li KY, Guan YZ, Krnjević K, Ye JH: Propofol facilitates glutamatergic transmission to neurons of the ventrolateral preoptic nucleus. ANESTHESIOLOGY 2009; 111:1271–8
19. Lu J, Nelson LE, Franks N, Maze M, Chamberlin NL, Saper CB: Role of endogenous sleep-wake and analgesic systems in anesthesia. J Comp Neurol 2008; 508:648–62
20. Nelson LE, Guo TZ, Lu J, Saper CB, Franks NP, Maze M: The sedative component of anesthesia is mediated by GABA(A) receptors in an endogenous sleep pathway. Nat Neurosci 2002; 5:979–84
21. Nelson LE, Lu J, Guo T, Saper CB, Franks NP, Maze M: The alpha2-adrenoceptor agonist dexmedetomidine converges

on an endogenous sleep-promoting pathway to exert its sedative effects. Anesthesiology 2003; 98:428–36

22. Moore JT, Chen J, Han B, Meng QC, Veasey SC, Beck SG, Kelz MB: Direct activation of sleep-promoting VLPO neurons by volatile anesthetics contributes to anesthetic hypnosis. Curr Biol 2012; 22:2008–16
23. Gong H, McGinty D, Guzman-Marin R, Chew KT, Stewart D, Szymusiak R: Activation of c-fos in GABAergic neurones in the preoptic area during sleep and in response to sleep deprivation. J Physiol 2004; 556(Pt 3):935–46
24. Fang Z, Laster MJ, Ionescu P, Koblin DD, Sonner J, Eger EI II, Halsey MJ: Effects of inhaled nonimmobilizer, proconvulsant compounds on desflurane minimum alveolar anesthetic concentration in rats. Anesth Analg 1997; 85:1149–53
25. Perouansky M: Non-immobilizing inhalational anesthetic-like compounds. Handb Exp Pharmacol 2008:209–23
26. Perouansky M, Pearce RA: Effects on synaptic inhibition in the hippocampus do not underlie the amnestic and convulsive properties of the nonimmobilizer 1,2-dichlorohexafluorocyclobutane. Anesthesiology 2004; 101:66–74
27. Sonner J, Li J, Eger EI II: Desflurane and nitrous oxide, but not nonimmobilizers, affect nociceptive responses. Anesth Analg 1998; 86:629–34
28. Dutton RC, Maurer AJ, Sonner JM, Fanselow MS, Laster MJ, Eger EI II: Short-term memory resists the depressant effect of the nonimmobilizer 1-2-dichlorohexafluorocyclobutane (2N) more than long-term memory. Anesth Analg 2002; 94:631–9
29. Mihic SJ, McQuilkin SJ, Eger EI II, Ionescu P, Harris RA: Potentiation of gamma-aminobutyric acid type A receptor-mediated chloride currents by novel halogenated compounds correlates with their abilities to induce general anesthesia. Mol Pharmacol 1994; 46:851–7
30. Dildy-Mayfield JE, Eger EI II, Harris RA: Anesthetics produce subunit-selective actions on glutamate receptors. J Pharmacol Exp Ther 1996; 276:1058–65
31. Liachenko S, Tang P, Somogyi GT, Xu Y: Comparison of anaesthetic and non-anaesthetic effects on depolarization-evoked glutamate and GABA release from mouse cerebrocortical slices. Br J Pharmacol 1998; 123:1274–80
32. Eckenhoff MF, Chan K, Eckenhoff RG: Multiple specific binding targets for inhaled anesthetics in the mammalian brain. J Pharmacol Exp Ther 2002; 300:172–9
33. Shiraishi M, Harris RA: Effects of alcohols and anesthetics on recombinant voltage-gated Na^+ channels. J Pharmacol Exp Ther 2004; 309:987–94
34. Kelz MB, Sun Y, Chen J, Cheng Meng Q, Moore JT, Veasey SC, Dixon S, Thornton M, Funato H, Yanagisawa M: An essential role for orexins in emergence from general anesthesia. Proc Natl Acad Sci U S A 2008; 105:1309–14
35. Sun Y, Chen J, Pruckmayr G, Baumgardner JE, Eckmann DM, Eckenhoff RG, Kelz MB: High throughput modular chambers for rapid evaluation of anesthetic sensitivity. BMC Anesthesiol 2006; 6:13
36. Hoffman GE, Le WW: Just cool it! Cryoprotectant anti-freeze in immunocytochemistry and *in situ* hybridization. Peptides 2004; 25:425–31
37. Ionescu P, Eger EI II, Trudell J: Direct determination of oil/saline partition coefficients. Anesth Analg 1994; 79:1056–8
38. Chesney MA, Perouansky M, Pearce RA: Differential uptake of volatile agents into brain tissue *in vitro*. Measurement and application of a diffusion model to determine concentration profiles in brain slices. Anesthesiology 2003; 99:122–30
39. Wei H, Kang B, Wei W, Liang G, Meng QC, Li Y, Eckenhoff RG: Isoflurane and sevoflurane affect cell survival and BCL-2/BAX ratio differently. Brain Res 2005; 1037:139–47
40. Franklin KBJ, Paxinos G: The Mouse Brain in Stereotaxic Coordinates, 3rd edition. New York, Elsevier Academic Press, 2008
41. Suntsova N, Guzman-Marin R, Kumar S, Alam MN, Szymusiak R, McGinty D: The median preoptic nucleus reciprocally modulates activity of arousal-related and sleep-related neurons in the perifornical lateral hypothalamus. J Neurosci 2007; 27:1616–30
42. Gaus SE, Strecker RE, Tate BA, Parker RA, Saper CB: Ventrolateral preoptic nucleus contains sleep-active, galaninergic neurons in multiple mammalian species. Neuroscience 2002; 115:285–94
43. Imas OA, Ropella KM, Ward BD, Wood JD, Hudetz AG: Volatile anesthetics enhance flash-induced gamma oscillations in rat visual cortex. Anesthesiology 2005; 102:937–47
44. Ferrarelli F, Massimini M, Sarasso S, Casali A, Riedner BA, Angelini G, Tononi G, Pearce RA: Breakdown in cortical effective connectivity during midazolam-induced loss of consciousness. Proc Natl Acad Sci U S A 2010; 107:2681–6
45. Lewis LD, Weiner VS, Mukamel EA, Donoghue JA, Eskandar EN, Madsen JR, Anderson WS, Hochberg LR, Cash SS, Brown EN, Purdon PL: Rapid fragmentation of neuronal networks at the onset of propofol-induced unconsciousness. Proc Natl Acad Sci U S A 2012; 109:E3377–86
46. Cimenser A, Purdon PL, Pierce ET, Walsh JL, Salazar-Gomez AF, Harrell PG, Tavares-Stoeckel C, Habeeb K, Brown EN: Tracking brain states under general anesthesia by using global coherence analysis. Proc Natl Acad Sci U S A 2011; 108:8832–7
47. Lee U, Ku S, Noh G, Baek S, Choi B, Mashour GA: Disruption of frontal-parietal communication by ketamine, propofol, and sevoflurane. Anesthesiology 2013; 118:1264–75
48. Li D, Voss LJ, Sleigh JW, Li X: Effects of volatile anesthetic agents on cerebral cortical synchronization in sheep. Anesthesiology 2013; 119:81–8
49. Jordan D, Ilg R, Riedl V, Schorer A, Grimberg S, Neufang S, Omerovic A, Berger S, Untergehrer G, Preibisch C, Schulz E, Schuster T, Schröter M, Spoormaker V, Zimmer C, Hemmer B, Wohlschläger A, Kochs EF, Schneider G: Simultaneous electroencephalographic and functional magnetic resonance imaging indicate impaired cortical top-down processing in association with anesthetic-induced unconsciousness. Anesthesiology 2013; 119:1031–42
50. Massimini M, Ferrarelli F, Huber R, Esser SK, Singh H, Tononi G: Breakdown of cortical effective connectivity during sleep. Science 2005; 309:2228–32
51. Pace-Schott EF, Hobson JA: The neurobiology of sleep: Genetics, cellular physiology and subcortical networks. Nat Rev Neurosci 2002; 3:591–605
52. Arnulf I, Ferraye M, Fraix V, Benabid AL, Chabardès S, Goetz L, Pollak P, Debû B: Sleep induced by stimulation in the human pedunculopontine nucleus area. Ann Neurol 2010; 67:546–9
53. Mashour GA: Integrating the science of consciousness and anesthesia. Anesth Analg 2006; 103:975–82
54. Bonhomme V, Boveroux P, Vanhaudenhuyse A, Hans P, Brichant JF, Jaquet O, Boly M, Laureys S: Linking sleep and general anesthesia mechanisms: This is no walkover. Acta Anaesthesiol Belg 2011; 62:161–71
55. Joiner WJ, Friedman EB, Hung HT, Koh K, Sowcik M, Sehgal A, Kelz MB: Genetic and anatomical basis of the barrier separating wakefulness and anesthetic-induced unresponsiveness. PLoS Genet 2013; 9:e1003605
56. Kottler B, Bao H, Zalucki O, Imlach W, Troup M, van Alphen B, Paulk A, Zhang B, van Swinderen B: A sleep/wake circuit controls isoflurane sensitivity in Drosophila. Curr Biol 2013; 23:594–8
57. Kandel L, Chortkoff BS, Sonner J, Laster MJ, Eger EI II: Nonanesthetics can suppress learning. Anesth Analg 1996; 82:321–6
58. Franks NP, Lieb WR: Selective actions of volatile general anaesthetics at molecular and cellular levels. Br J Anaesth 1993; 71:65–76

59. Eger EI II, Koblin DD, Sonner J, Gong D, Laster MJ, Ionescu P, Halsey MJ, Hudlicky T: Nonimmobilizers and transitional compounds may produce convulsions by two mechanisms. Anesth Analg 1999; 88:884–92
60. Eger EI II, Gong D, Xing Y, Raines DE, Flood P: Acetylcholine receptors and thresholds for convulsions from flurothyl and 1,2-dichlorohexafluorocyclobutane. Anesth Analg 2002; 95:1611–5
61. Scammell T, Gerashchenko D, Urade Y, Onoe H, Saper C, Hayaishi O: Activation of ventrolateral preoptic neurons by the somnogen prostaglandin D2. Proc Natl Acad Sci U S A 1998; 95:7754–9
62. Scammell TE, Gerashchenko DY, Mochizuki T, McCarthy MT, Estabrooke IV, Sears CA, Saper CB, Urade Y, Hayaishi O: An adenosine A2a agonist increases sleep and induces Fos in ventrolateral preoptic neurons. Neuroscience 2001; 107:653–63
63. Gallopin T, Luppi PH, Cauli B, Urade Y, Rossier J, Hayaishi O, Lambolez B, Fort P: The endogenous somnogen adenosine excites a subset of sleep-promoting neurons *via* A2A receptors in the ventrolateral preoptic nucleus. Neuroscience 2005; 134:1377–90
64. Gvilia I, Turner A, McGinty D, Szymusiak R: Preoptic area neurons and the homeostatic regulation of rapid eye movement sleep. J Neurosci 2006; 26:3037–44
65. Gvilia I, Xu F, McGinty D, Szymusiak R: Homeostatic regulation of sleep: A role for preoptic area neurons. J Neurosci 2006; 26:9426–33
66. Uschakov A, McGinty D, Szymusiak R, McKinley MJ: Functional correlates of activity in neurons projecting from the lamina terminalis to the ventrolateral periaqueductal gray. Eur J Neurosci 2009; 30:2347–55
67. Kotani N, Akaike N: The effects of volatile anesthetics on synaptic and extrasynaptic GABA-induced neurotransmission. Brain Res Bull 2013; 93:69–79
68. Pan JZ, Xi J, Eckenhoff MF, Eckenhoff RG: Inhaled anesthetics elicit region-specific changes in protein expression in mammalian brain. Proteomics 2008; 8:2983–92
69. Correa AM: Gating kinetics of Shaker K^+ channels are differentially modified by general anesthetics. Am J Physiol 1998; 275(4 Pt 1):C1009–21
70. Antunes LM, Golledge HD, Roughan JV, Flecknell PA: Comparison of electroencephalogram activity and auditory evoked responses during isoflurane and halothane anaesthesia in the rat. Vet Anaesth Analg 2003; 30:15–23
71. Hudetz AG: Effect of volatile anesthetics on interhemispheric EEG cross-approximate entropy in the rat. Brain Res 2002; 954:123–31
72. Gompf H, Chen J, Sun Y, Yanagisawa M, Aston-Jones G, Kelz MB: Halothane-induced hypnosis is not accompanied by inactivation of orexinergic output in rodents. Anesthesiology 2009; 111:1001–9
73. Maurer AJ, Sessler DI, Eger EI II, Sonner JM: The nonimmobilizer 1,2-dichlorohexafluorocyclobutane does not affect thermoregulation in the rat. Anesth Analg 2000; 91:1013–6
74. Gvilia I, Angara C, McGinty D, Szymusiak R: Different neuronal populations of the rat median preoptic nucleus express c-fos during sleep and in response to hypertonic saline or angiotensin-II. J Physiol 2005; 569(Pt 2):587–99
75. Sakai K: Sleep-waking discharge profiles of median preoptic and surrounding neurons in mice. Neuroscience 2011; 182:144–61
76. Pick J, Chen Y, Moore JT, Sun Y, Wyner AJ, Friedman EB, Kelz MB: Rapid eye movement sleep debt accrues in mice exposed to volatile anesthetics. Anesthesiology 2011; 115:702–12
77. Weber B, Schaper C, Bushey D, Rohlfs M, Steinfath M, Tononi G, Cirelli C, Scholz J, Bein B: Increased volatile anesthetic requirement in short-sleeping Drosophila mutants. Anesthesiology 2009; 110:313–6
78. Schaper C, Höcker J, Böhm R, Roeder T, Bein B: The shaker potassium channel is no target for xenon anesthesia in short-sleeping *Drosophila melanogaster* mutants. ScientificWorldJournal 2012; 2012:373709
79. Franks NP, Zecharia AY: Sleep and general anesthesia. Can J Anaesth 2011; 58:139–48
80. Mashour GA, Pal D: Interfaces of sleep and anesthesia. Anesthesiol Clin 2012; 30:385–98
81. Pal D, Lipinski WJ, Walker AJ, Turner AM, Mashour GA: State-specific effects of sevoflurane anesthesia on sleep homeostasis: Selective recovery of slow wave but not rapid eye movement sleep. Anesthesiology 2011; 114:302–10
82. Mashour GA, Lipinski WJ, Matlen LB, Walker AJ, Turner AM, Schoen W, Lee U, Poe GR: Isoflurane anesthesia does not satisfy the homeostatic need for rapid eye movement sleep. Anesth Analg 2010; 110:1283–9
83. Nelson AB, Faraguna U, Tononi G, Cirelli C: Effects of anesthesia on the response to sleep deprivation. Sleep 2010; 33:1659–67