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Activation of Parabrachial Nucleus Glutamatergic Neurons Accelerates Reanimation from Sevoflurane Anesthesia in Mice

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ABSTRACT

Background: The parabrachial nucleus (PBN), which is a brainstem region containing glutamatergic neurons, is a key arousal nucleus. Injuries to the area often prevent patient reanimation. Some studies suggest that brain regions that control arousal and reanimation are a key part of the anesthesia recovery. Therefore, we hypothesize that the PBN may be involved in regulating emergence from anesthesia.

Methods: We investigated the effects of specific activation or inhibition of PBN glutamatergic neurons on sevoflurane general anesthesia using the chemogenetic "designer receptors exclusively activated by designer drugs" approach. Optogenetic methods combined with polysomnographic recordings were used to explore the effects of transient activation of PBN glutamatergic neuron on sevoflurane anesthesia. Immunohistochemical techniques are employed to reveal the mechanism by which PBN regulated sevoflurane anesthesia.

Results: Chemogenetic activation of PBN glutamatergic neurons by intraperitoneal injections of clozapine-N-oxide decreased emergence time (mean ± SD, control *vs.* clozapine-N-oxide, 55 ± 24 *vs.* 15 ± 9 s, $P = 0.0002$) caused by sevoflurane inhalation and prolonged induction time (70 ± 15 *vs.* 109 ± 38 s, n = 9, $P = 0.012$) as well as the ED50 of sevoflurane (1.48 *vs.* 1.60%, $P = 0.0002$), which was characterized by a rightward shift of the loss of righting reflex cumulative curve. In contrast, chemogenetic inhibition of PBN glutamatergic neurons slightly increased emergence time (56 ± 26 *vs.* 87 ± 26 s, n = 8, $P = 0.034$). Moreover, instantaneous activation of PBN glutamatergic neurons expressing channelrhodopsin-2 during steady-state general anesthesia with sevoflurane produced electroencephalogram evidence of cortical arousal. Immunohistochemical experiments showed that activation of PBN induced excitation of cortical and subcortical arousal nuclei during sevoflurane anesthesia.

Conclusions: Activation of PBN glutamatergic neurons is helpful to accelerate the transition from general anesthesia to an arousal state, which may provide a new strategy in shortening the recovery time after sevoflurane anesthesia.

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- The parabrachial nucleus is a brainstem region involved in arousal.
- Brain regions involved in arousal regulate anesthetic induction and emergence.

What This Article Tells Us That Is New

- Using chemogenetic techniques, activation of parabrachial nucleus glutamatergic neurons prolonged anesthetic induction and hastened emergence in mice. Inhibition of these neurons provided opposite effects.
- Modulating the activity of arousal centers may provide an approach to controlling the duration of general anesthesia.

Anesthetics have been critical tools in modern medicine for nearly 171 yr.[1] General anesthesia is similar to a drug-induced transient coma-like state. In the United States, nearly 60,000 patients receive general anesthesia each day for surgical and medical procedures,[2] but it is not known how these drugs impact hypnosis. Therefore, there is no effective treatment for patients with delayed emergence from anesthesia. Basically, the mechanisms of general anesthesia have been thought to be multiple molecular targets of anesthesia.[3,4] Thus, an effective way to characterize how anesthetics act in the context of neural anatomy and network connectivity may help elucidate the mechanism of general anesthesia.

Numerous sites in the brain have been shown to promote arousal, such as the basal forebrain, ventral tegmental area, tuberomammillary nucleus, and locus coeruleus.[5] Pharmacologic activation of these areas has been reported to produce reanimation during general anesthesia.[6–9] These results have led to the increasingly popular theory that anesthesia may induce unconsciousness by acting on endogenous arousal neural circuitry. However, whether other arousal-promoting nuclei are involved in general anesthesia remains unclear. Therefore, we aimed to improve the understanding of the mechanism of anesthetic recovery by further studying the arousal system.

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Recent evidence has suggested that the parabrachial nucleus (PBN) is a predominant arousal-active nucleus containing glutamatergic neurons.[10] Studies have found that selective deletion of the vesicular glutamate transporter-2 (Vglut2) gene in PBN neurons increases non–rapid eye movement sleep during the dark period.[10] Lesions in the PBN also lead to increases in non–rapid eye movement and rapid eye movement sleep during the night in rats.[11] Moreover, PBN neurons project to many arousal-promoting areas in the brain, including the basal forebrain, lateral hypothalamus, thalamus, and cortex.[12,13] These regions have been shown to be involved in general anesthesia.[14–16] Here, we hypothesized that the PBN glutamatergic neurons may be involved in regulating emergence from general anesthesia, and this effect may be related to the activation of the downstream arousal nucleus.

In the present study, we used chemogenetics and optogenetics to explore the role of PBN glutamatergic neurons in regulating sevoflurane general anesthesia states. Utilizing Vglut2-Cre transgenic mice in which Cre recombinase is expressed exclusively in glutamatergic neurons,[17] we selectively expressed modified G protein–coupled muscarinic receptors (excitatory hM3Dq receptors and inhibitory hM4Di receptors) and channelrhodopsin-2 (ChR2) in PBN glutamatergic neurons. Then, we used chemogenetics to assess effects of continuous activation or inhibition of PBN glutamatergic neurons on regulation of sevoflurane inhalation anesthesia states. In addition, optogenetics, which uses light to activate ion channels and modulate ionic conductance of neuronal membranes, provides precise temporal control of specific neuronal populations *in vivo*. Thus, it allows us to observe effects of transient manipulation of neuronal activity on general anesthesia in mice. We also examined expression of c-Fos (a well-known indicator of rapid and transient neuronal activity) in the cortex and subcortical arousal nuclei after PBN activation in glutamic acid decarboxylase-67 (GAD-67)–green fluorescent protein (GPF) knock-in mice during sevoflurane anesthesia. The results suggest that PBN glutamatergic neurons play a critical role in general anesthesia.

Materials and Methods

Animals

Adult male Vglut2-IRES-Cre mice (8 to 10 weeks, 22 to 25 g) and GAD-67-GPF knock-in mice (8 to 10 weeks, 22 to 25 g) on a C57BL/6J background were used for all experiments. Vglut2-IRES-Cre mice expressing Cre recombinase under control of the *Vglut2* gene promoter were provided by Dr. Ji Hu (Shanghai Tech University, Shanghai, China). GAD-67–GFP knock-in mice coexpressing enhanced GFP and γ-aminobutyric acid (GABA) synthesizing enzyme, GAD-67, were provided by Yuchio Yanagawa, Ph.D. (Gunma University, Maebashi, Japan). Animals were housed individually in the Shanghai Model Organisms Center, Inc., Shanghai, China, at an ambient temperature of 22 ± 0.5°C, with relative humidity of 60 ± 2%, under an automatically controlled 12-h light/12-h dark cycle (lights on at 07:00, illumination intensity ≈ 100 lux),[18] and with free access to food and water. Experimental protocols were approved by the Medical Experimental Animal Administrative Committee of Fudan University, Shanghai, China. Every effort was made to minimize the number of animals used as well as their pain and discomfort. For one section of the experiment, we designed animal code numbers and used online randomization tools (https://www.random.org/lists/) to randomize mice into different groups.

Chemicals

Sevoflurane was purchased from the HengRui Pharmaceutical Co., Ltd. (China). Rabbit polyclonal anti–c-Fos antibody was purchased from Millipore (catalog No. ABE457, USA), and fluorescent goat anti-rabbit IgG was obtained from Life Technologies Corporation (catalog No. A-21206, USA). Clozapine-N-oxide (CNO) was purchased from LKT Labs (USA) and dissolved in sterile saline before use.

Surgery

Mice were anesthetized with chloral hydrate (5% in saline, 360 mg/kg) and placed in a stereotaxic apparatus (RWD Life Science, China). To selectively express Cre-dependent hM3Dq or hM4Di receptors in PBN glutamatergic neurons, we used adeno-associated virus (AAV) as a vector. hSyn-DIO-hM3Dq-mCherry-AAV or hSyn-hM4Di-mCherry-AAV was delivered bilaterally into the PBN (coordinates: posterior = 5.2 mm and lateral = 1.2 mm from bregma; 2.2 mm below the endocranium, as per the mouse atlas of Paxinos and Franklin) of Vglut2-Cre mice.[19–22] In a separate group of Vglut2-Cre mice, hSyn-DIO-ChR2-mCherry-AAV was microinjected into the PBN to selectively express Cre-dependent ChR2. Injections of the viral vector (35 to 50 nl) were performed using a compressed air delivery system as previously described.[22,24]

Three weeks after injection, mice were chronically implanted with electrodes for polysomnographic recordings of electroencephalograms and electromyograms as described previously.[25] For optogenetic stimulation, the fiber optic cannula (200 μm diameter; Newton Inc., China) was placed in the PBN (posterior = 5.2 mm and lateral = 1.2 mm from

bregma; 2.2 mm below the endocranium) and fixed to the skull using dental cement. The scalp wound was closed with surgical sutures, and each mouse was kept in a warm environment until it resumed normal activity as previously described.[26,27]

Spectral Analysis of Electroencephalogram Data

Electroencephalogram and electromyogram signals were amplified and filtered (electroencephalogram, 0.5 to 25 Hz; electromyogram, 20 to 200 Hz), digitized at a sampling rate of 128 Hz, and recorded using SleepSign for Animal (Kissei Comtec, Japan).[28–30] Electroencephalogram power spectra were computed for consecutive 10-s epochs within the frequency range of 0.5 to 25 Hz (0.5-Hz bins for 10 s) using a fast Fourier transform routine. Electroencephalogram changes from 30-s epochs within 100 s before and after optical stimulation were analyzed. Electroencephalogram frequency bands (low δ, 0.5 to 2.5 Hz; θ, 2.5 to 7.0 Hz; α, 7.0 to 13.0 Hz; β, 13.0 to 20 Hz) were based on visual inspection of anesthetic-sensitive frequencies as demonstrated previously.[31]

Anesthetic Response to Sevoflurane in Vglut2-Cre Mice

Loss of righting reflex (LORR) was used as a behavioral test to investigate hypnotic properties of sevoflurane according to previously described methods.[32] To determine the sevoflurane concentration that induced LORR, each mouse was placed in a small acrylic glass chamber ($12 \times 12 \times 12\ cm^3$) connected to a sevoflurane vaporizer (Vapor2000; Dräger Medical Systems, Inc., Germany) with 1.5 l/min flow of 100% oxygen. The outflow from the chamber was connected to an infrared gas analyzer (Infinity Vista XL; Dräger Medical Systems, Inc., Germany). Sevoflurane was administered to the chamber starting at a concentration of 1.2%, and concentration was increased in increments of 0.1% until LORR occurred. The concentration of sevoflurane reached a constant value less than 15 min after a change of dose as verified by the infrared gas analyzer. Each concentration of anesthetic was maintained for a minimum equilibration period of 15 min, after which the chamber was rotated to place the mouse on its back. A mouse was considered to show LORR if it did not turn onto all four feet within 30 s, and this was confirmed by a second trial. The percentage of mice showing LORR at each dose of sevoflurane was established for the CNO and vehicle groups, and ED50 was estimated from the dose–response equation described in the section "Statistical Analysis." To assess sevoflurane induction and emergence, the time to LORR and time for recovery of the righting reflex were investigated after 2% sevoflurane was given. Each mouse was placed individually in the small acrylic glass chamber, after which 2% sevoflurane was delivered with 1.5 l/min oxygen. At 15-s intervals, the chamber was rotated to place the mouse on its back, and the ability to right itself was assessed. The time to LORR (induction time) was defined as the time at which a mouse first lost its ability to right itself for more than 30 s from the time of sevoflurane onset. After a 30-min exposure to 2% sevoflurane, the mouse was removed from the chamber and placed in a supine position in room air. Emergence time was defined as the time at which a mouse righted itself (all four feet on the floor) from the time of removal from the sevoflurane chamber. The investigator was blinded to the allocated groups.

Optogenetic Stimulation *In Vivo*

The optical fiber cannula was attached to a rotating joint (FRJ_FC-FC, Doric Lenses, Canada) to relieve torque. The joint was connected *via* a fiber to a 473-nm blue laser diode (Newton Inc., China). Light pulses were generated through a stimulator (SEM-7103, Nihon Kohden, Japan) and output *via* an isolator (ss-102J, Nihon Kohden). For photostimulation, we used programed light pulse trains (5-ms pulses at 30 Hz for 30 s) and recorded electroencephalogram/electromyogram during the experiments. Light intensity was tested by a power meter (PM10, Coherent, USA) before each experiment and calibrated to emit 20 to 30 mW/mm^2 from the tip of the optical fiber cannula.[19] No *a priori* statistical power calculation was conducted. In this part of the experiment, we used sample sizes that indicated as sufficient to identify biologically meaningful differences in the previous studies.[31]

c-Fos Immunohistochemistry

We bilaterally injected AAV vectors containing excitatory hM3Dq receptors (CMV-hM3Dq-mCherry-AAV) into the PBN of GAD-67–GFP knock-in mice. Three weeks after virus injection, mice were pretreated with either vehicle or CNO 1 mg/kg (intraperitoneally) and placed in an acrylic glass chamber with inflow of 100% oxygen. After 1 h of conditioning, 2% sevoflurane was administered to the chamber for 2 h. After sevoflurane anesthesia, all animals were killed for immunohistochemical experiments. One series of sections was processed for c-Fos staining as described previously.[20,33,34]

The numbers of c-Fos–positive neurons and GFP-positive neurons in the prefrontal cortex and motor cortex were counted at +1.94 mm and +0.62 mm from bregma, respectively. The numbers of c-Fos–positive neurons and GFP-positive neurons in the basal forebrain and lateral

hypothalamus were counted at −0.10 and −2.18 mm from bregma, respectively. The average number of c-Fos–positive neurons of the nucleus in each hemisphere was analyzed statistically as described.[33] The investigator was blinded to the allocated groups.

Statistical Analysis

In this study, we injected virus into the PBN in 83 mice. After histologic tests were carried out to identify the position of virus infection, 51 mice with inaccurate injection positions were excluded, and the other 32 mice were included into the data analysis. Graph Pad Prism 7.0 (GraphPad Software, USA) and SPSS 16.0 software (IBM Corp., USA) were used for statistical evaluation. LORR dose–response data were curve-fitted by nonlinear regression (Sigmoidal, Four Parameter Logistic) with Graph Pad Prism to determine the ED50. Group differences in c-Fos numbers, onset of LORR, return of the righting reflex, and electroencephalogram power spectrum were compared using independent-samples Student's *t* tests. Before analysis, all data underwent the Kolmogorov–Smirnov normality test and Levene test; results showed that all data from different groups of mice satisfied the normality and homogeneity of variance. The sample size calculations in experiments were determined by power analysis and previous study. Data are expressed as mean ± SD. $P < 0.05$ was considered statistically significant.

Results

Effects of Chemogenetic Activation of Glutamatergic Neurons in the PBN on Sevoflurane Sensitivity, Induction, and Emergence

To selectively activate PBN glutamatergic neurons by chemogenetics, we bilaterally injected DIO-hM3Dq-mCherry-AAV into the PBN of Vglut2-IRES-Cre mice (fig. 1A). Heat maps show maximum overlap of hM3Dq expression in the PBN from eight mice (fig. 1B). Bath application of CNO (5 μM) elicited recurrent bursting in neurons from acute slices of mice expressing hM3Dq in the PBN (fig. 1C). Robust fluorescence of mCherry was observed in the PBN (fig. 1, D and F). Very little c-Fos protein was detected in neurons expressing mCherry after vehicle injection (fig. 1E). In contrast, CNO injection greatly increased c-Fos protein expression (fig. 1G) in mCherry-expressing neurons, which confirmed ligand-induced activation of PBN glutamatergic neurons expressing hM3Dq *in vivo*.

To investigate behavioral consequences of PBN hM3Dq activation, we first examined anesthetic sensitivity of sevoflurane by increasing sevoflurane concentration from 1.2 to 2.0%. All animals underwent systemic delivery of vehicle or CNO (1 mg/kg, intraperitoneally) for 60 min before the test. No mice showed LORR at 1.3% or lower sevoflurane concentration. However, at 1.5% sevoflurane, five of nine mice in the vehicle group but only one of nine mice in the CNO group (PBN activation) showed LORR. At 1.6%, all vehicle group mice but only four of nine mice in the CNO group showed LORR. A dose–response plot of percent LORR *versus* sevoflurane concentration was right-shifted for PBN activation compared with vehicle control mice (fig. 1I). The concentration at which half the mice showed LORR, or ED50 (LORR), was 1.48% in the vehicle group (n = 9) and 1.60% in the CNO group (n = 9). The ED50 (LORR) was different between the CNO and control groups ($P = 0.0002$), which indicated lower sevoflurane sensitivity in PBN activation mice. When exposed to 2% sevoflurane, CNO group mice showed a significantly longer LORR onset (induction) time that averaged 109 ± 38 s (n = 9) as compared with 70 ± 15 s ($P = 0.012$, n = 9, fig. 1H) in control mice. When returned to room air after exposure to 2% sevoflurane for 30 min, time to recover the righting reflex (emergence time) was shorter in CNO group mice (15 ± 9 s, n = 9) compared with control mice (55 ± 24 s, $P = 0.0002$, n = 9; fig. 1J). These results suggest that activation of glutamatergic neurons in the PBN may decrease sevoflurane sensitivity and accelerate reanimation after sevoflurane anesthesia.

Chemogenetic Inhibition of Glutamatergic Neurons in the PBN Prolonged Time to Emergence

To test effects of selectively inhibiting PBN glutamatergic neurons on sevoflurane anesthesia, we bilaterally injected DIO-hM4Di-mCherry-AAV into the PBN of Vglut2-IRES-Cre mice (fig. 2A). Heat maps show hM4Di expression profiles of eight mice, with maximum overlap in red (fig. 2B). Immunofluorescence staining showed that mCherry was specifically expressed in the PBN (fig. 2, D and F). CNO decreased expression of c-Fos in the PBN region (fig. 2, E and G), which confirmed ligand-induced inhibition of PBN glutamatergic neurons expressing hM4Di *in vivo*.

To determine effects of attenuating endogenous activity of PBN glutamatergic neurons on sevoflurane anesthesia, we examined anesthetic sensitivity as previously described. The ED50 (LORR) was 1.46% in the vehicle group (n = 8) and 1.38% in the CNO group (n = 8). The ED50 (LORR) was different between the CNO and control groups ($P = 0.021$; fig. 2I). The induction time was not significantly different between the CNO group (76 ± 21 s, n = 8) and control group (65 ± 15 s, $P = 0.244$, n = 8, fig. 2H). However, time to

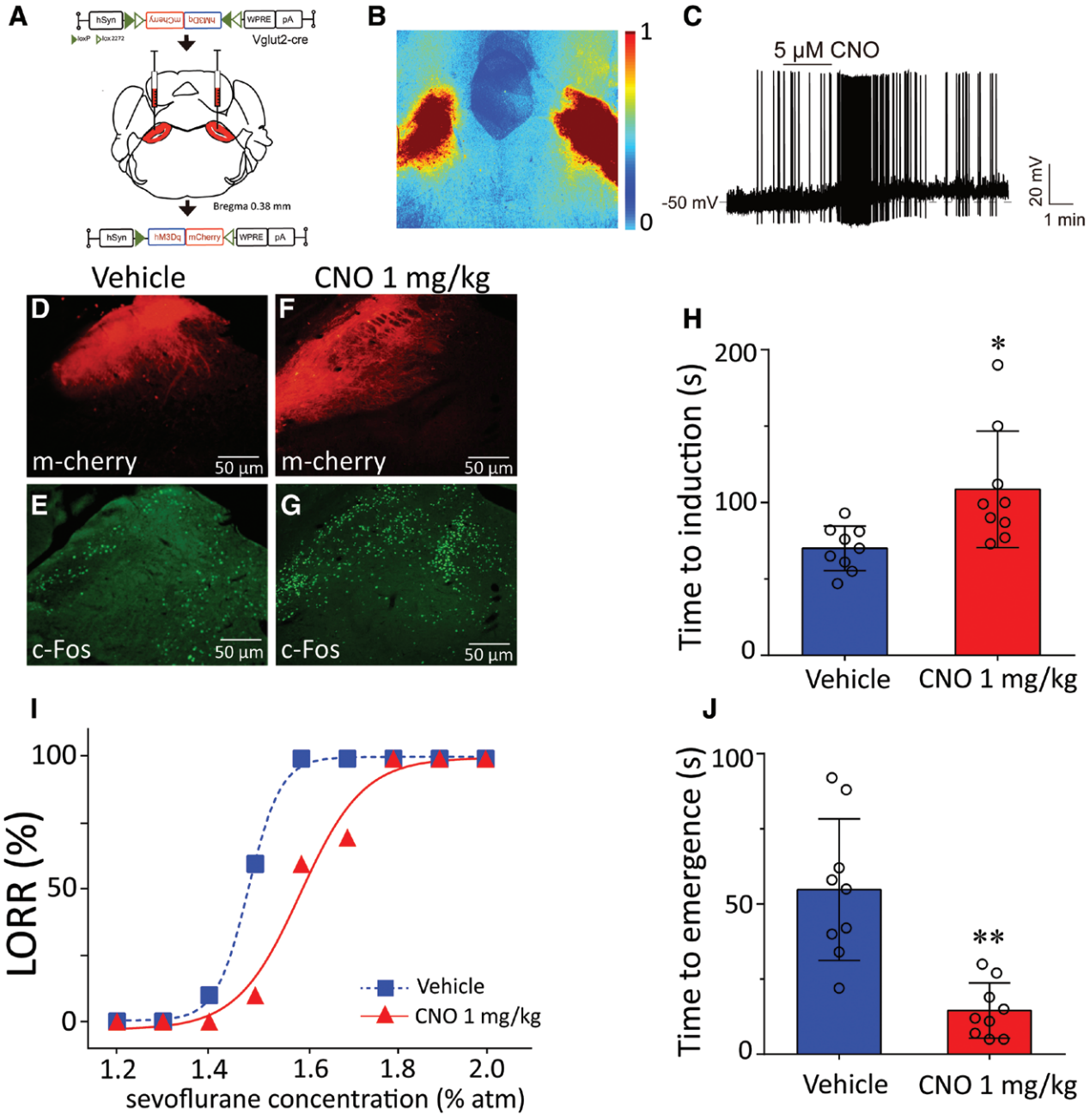

Fig. 1. Effects of chemogenetic activation of glutamatergic neurons in the parabrachial nucleus (PBN) on sevoflurane sensitivity, induction, and emergence time. (*A*) Coronal section showing the injection target of PBN in vesicular glutamate transporter-2 (Vglut2)–Cre mice and schematic representation of hM3Dq vectors injected under control of the hSyn promoter. (*B*) Heat map generated from eight injected mice. *Red* indicates the area of maximum overlap of hM3Dq expression across the eight animals. (*C*) Whole-cell current clamp recordings of PBN neurons expressing hM3Dq showing increased firing responses to clozapine-N-oxide (CNO; 5 μM) applied *in vitro.* (*D–G*) Representative images of mCherry (*red*) and c-Fos (*green*) expression in the PBN after injection of vehicle or CNO (1 mg/kg). *(H)* Induction time with exposure to 2% sevoflurane after treatment with vehicle or CNO, respectively. (*I*) Dose–response plot of cumulative percentage of mice showing that loss of righting reflex (LORR) between PBN activation and control mice. *Horizontal axis* is the dose of sevoflurane in percentage atmosphere concentration (% atm). (*J*) Emergence time with exposure to 2% sevoflurane after treatment with vehicle or CNO, respectively. Values represent mean ± SD (n = 9), **P* < 0.05 or ***P* < 0.01 indicates significantly different from the vehicle group as assessed by unpaired Student's *t* test. *Scale bars* = 50 μm.

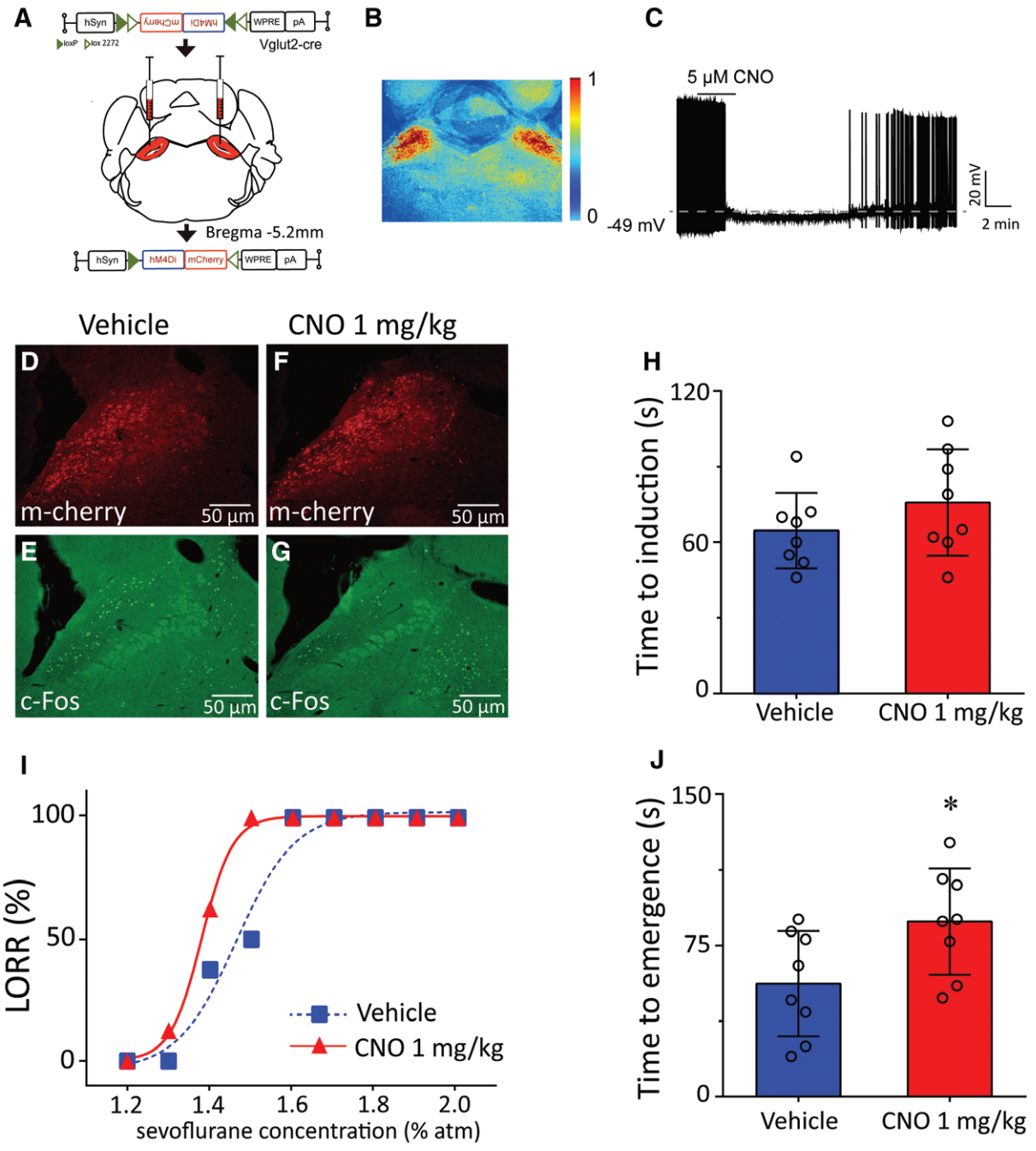

Fig. 2. Chemogenetic inhibition of glutamatergic neurons in the parabrachial nucleus (PBN) prolonged emergence time from sevoflurane anesthesia. (*A*) Coronal section showing the injection target of the PBN in vesicular glutamate transporter-2 (Vglut2)-Cre mice and schematic representation of hM4Di vectors injected under control of the hSyn promoter. (*B*) Heat map generated from eight injected mice. *Red* indicates the area of maximum overlap of hM4Di expression across the eight animals. (*C*) Whole-cell current clamp recordings of PBN neurons expressing hM4Di showing suppressed firing responses to clozapine-N-oxide (CNO; 5 μM) applied *in vitro*. (*D–G*) Representative images of mCherry (*red*) and c-Fos (*green*) expression in the PBN after injection of vehicle or CNO (1 mg/kg). *(H)* Induction times with exposure to 2% sevoflurane after treatment with vehicle or CNO, respectively. (*I*) Dose–response plot of cumulative percentage of mice showing that loss of righting reflex (LORR) between PBN activation and control mice. *Horizontal axis* is the dose of sevoflurane in percentage atmosphere concentration (% atm). (*J*) Emergence times with exposure to 2% sevoflurane after treatment with vehicle or CNO, respectively. Values represent mean ± SD (n = 8), **P* < 0.05 indicates significantly different from the vehicle group as assessed by unpaired Student's *t* test. *Scale bars* = 50 μm.

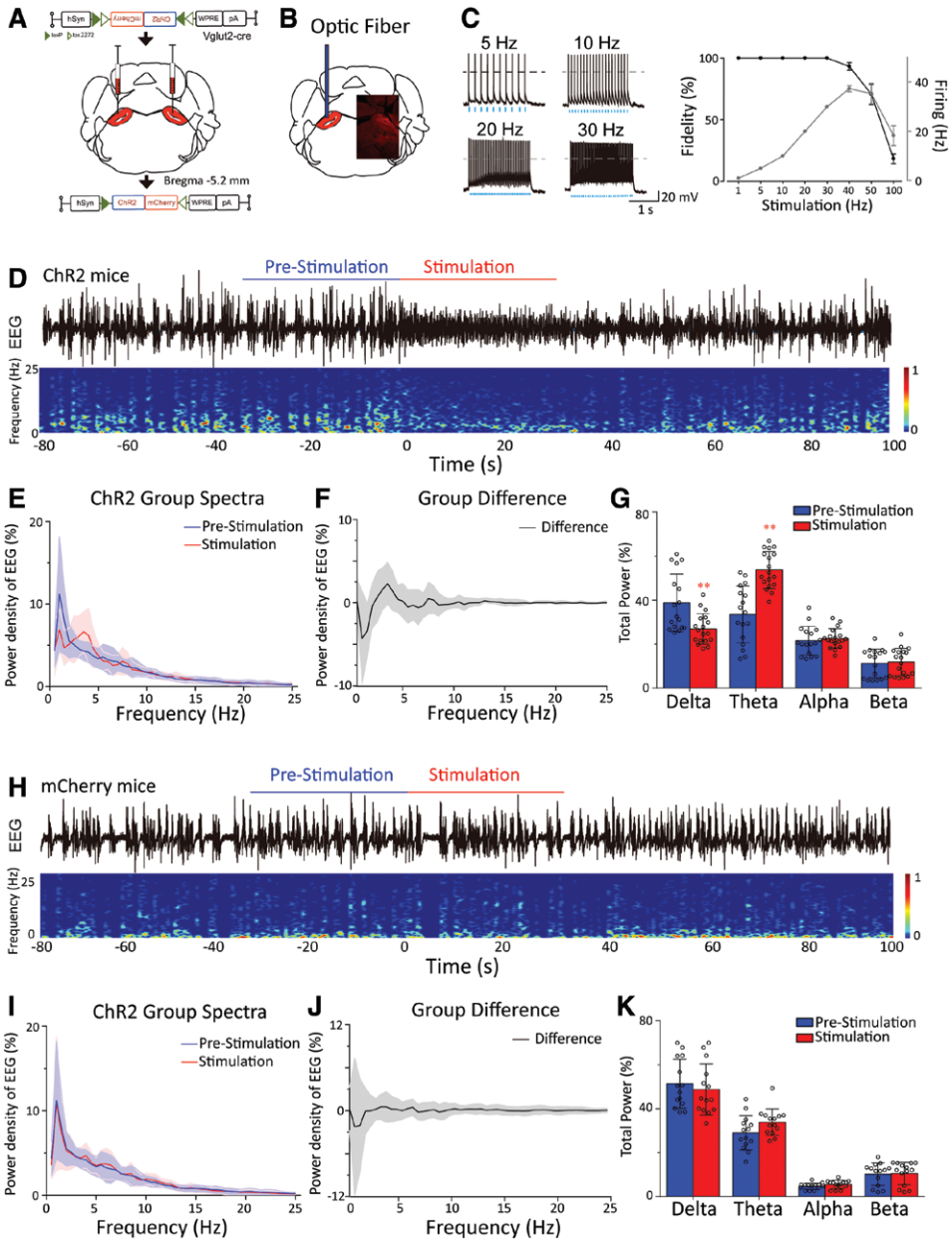

Fig. 3. Photostimulation of parabrachial nucleus (PBN) glutamatergic neurons induced cortical arousal during sevoflurane inhalation anesthesia. (*A* and *B*) Schematic representation showing the location of the optic fiber in the PBN and a coronal brain section with mCherry fluorescence to confirm that channelrhodopsin-2 (ChR2) protein was expressed in the PBN. (*C*) Neuronal firing showing effective entrainment up to 30 Hz photostimulation of PBN neurons. (*D*) Typical examples of electroencephalogram and electroencephalogram power spectral in a mouse injected with AAV-DIO-ChR2-mCherry. Time 0 indicates the beginning of photostimulation during continuous sevoflurane anesthesia. The *blue and red lines* indicate the 30-s intervals used for power spectral density comparison. (*E*) Normalized group power spectral densities from PBN-ChR2 or PBN-mCherry mice with prephotostimulation (*blue*) and photostimulation (*red*). *Shaded areas* represent mean ± SD. (*F*) Difference between prephotostimulation and photostimulation power spectra. (*G*) Photostimulation decreased relative electroencephalogramδpower and increased θpower in PBN-ChR2 mice but not in PBN-mCherry mice (*K*). (*H*) Typical examples of electroencephalogram and electroencephalogram power spectral in a mouse injected with AAV-DIO- mCherry. Time 0 indicates the beginning of photostimulation during continuous sevoflurane anesthesia. The *blue and red lines* indicate the 30-s intervals used for power spectral density comparison. (*I*) Normalized group power spectral densities from PBN-ChR2 or PBN-mCherry mice with prephotostimulation (*blue*) and photostimulation (*red*). *Shaded areas* represent mean ± SD. (*J*) Difference between prephotostimulation and photostimulation power spectra. (*K*) Photostimulation decreased relative electroencephalogramδpower and increased θpower in PBN-mCherry mice but not in PBN-ChR2 mice (*G*). $^{**}P < 0.01$ indicates comparison with the prestimulation group as assessed by unpaired Student's *t* test. Data are presented as mean ± SD (n = 14 to 17).

recover the righting reflex (emergence time) was slightly longer in CNO group mice (87 ± 26 s, n = 8) compared with control mice (56 ± 26 s, P = 0.034, n = 8, fig. 2J). These results show that inhibition of PBN glutamatergic neurons by CNO changed the sevoflurane anesthesia recovery.

Photostimulation of PBN Glutamatergic Neurons Induced Cortical Arousal during Sevoflurane General Anesthesia

To explore effects of transient manipulation of PBN glutamatergic neuron activity during sevoflurane anesthesia in mice, ChR2, a blue light-gated cation channel, was expressed in glutamatergic neurons by injecting hSyn-DIO-ChR2-mCherry-AAV into the PBN ofVglut2-Cre mice (fig. 3A). For whole-cell current clamp recording, trains of brief blue light pulses entrained firing of PBN ChR2-expressing neurons up to 50 Hz with high-frequency fidelity between 1 Hz and 30 Hz (fig. 3B)

We stimulated the PBN *in vivo* in a PBN-ChR2 mouse with 5-ms pulses of blue light at 30 Hz during 2% sevoflurane anesthesia. Photostimulation caused a rapid decrease in electroencephalogram amplitude and an increase in electroencephalogram frequency (fig. 3C). The time-frequency domain spectrogram in figure 3D (computed from the electroencephalogram in fig. 3C) shows rapid loss of low δ (0.5 to 2.5 Hz) power during the 30 s of photostimulation. In group comparisons of power spectral densities from 30 s before photostimulation (blue line) and 30 s during photostimulation (red line) of PBN glutamatergic neurons, we found a significant transformation between base and photostimulation spectral frequencies (fig. 3, D and E). Low δ power significantly decreased during PBN photostimulation (0.27 ± 0.07%, n = 17) compared with prestimulation (0.39 ± 0.13%, P = 0.002, n = 17). However, θ (2.5 to 7 Hz) power increased during photostimulation (0.54 ± 0.08%, n = 17) compared with controls (0.33 ± 0.13%, P < 0.0001, n = 17, fig. 3F). These findings suggest that selective activation of PBN glutamatergic neurons is sufficient to elicit cortical electroencephalogram changes consistent with arousal during continuous deep (2%) sevoflurane anesthesia.

To exclude possible effects of photostimulation, we injected hSyn-DIO-mCherry-AAV into the PBN of Vglut2-Cre mice. Figure 3G shows a representative raw electroencephalogram during stimulation in a PBN-mCherry mouse. During stimulation, there was no change in electroencephalogram amplitude or frequency. Figure 3, H–J, shows power spectral densities obtained prestimulation and during the stimulation in PBN-mCherry mice. The large degree of overlap between the 95% CIs subjectively indicates no significant differences between the two spectral densities.

Activation of PBN Glutamatergic Neurons Increased c-Fos Expression of Cortical and Subcortical Arousal Nuclei during Sevoflurane Anesthesia

To explore the mechanism of cortical arousal by PBN glutamatergic neurons during sevoflurane anesthesia, numbers of c-Fos–positive neurons in the prefrontal cortex and motor cortex were determined after activation of PBN-hM3Dq neurons by CNO in GAD-67–GFP knock-in mice.The transgenic mice coexpressed enhanced GFP and GABA-synthesizing enzyme GAD-67, which characterizes γ-aminobutyric acid–mediated neurons in the central nervous system.[35] Figure 4, parts C–F, and c–f, shows representative photomicrographs of c-Fos expression after sevoflurane anesthesia in the prefrontal cortex and motor cortex of PBN-hM3Dq mice treated with vehicle or CNO (1 mg/kg). Robust GFP was observed in these regions (fig. 4, parts c1–f1). Analysis of the number of c-Fos–immunoreactive nuclei showed that CNO increased expression of c-Fos in the prefrontal cortex and motor cortex by 93% (CNO group: 83 ± 10, vehicle group: 43 ± 26, P = 0.012, n = 5) and 65% (CNO group: 41 ± 11; vehicle group: 25 ± 9, P = 0.029, n = 5), respectively, relative to the vehicle group. Figure 4, parts c2–c4, shows representative photomicrographs of c-Fos/GABA double-labeled neurons in the prefrontal cortex and motor cortex. We calculated the percentage of c-Fos + GABA/c-Fos and found that CNO decreased the percentage in the prefrontal cortex group by 60% (CNO group: 0.08 ± 0.03; vehicle group: 0.22 ± 0.10, P = 0.019, n = 5) compared with the vehicle group.These results suggest that activation of PBN glutamatergic neurons significantly increases excitability of non—γ-aminobutyric acid–mediated neurons in the prefrontal cortex and motor cortex.

In addition, we observed activation of PBN glutamatergic neurons in subcortical arousal nuclei. Figure 5, B, C, F, G, b, c, f, and g, shows representative photomicrographs of c-Fos expression after sevoflurane anesthesia in the basal forebrain and lateral hypothalamus of PBN-hM3Dq mice treated with vehicle or CNO (1 mg/kg). CNO injection significantly increased the number of c-Fos–positive neurons in the basal forebrain and lateral hypothalamus by 2.8-fold (CNO group: 17 ± 5; vehicle group: 6 ± 2, P = 0.0021, n = 5) and 4.6-fold (CNO group: 106 ± 56; vehicle group: 23 ± 13, P = 0.012, n = 5), respectively, relative to vehicle injection. These results suggest that stimulation of PBN glutamatergic neurons increases activation of cortical and subcortical arousal nuclei, which may accelerate the transition from general anesthesia to an arousal state.

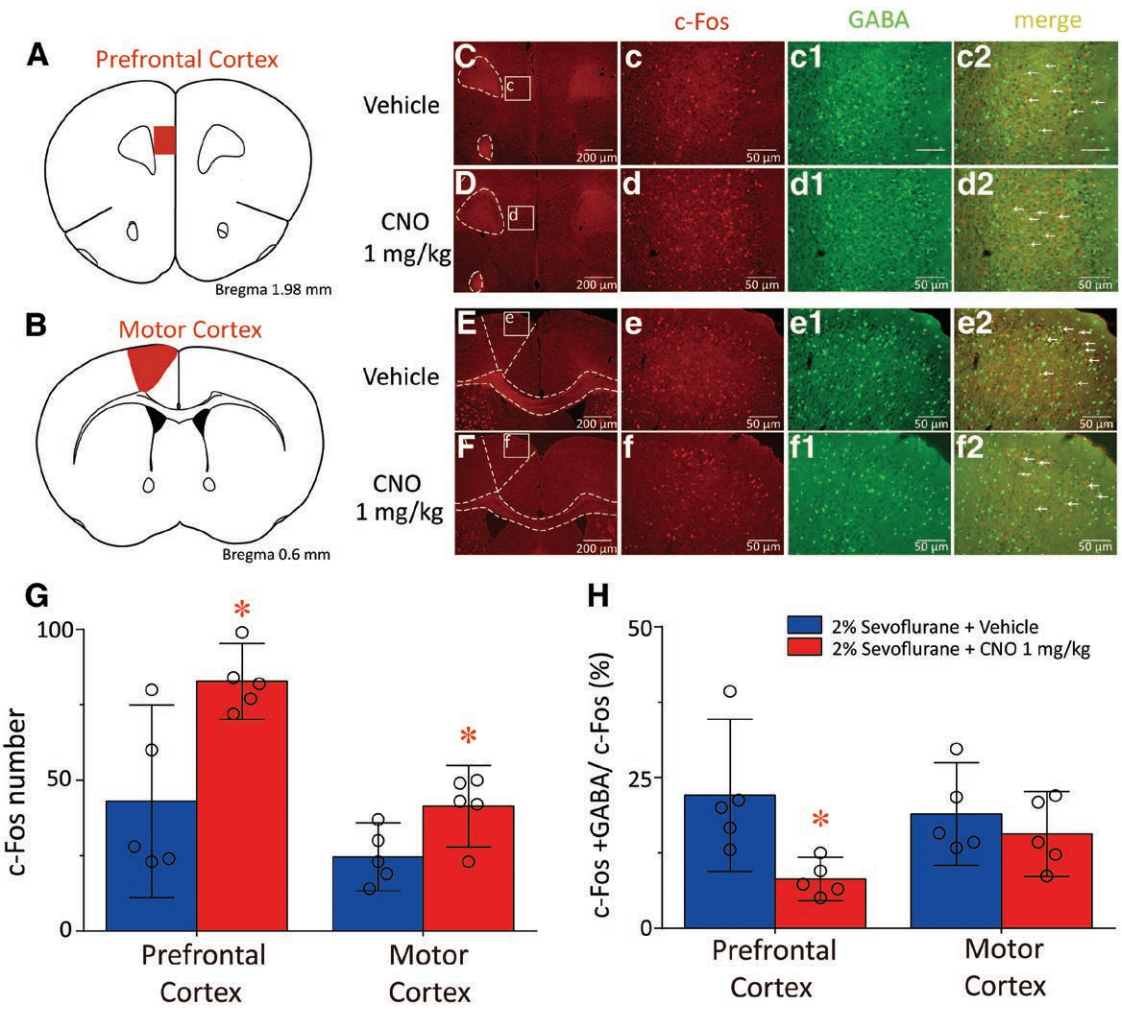

Fig. 4. Activation of parabrachial nucleus (PBN) glutamatergic neurons increased cortical c-Fos expression during sevoflurane inhalation anesthesia. (*A* and *B*) Position of the prefrontal cortex and motor cortex in the mouse brain atlas. (*C–F*) Representative photomicrographs of c-Fos expression in the prefrontal cortex (*C*, *D*, *c*, and *d*) and motor cortex (*E*, *F*, *e*, and *f*). (*c–f*) Higher magnification views of the rectangular areas marked in *c–f* from *C–F*. (*c1–f1*) Green fluorescent protein (GFP) represents the distribution of γ-aminobutyric acid (GABA) neurons in the prefrontal cortex and motor cortex. (*c2–f2*) Immunofluorescent double-label for GABA (*green*) and c-Fos (*red*). (*G* and *H*) Amount of c-Fos expression and the percentage of double-labeled neurons in the prefrontal cortex and motor cortex of mice administered vehicle or clozapine-N-oxide (CNO) after treatment with 2% sevoflurane inhalation. Values represent mean ± SD (n = 4 to 5), *$P < 0.05$ indicates significantly different from the vehicle group as assessed by unpaired Student's *t* test.

Discussion

In this study, we manipulated PBN glutamatergic neurons to elucidate regulatory effects on sevoflurane inhalation anesthesia in mice. The results showed that, compared with control mice, cell-specific activation of PBN glutamatergic neurons by chemogenetics resulted in a higher ED50 for LORR, longer induction time, and shorter emergence time associated with sevoflurane anesthesia. Moreover, using an optogenetic approach, instantaneous activation of PBN glutamatergic neurons produced cortical arousal during sevoflurane anesthesia. Conversely, inhibition of PBN glutamatergic neurons slightly prolonged emergence time. Our results demonstrate that glutamatergic transmission from the PBN alone is sufficient to modulate the anesthetic state of sevoflurane.

Loss of awareness is an essential component of general anesthesia and sleep. Therefore, it has been suggested that overlapping neural mechanisms may underlie these states. A series of studies confirmed that specific activation of arousal nuclei, such as the ventral tegmental area and locus coeruleus, not only interrupts sleep and induces arousal,[36,37] but also induces reanimation from general anesthesia.[31,38] Recent studies have found that the PBN

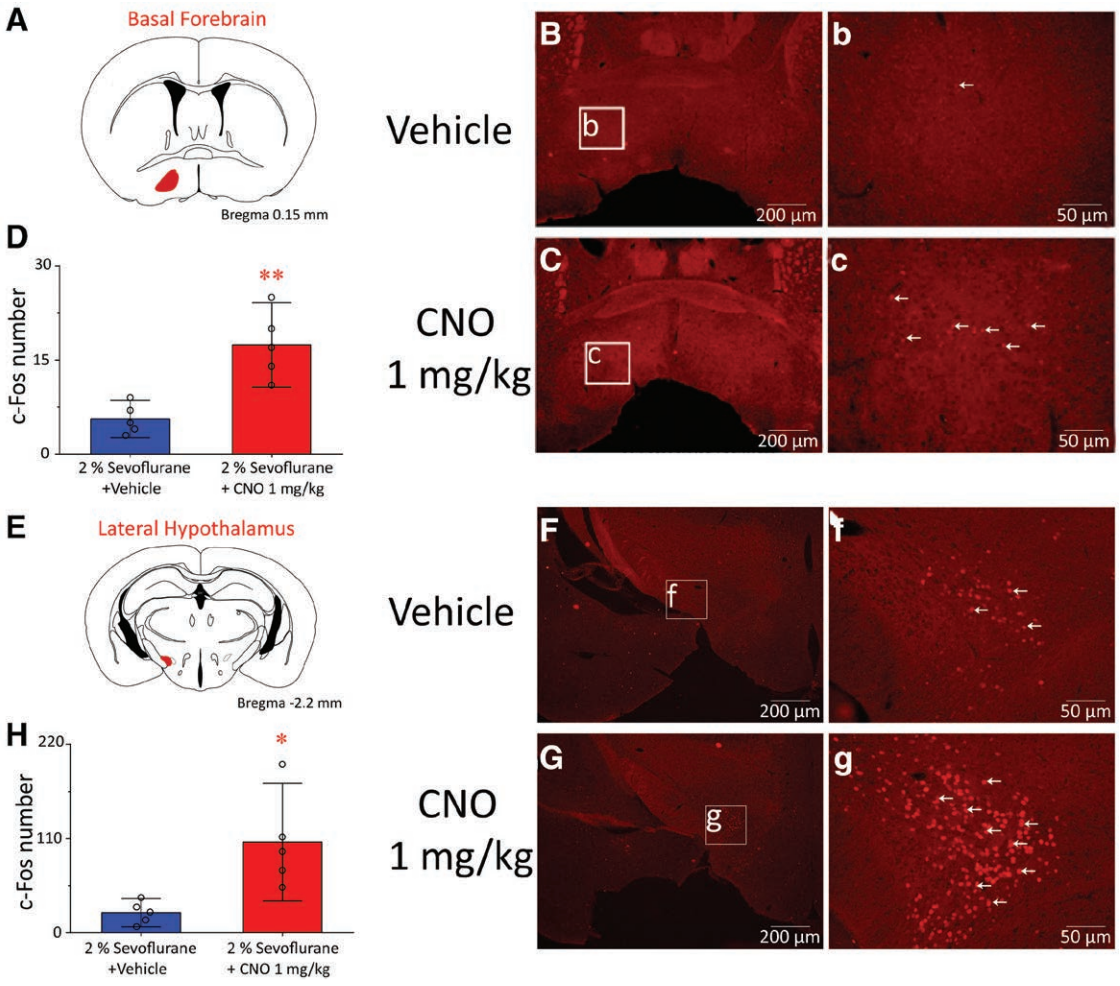

Fig. 5. (*A*) Position of the basal forebrain in the mouse brain atlas. (*B* and *C*) Representative photomicrographs of c-Fos expression in the basal forebrain. (*b* and *c*) Higher magnification views of the boxed areas marked in *b* and *c* of *B* and *C*. (*D*) Amount of c-Fos expression in the basal forebrain of mice administration vehicle or clozapine-N-oxide (CNO) after treatment with 2% sevoflurane inhalation. (*E*) Position of the lateral hypothalamus in the mouse brain atlas. (*F* and *G*) Representative photomicrographs of c-Fos expression in the lateral hypothalamus. (*f* and *g*) Higher magnification views of the boxed areas marked in *f* and *g* of *F* and *G*. (*H*) Amount of c-Fos expression in the lateral hypothalamus of mice administration vehicle or clozapine-N-oxide (CNO) after treatment with 2% sevoflurane inhalation. Values represent mean ± SD (n = 5). $*P < 0.05$ or $**P < 0.01$ indicates significantly different from the vehicle group as assessed by unpaired Student's *t* test.

plays a pivotal role in maintenance of wakefulness.[10,11] Electrical activation of the PBN is sufficient to induce reanimation of the righting reflex and to produce a significant decrease in electroencephalogram delta power.[39] However, manipulations of PBN function in rats used electrical stimulation that did not allow selective manipulation of PBN glutamatergic neurons. Thus, stimulation could have affected the locus coeruleus, which is the principal site for brain synthesis of norepinephrine near the PBN. Norepinephrine is known to be a key modulator of arousal from sleep,[40] and locus coeruleus–norepinephrine is one of the systems that regulates emergence from volatile inhalation anesthetics.[31]

Therefore, to clarify the role of PBN glutamatergic neurons in sevoflurane anesthesia, new approaches that can cell-selectively manipulate PBN glutamatergic neurons are needed. Designer receptors exclusively activated by designer drugs (DREADDs) are novel tools that provide distinct advantages over traditional methods used to study anesthetic-induced unconsciousness and recovery of consciousness in rodents. Unlike local and systemic drug administration,[41,42] targeted brain lesions,[6,43] and electrical stimulation,[44] which recruits multiple pathways, we could reversibly manipulate PBN glutamatergic neurons through synthetic Galpha (q) or Galpha (i) signaling using DREADD receptors.

Our results found that mice with activation of PBN glutamatergic neurons showed longer induction times, shorter emergence times, and a rightward shift of the LORR *versus* sevoflurane concentration dose–response curve as compared with control mice. In contrast, chemogenetic inhibition of PBN glutamatergic neurons produced a slight effect on the emergence time and sevoflurane sensitivity. Under baseline conditions, inhalation of sevoflurane inhibits the activity of neurons in the brain, including the PBN. Using chemogenetics to activate PBN neurons exhibited a strong antagonistic effect on anesthetic efficacy. However, inhibition of PBN neurons by CNO did not greatly change sevoflurane sensitivity because sevoflurane also inhibited the neurons.

Although chemogenetic activation of the PBN glutamatergic neurons changed the behavior response during general anesthesia, optogenetics did not induce the same response. After the experiment, we checked the expression of the hM3Dq and ChR2 in the PBN neurons and found that they did not show any difference in transfection efficiency. Therefore, why optical stimulation did not produce significant changes in behaviors may be attributed to the limitations of optogenetic approach applied to the PBN. The manipulability of the size or number of neurons in the brain is not enough because of the absorption, scattering, and distance-dependent decay of light through brain tissue and subsequent attenuation of light delivered to the target region.[45] Because of this limitation, transient blue light did not activate enough PBN neurons infected with ChR2, so that optical activation of the PBN could not induce behavioral arousal during general anesthesia.

The observed increases in cortical activity after optical stimulation suggest that activation of PBN glutamatergic neurons is sufficient to induce cortical arousal during general anesthesia. Several studies have shown that PBN glutamatergic neurons project directly to the prelimbic cortex.[12,46] Our immunohistochemical results demonstrated that activation of PBN glutamatergic neurons by DREADD increased expression of c-Fos in the prelimbic cortex during sevoflurane anesthesia. Cortical areas to which PBN neurons do not project, such as the motor cortex, also showed a marked increase in excitability. Therefore, we hypothesize that activation of PBN glutamatergic neurons induces cortical arousal not only through release of glutamate in the cortex from PBN nerve terminals, but also through activation of subcortical structures that modulate activity of the cortex during general anesthesia.

The lateral hypothalamus and basal forebrain, which are two important downstream regions of the PBN, have been reported to regulate sleep–wake behaviors and cortical excitability.[23,47,48] More importantly, previous studies have suggested that these regions are involved in general anesthesia. Kelz *et al.*[32] reported that genetic knockout of lateral hypothalamus orexinergic neurons delayed emergence. Selective lesion of histaminergic neurons in the tuberomammillary nucleus area enhanced the anesthesia response to isoflurane.[6] Furthermore, 192 immunoglobulin G-saporin selective lesion of cholinergic neurons in the basal forebrain was shown to enhance the response to general anesthetics.[14,49] We found that activation of PBN neurons increased c-Fos expression in the lateral hypothalamus and basal forebrain during general anesthesia. These results suggest that activation of PBN neurons to drive subcortical arousal nuclei may be an important mechanism for PBN regulation of cortical arousal and general anesthesia.

Conclusions

In summary, stimulation of PBN glutamatergic neurons is sufficient to alter efficiency of sevoflurane and accelerate the transition from general anesthesia to an arousal state. This effect may be attributable to activation of cortical and subcortical arousal nuclei. These results suggest that activating the PBN arousal circuit may provide a new strategy in treatment of delayed emergence from sevoflurane anesthesia.

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Competing Interests

The authors declare no competing interests.

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