

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

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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-naïve 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 heterogeneous 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

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.

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.

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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 $37.1 \pm 0.5^\circ\text{C}$ throughout the exposure. Inhaled exposure occurred for 2 h with 1.2% isoflurane (administered *via* a Dräger model 19.1 isoflurane vaporizer; Dräger Medical Inc., Telford, PA), 1.0% halothane (administered *via* a Dräger model 19.1 halothane vaporizer), 3.2% F6 (administered *via* a Dräger 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 EC₉₉ 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 anti-freeze 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 NaH₂PO₄, 1.0 mM MgSO₄, 2.0 mM CaCl₂, 10 mM glucose, and 26 mM NaHCO₃) bubbled with 95% O₂–5% CO₂. 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 NaH₂PO₄, 1.0 mM MgSO₄, 2.0 mM CaCl₂, 10 mM glucose, and 26 mM NaHCO₃)

bubbled with 95% O₂–5% CO₂. 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 C₁₈ 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 NaH₂PO₄ 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% H₂O₂, 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 \pm 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-naïve 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

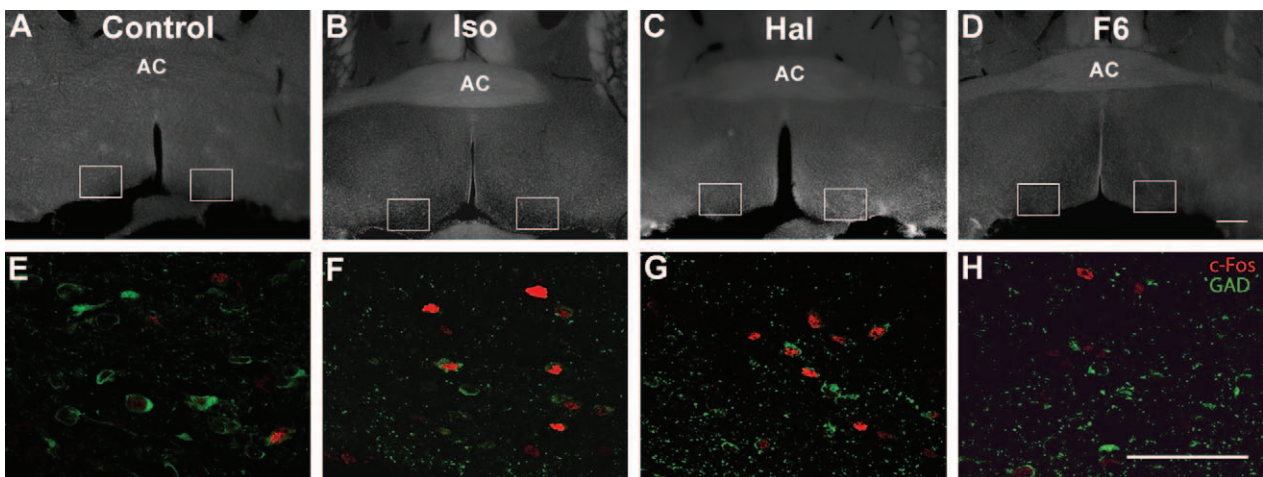


Fig. 1. Representative images showing immunofluorescence-labeled neurons in the ventrolateral preoptic area of mice after a 2-h *in vivo* drug exposure. White boxes in A–D indicate the region of interest, shown at higher magnification in E–H for mice exposed to (E) 100% oxygen, (F) 1.2% isoflurane in 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 μ m and applies to A–D; scale bar in H is 60 μ m and applies to E–H.

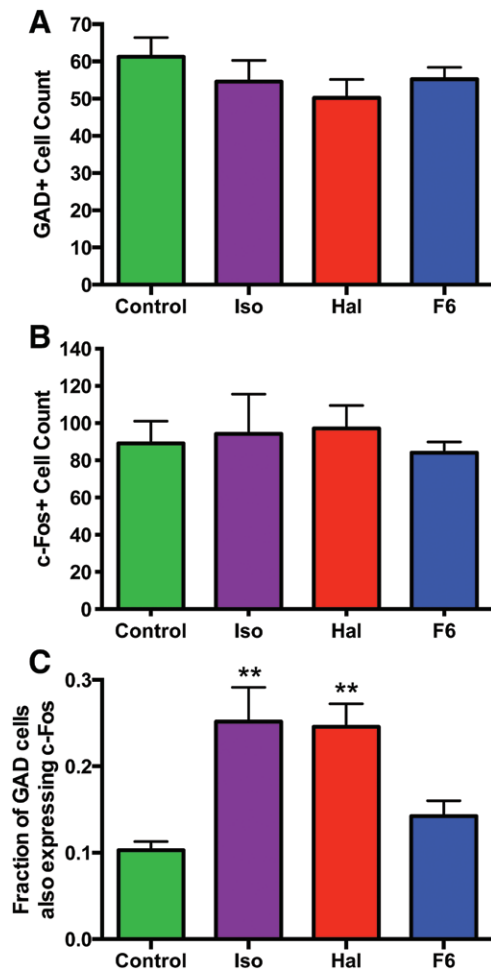


Fig. 2. Quantification of (A) glutamic acid decarboxylase (GAD)-labeled neurons, (B) c-Fos-labeled neurons, and (C) the fraction of GAD-labeled neurons that also express c-Fos in the ventrolateral preoptic area (VLPO) after a 2-h *in vivo* anesthetic or oxygen exposure. Control, 100% oxygen (n = 10); 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-naïve 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 traits.^{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

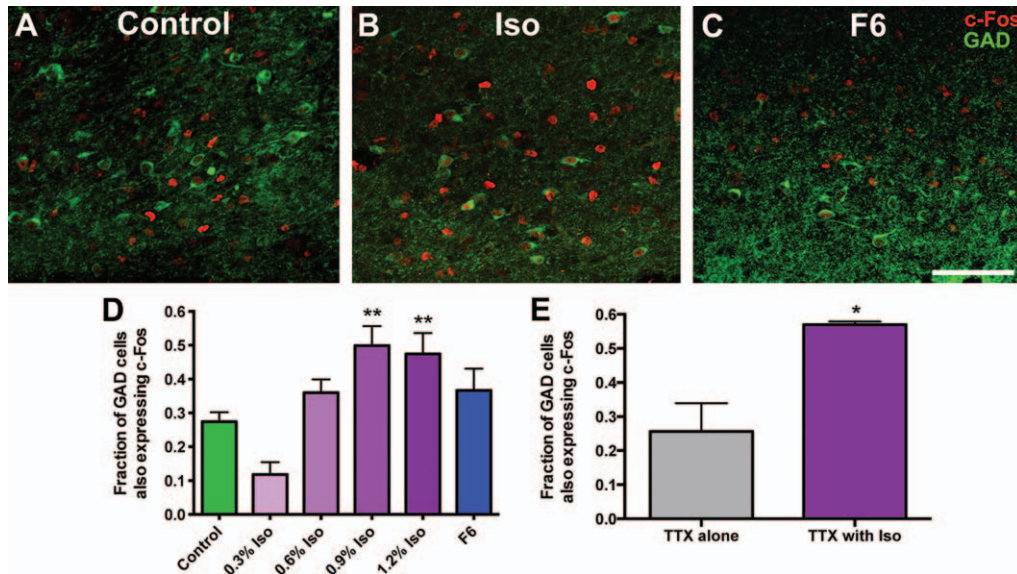


Fig. 3. Representative immunofluorescent images of the ventrolateral preoptic area (VLPO) after a 2-h *ex vivo* exposure to (A) oxygenated artificial cerebrospinal fluid (control), (B) 1.2% isoflurane in oxygenated artificial cerebrospinal fluid, and (C) 24 μM F6 in oxygenated artificial cerebrospinal 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 isoflurane²² 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

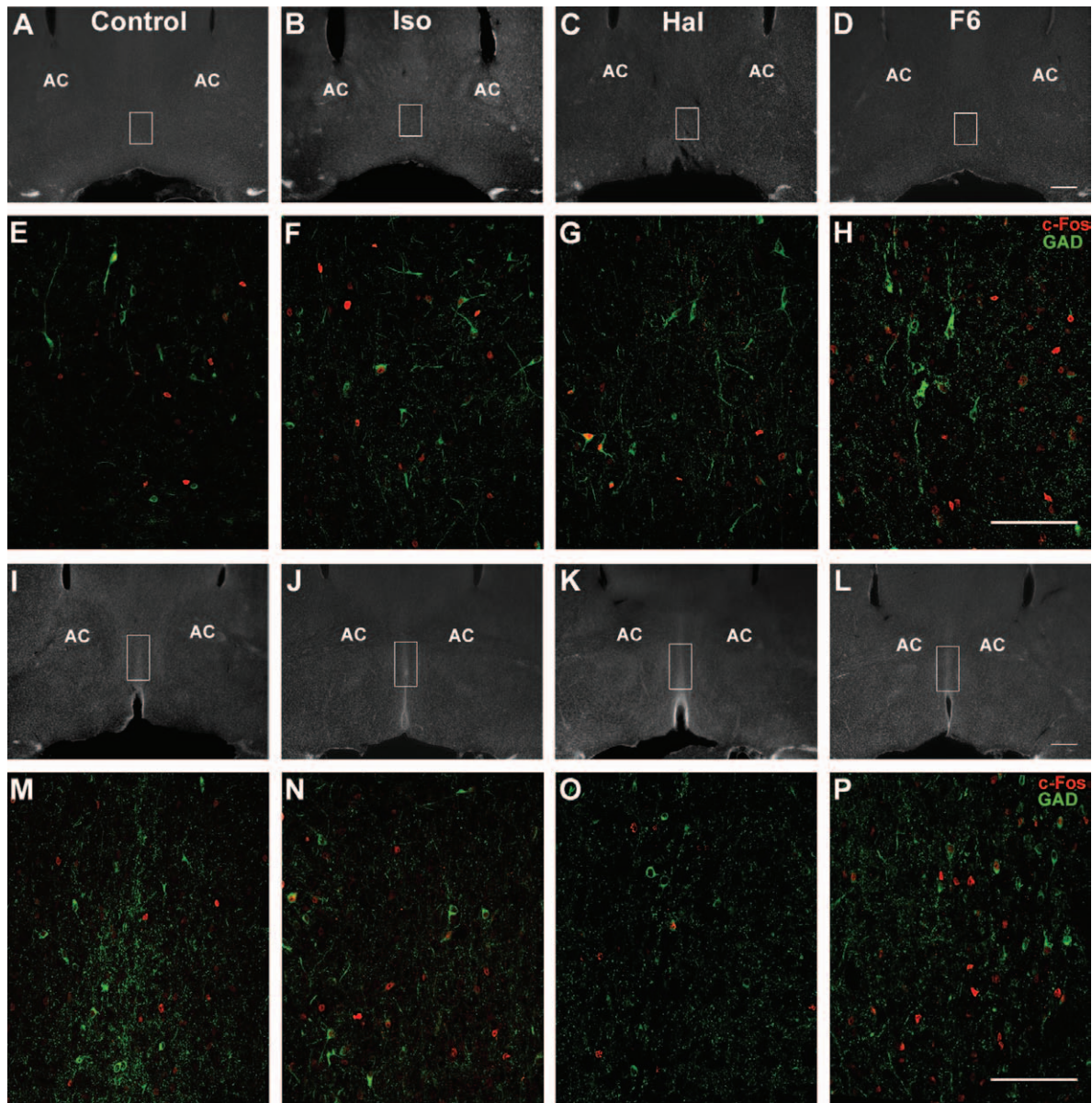


Fig. 4. Representative images showing immunofluorescence-labeled neurons in the median preoptic area (MnPO) of mice after a 2-h *in vivo* drug exposure. *White boxes* in *A–D* indicate the rostral region of interest, shown at higher magnification in *E–H* for mice exposed to (*E*) 100% oxygen, (*F*) 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.

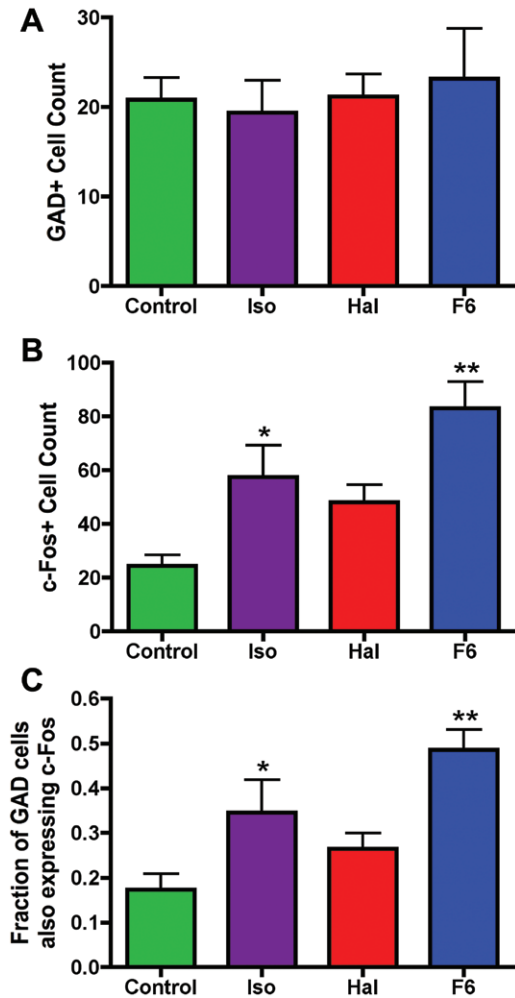


Fig. 5. Quantification of (A) glutamic acid decarboxylase (GAD)-labeled neurons, (B) c-Fos-labeled neurons, and (C) the fraction of GAD-labeled neurons that also express c-Fos in the median preoptic area (MnPO) after a 2-h *in vivo* anesthetic or oxygen exposure. Control, 100% oxygen (n = 12); isoflurane, 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. * $P < 0.05$; ** $P < 0.01$.

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 non-hypnotic 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

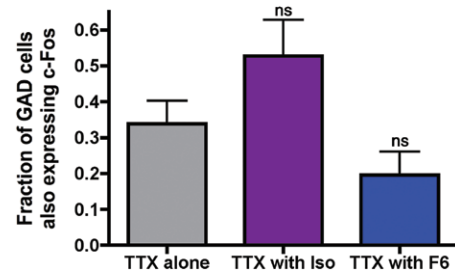


Fig. 6. Quantification of the fraction of glutamic acid decarboxylase (GAD)-labeled neurons that also express c-Fos in the median preoptic area (MnPO) after a 2-h *ex vivo* exposure in oxygenated artificial cerebrospinal fluid to tetrodotoxin (TTX, n = 15) alone or in combination with 1.2% isoflurane (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.

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 neurons⁶⁴ 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.

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Competing Interests

The authors declare no competing interests.

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