HHS Public Access

Author manuscript

Anesth Analg. Author manuscript; available in PMC 2023 June 01.

Published in final edited form as:

Anesth Analg. 2022 June 01; 134(6): 1140–1152. doi:10.1213/ANE.0000000000006011.

Inactivation of Prefrontal Cortex Attenuates Behavioral Arousal Induced by Stimulation of Basal Forebrain During Sevoflurane Anesthesia

Jon G. Dean, Ph.D.[*,†,‡], **Christopher W. Fields, B.S.**[*], **Michael A. Brito, Ph.D.**[*,‡,§], **Brian H. Silverstein, Ph.D.**[*,‡], **Chloe Rybicki-Kler, B.S.**[*], **Anna M. Fryzel, M.S.**[*], **Trent Groenhout, B.A.**[*], **Tiecheng Liu, M.D.**[*], **George A. Mashour, M.D., Ph.D.**[*,‡,§], **Dinesh Pal, Ph.D.**[*,†,‡,§]

[*]Department of Anesthesiology, University of Michigan, 7433 Medical Science Building I, 1150 West Medical Center Drive, Ann Arbor, MI 48109-5615, USA

[†]Department of Molecular and Integrative Physiology, University of Michigan, 7744 Medical Science Building II, 1137 East Catherine Street, Ann Arbor, MI 48109-5622, USA

[‡]Center for Consciousness Science, University of Michigan, Ann Arbor, MI 48109, USA

[§]Neuroscience Graduate Program, University of Michigan, 4137 Undergraduate Science Building, 204 Washtenaw Avenue, Ann Arbor, MI 48109-2215, USA

Abstract

Background: Cholinergic stimulation of prefrontal cortex can reverse anesthesia. Conversely, inactivation of prefrontal cortex can delay emergence from anesthesia. Prefrontal cortex receives cholinergic projections from basal forebrain, which contains wake-promoting neurons. However, the role of basal forebrain cholinergic neurons in arousal from the anesthetized state requires refinement and it is currently unknown if the arousal-promoting effect of basal forebrain is mediated through prefrontal cortex. To address these gaps in knowledge, we implemented a

Corresponding Author: Dinesh Pal, Ph.D., dineshp@med.umich.edu.

Author Contribution to the Manuscript

Name: Jon G. Dean, Ph.D.
Contribution: This author helped design the study, conduct the experiments, analyze and interpret the data, and write the manuscript.
Name: Christopher W. Fields, B.S.
Contribution: This author helped conduct the experiments, analyze and interpret the data, and write the manuscript.
Name: Michael A. Brito, Ph.D.
Contribution: This author helped design and conduct the experiments and write the manuscript.
Name: Brian H. Silverstein, Ph.D.
Contribution: This author helped analyze and interpret the data, and write the manuscript.
Name: Chloe Rybicki-Kler, B.S.
Contribution: This author helped conduct the experiments, analyze the data, and write the manuscript.
Name: Anna M. Fryzel, M.S.
Contribution: This author helped conduct the experiments, analyze the data, and write the manuscript.
Name: Trent Groenhout, B.A.
Contribution: This author helped conduct the experiments and write the manuscript.
Name: Tiecheng Liu, M.D.
Contribution: This author helped conduct the experiments and write the manuscript.
Name: George A. Mashour, M.D., Ph.D.
Contribution: This author helped design the study, analyze and interpret the data, and write the manuscript.
Name: Dinesh Pal, Ph.D.
Contribution: This author helped design the study, analyze and interpret the data, and write the manuscript.

Conflicts of Interests/Financial Disclosures: NONE

novel approach to the use of chemogenetic stimulation and tested the role of basal forebrain cholinergic neurons in behavioral arousal during sevoflurane anesthesia. Next, we investigated the effect of tetrodotoxin-mediated inactivation of prefrontal cortex on behavioral arousal produced by electrical stimulation of basal forebrain during sevoflurane anesthesia.

Methods: Adult male and female transgenic rats [*Long-Evans-Tg(ChAT-Cre)5.1 Deis;* n = 22] were surgically prepared for expression of excitatory hM3D(Gq) receptors or mCherry in basal forebrain cholinergic neurons, and activation of these neurons by local delivery of Compound 21, an agonist for hM3D(Gq) receptors. The transgenic rats were fitted with microdialysis probes for agonist delivery into basal forebrain and simultaneous prefrontal acetylcholine measurement. Adult male and female Sprague Dawley rats were surgically prepared for bilateral electrical stimulation of basal forebrain and tetrodotoxin infusion (156 μM, 500nL) into prefrontal cortex (n = 9) or bilateral electrical stimulation of piriform cortex (n = 9) as an anatomical control. All rats were implanted with electrodes to monitor the electroencephalogram. Heart- and respiration rate was monitored using non-invasive sensors. A six-point scale was used to score behavioral arousal (0=no arousal, 5=return of righting reflex).

Results: Compound 21 delivery into basal forebrain of rats with hM3D(Gq) receptors during sevoflurane anesthesia produced increases in arousal score [$P < .001$, CI: 1.80 – 4.35], heart rate [$P < .001$, CI: 36.19 – 85.32], respiration rate [$P < .001$, CI: 22.81 – 58.78], theta/delta ratio [$P = .008$, CI: 0.028 – 0.16], and prefrontal acetylcholine [$P < .001$, CI: 1.73 – 7.46]. Electrical stimulation of basal forebrain also produced increases in arousal score [$P < .001$, CI: 1.85 – 4.08], heart rate [$P = .018$, CI: 9.38 – 98.04], respiration rate [$P < .001$, CI: 24.15 – 53.82], and theta/delta ratio [$P = .020$, CI: 0.019 – 0.22], which were attenuated by tetrodotoxin-mediated inactivation of prefrontal cortex.

Conclusions: This study validates the role of basal forebrain cholinergic neurons in behavioral arousal and demonstrates that the arousal-promoting effects of basal forebrain are mediated in part through prefrontal cortex.

Introduction

Cholinergic stimulation of prefrontal cortex – via local carbachol delivery – in sevoflurane-anesthetized rats induces a wake-like state despite the continued administration of sevoflurane anesthesia.[1] Similarly, cholinergic stimulation of prefrontal cortex during slow-wave sleep decreases the latency to wakefulness and increases the time spent in wakefulness.[2] Conversely, tetrodotoxin-mediated inactivation of prefrontal cortex, but not parietal cortex, delays emergence from sevoflurane anesthesia.[3] Notably, reversal of general anesthesia after carbachol delivery into prefrontal cortex was associated with an increase in local acetylcholine levels,[1] which is consistent with a wide array of studies demonstrating high cortical acetylcholine levels in association with cortical activation and wakefulness.[4–10] In contrast, cortical acetylcholine levels are known to be low during slow-wave sleep and ether- or propofol-induced unconsciousness,[4–10] and decreases in endogenous forebrain acetylcholine levels increase anesthetic sensitivity.[11]

The primary source of acetylcholine in prefrontal cortex is cholinergic neurons in basal forebrain,[12,13] which have been shown to be active during the wake state and which are

known to produce behavioral arousal.[14–17] Although manipulation of both prefrontal cortex and basal forebrain have been shown to produce behavioral arousal,[1,2,14,16,17] the direct cholinergic projections from basal forebrain to prefrontal cortex,[12,13] and the increase in prefrontal acetylcholine in association with wakefulness,[1,4–10] suggest that the prefrontal cortex might gate the arousal-promoting effect of basal forebrain. However, there is no direct evidence for an interplay between prefrontal cortex and basal forebrain in behavioral arousal. Furthermore, although recent studies showed a role for basal forebrain cholinergic neurons in passive emergence from anesthesia,[18,19] a direct role of cholinergic basal forebrain neurons in reversing the state of anesthesia has not been demonstrated.

Therefore, in this study, we first investigated if chemogenetic activation of cholinergic neurons in basal forebrain was sufficient to reverse the state of general anesthesia and produce behavioral arousal in the continued presence of sevoflurane anesthesia. Next, we conducted electrical stimulation of basal forebrain in sevoflurane-anesthetized rats with or without concurrent inactivation of prefrontal cortex to determine if the arousal-promoting effects of basal forebrain are mediated through prefrontal cortex. We demonstrate that chemogenetic activation of basal forebrain cholinergic neurons in sevoflurane-anesthetized rats induces a wake-like state, and inactivation of prefrontal cortex attenuates behavioral arousal induced by electrical stimulation of basal forebrain.

Methods

Rats

The experiments were approved by the Institutional Animal Care and Use Committee at the University of Michigan (Ann Arbor, Michigan) and were in compliance with the Guide for the Care and Use of Laboratory Animals (Ed 8, National Academies Press) and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. The rats were maintained on a 12 hours light/12 hours dark cycle (lights ON at 8:00 AM) with ad libitum food and water in a temperature-controlled animal care and housing facility at the University of Michigan. To take advantage of the chemogenetic cell-specific targeting, we used adult male and female transgenic rats [*Long-Evans-Tg(ChAT-Cre)5.1 Deis,* referred herein as the ChAT-Cre rats, ~ 6 months age, n = 22][20] that express Cre recombinase in cholinergic neurons (Rat Resource and Research Center, Missouri, USA). We infused 500 nL of *AAV5:pAAV-hSyn-DIO-hM3D(Gq)-mCherry* bilaterally into the basal forebrain of these rats (n = 18) to express excitatory hM3D(Gq) receptors in the local cholinergic neurons, which were subsequently activated with Compound 21 (C21), a selective agonist for hM3D(Gq) receptors.[21–24] Of these 18 rats, 13 were used in chemogenetic experiments wherein C21 was dialyzed directly into basal forebrain while 5 rats received C21 as intravenous bolus. A subgroup of ChAT-Cre rats (n = 4) and a wild type male Long-Evans rat received *AAV5:pAAV-hSyn-DIO-mCherry* via bilateral injection (500 nL) into basal forebrain to express mCherry, the fluorescent protein/tag, in the local cholinergic neurons. The electrical stimulation experiments were conducted in adult male and female non-transgenic Sprague Dawley rats (n=18, 300–350 g, Charles River Laboratories).

Experimental Design

Chemogenetic activation of basal forebrain cholinergic neurons—The experimental design is illustrated in Figure 1. The rats (n = 10 male, 8 female) were connected to the electrophysiological recording system at least 30 minutes prior to the start of experimental sessions (9:30 AM – 10:30 AM), and microdialysis probes being perfused (2 μL/minute) with artificial cerebrospinal fluid were lowered into the basal forebrain (bilateral) and prefrontal cortex (unilateral). Baseline electroencephalographic data were collected for 50 minutes while keeping the behavioral state constant by introducing novel objects and gentle tapping on the recording chamber. Thereafter, sevoflurane administration was started and titrated (1.7–2.3%) to produce loss of righting reflex - a behavioral surrogate for unconsciousness in rodents - along with complete immobility and high-amplitude slow waves in electroencephalogram (EEG). Sevoflurane concentration was continuously monitored using anesthesia monitors (Datex Medical Instrumentation, Tewksbury, MA) connected to the anesthesia inlet and outlet ports of the recording chamber. At the onset of loss of righting reflex, a rectal probe connected to a far-infrared heating pad (Kent Scientific, Torrington, Connecticut) through a feedback temperature controller (Physitemp, Model RET-3) was positioned to monitor and maintain the body temperature at 37.0 ± 1°C. In addition, a pulse oximetry sensor (MouseOx, Starr Life Science, Oakmont, PA) was positioned on the foot or around the neck to record the changes in respiration rate, heart rate, and oxygen saturation levels during anesthesia.

After 50 minutes of sevoflurane administration, C21 was dialyzed (12.5 minutes) into the basal forebrain while the rats continued to inhale the same concentration of sevoflurane, which continued for 62.5 minutes. The EEG data were collected for another 50 minutes in post-sevoflurane period. Our pilot experiments showed that dialysis delivery of C21 at equimolar concentration (1.0 mM) during sevoflurane anesthesia produced profound respiratory depression in female, but not male rats (data not included). Therefore, we used 1.0 mM C21 in male rats and 0.5 mM C21 in female rats. Electroencephalographic recording and microdialysis were conducted simultaneously and continuously across the experimental session but the microdialysis samples were collected in 12.5-minute bins, as described in our recent publications.[1,10]

Electrical stimulation of basal forebrain with or without concurrent infusion of tetrodotoxin into prefrontal cortex—As illustrated in Figure 1, three experimental sessions were conducted in each rat (n = 4 male, 5 female), separated by at least one week: **Session 1 -** the rats were connected to the EEG recording and electrical stimulation system at least 30 minutes prior to the start of the experimental session (9:30 AM – 10:30 AM). The baseline electroencephalographic data collection, sevoflurane administration (1.7– 2.4%), and physiological monitoring were conducted as described earlier for the chemogenetic experiments. After 50 minutes of sevoflurane administration, a constant current electrical stimulation protocol was initiated to deliver stimuli bilaterally to the basal forebrain: 200 Hz square wave, 60 μA, 30 s ON, 30 s OFF (rest period) for 3 cycles after which the protocol was repeated for another 3 cycles, but the current amplitude was increased to 100 μA. The electrical stimulation parameters were informed by previous studies showing the effect of subcortical electrical stimulation on behavioral arousal[25,26] and pilot studies conducted

in our laboratory (data not included). After cessation of sevoflurane administration, the electroencephalographic data were collected for another 50 minutes.

Session 2 - forty minutes before the start of baseline electroencephalographic recording, the rats received 500 nL of 156 μM tetrodotoxin (TTX) bilaterally into the prefrontal cortex. Thereafter, the experimental protocol as described for session 1 was repeated. The concentration of TTX was based on previous studies.[27,28] **Session 3 -** the experimental protocol as described for session 1 was repeated to confirm that the electrical stimulation in session 1 did not cause damage to basal forebrain that may have resulted in the lack of arousal phenotype in session 2 experiments. One rat was excluded from analysis because of unusual resistance to sevoflurane anesthesia.

Electrical stimulation of piriform cortex—A separate group of rats with bipolar stimulation electrodes implanted in piriform cortex (n = 7 male, 2 female), a site adjacent to basal forebrain, was used as anatomical control. These rats underwent only one experimental session, which was the same as session 1 described above for the basal forebrain group. Two rats were excluded from analysis because the stimulation electrodes were found to be outside the target area.

Surgical and experimental procedures—The detailed surgical methods are provided in the Supplemental Digital Content. In brief, the ChAT-Cre rats were implanted with 15 stainless-steel screw electrodes to record EEG from across the cortical hemisphere. The electrodes were arranged in two columns along the rostral-caudal axis (interelectrode distance of 2 mm). The lateral distance from sagittal suture was 2 mm and 4 mm for the first and second electrode column, respectively. Thereafter, the rats received bilateral injection (500 nL) of either the excitatory or the control viral construct into substantia innominata region of the basal forebrain (Bregma: posterior 0.48 mm, mediolateral 2.0 mm, ventral 8.2 mm).[29] After the infusion of viral constructs, a pair of microdialysis guide tubes was implanted 1 mm above the substantia innominata for dialysis delivery of C21, and a single microdialysis guide tube was implanted 1 mm above the prefrontal cortex (Bregma: 3.0 mm anterior, 0.5 mm mediolateral, and 3.0 mm ventral) [29] for simultaneous quantification of local acetylcholine levels.

For the electrical stimulation studies, the rats were implanted with 15 stainless-steel screw electrodes arranged in two columns, one on either side (2 mm) of the sagittal suture along the rostral-caudal axis (interelectrode distance 2 mm). Thereafter, the rats were bilaterally implanted with bipolar wire electrodes either into substantia innominata or piriform cortex (from Bregma: 0.48 mm posterior, 5.0 mm mediolateral, and 9.0 mm ventral).[29] The rats with stimulation electrode in substantia innominata were also implanted with a pair of microinjection guide cannula 1 mm above the prefrontal cortex for bilateral infusion of tetrodotoxin. A stainless-steel screw implanted over the nasal sinus served as reference electrode for all EEG recordings.

Quantification of electroencephalographic activation and behavioral arousal —The electroencephalographic activation was quantified using power spectral changes in theta and delta bandwidths (theta/delta ratio), as described in Supplemental Digital

Content. Behavioral arousal was quantified using a six-level scoring scale (Table 1) that was modified from previous studies assessing responses to brain stimulation in rodents under anesthesia.[30,31] The arousal scores were quantified by investigators not directly involved in data collection and blinded to the experimental conditions.

Statistical analyses—Distribution of response variables were investigated graphically. All variables appeared to be normally distributed except acetylcholine, which was positively skewed and was therefore analyzed on logarithmic scale.

To compare between-rat arousal scores, we employed a linear regression controlling for sex, i.e., treatment (experimental versus control rats)-by-sex interaction. We used linear mixed models for the statistical comparison of within-rat (repeated) observations of prefrontal acetylcholine levels, theta/delta ratio, and heart- and respiration rates, before, during, and after C21 administration or electrical stimulation. The linear mixed models included an epoch-(wake, sevoflurane, C21/electrical stimulation, and recovery wake)-by-sex interaction term to investigate differential sex effects. The interaction term was not found to be statistically significant either in linear regression or linear mixed models. In cases with comparison of more than two epochs, Tukey's correction was applied to post-hoc pairwise comparisons (e.g., respiration rate between pre-stimulation vs. stimulation, pre-stimulation vs. post-stimulation, stimulation vs. post-stimulation).

The respiration and heart rates were statistically compared 300 s before and 300 s after the visible change in EEG following C21 delivery, and 300 s prior to, during (330 s), and 300 s following electrical stimulation of basal forebrain or piriform cortex. The effect of C21 delivery on prefrontal acetylcholine levels was assessed by statistical comparison of the following 12.5-minute epochs: 1) wakefulness – last epoch in the wake condition, 2) sevoflurane anesthesia – last epoch during sevoflurane exposure and before C21 delivery, 3) C21 administration – epoch following C21 administration and the appearance of first signs of arousal, and 4) recovery wake state – the second 12.5 minute epoch following sevoflurane cessation by which all the animals were ambulatory. For graphical purposes, the acetylcholine data were normalized and plotted as percent change with respect to baseline wake values. A P value of $< .05$ was considered statistically significant. The data in the results section are reported as mean ± standard deviation (sd) along with 95% confidence interval of ratios of the means (CI). The descriptive and inferential statistics for all experiments are provided in a tabular format in the Supplemental Digital Content, Tables S1–S10. Statistical analyses were conducted with **R** (Version 3.3.1) and in consultation with the *Consulting for Statistics, Computing and Analytics Research* unit at the University of Michigan. We designed the chemogenetic study to have 80% power (alpha = 0.05/6, Bonferroni correction for 6 pairwise tests) and to detect an effect size (difference in means divided by standard deviation of difference) of 1.2 or larger, predicted based on a recently published similar study from our laboratory.[1] The sample size for electrical stimulation study was based on similar, previously published rodent studies.[25,26,32]

Results

Chemogenetic stimulation of basal forebrain cholinergic neurons during sevoflurane anesthesia caused electroencephalographic and physiologic activation, increased prefrontal acetylcholine, and produced behavioral arousal

Histological analysis confirmed the expression of hM3D(Gq) receptors in basal forebrain cholinergic neurons, and localization of microdialysis sites in basal forebrain and prefrontal cortex (Figure 2). Bilateral delivery of C21 into basal forebrain - expressing hM3D(Gq) receptors in cholinergic neurons - of sevoflurane-anesthetized rats produced electroencephalographic activation and increased the theta/delta ratio [$P = .008$, CI: 0.028 – 0.16] (Figure 3A). There was no statistical change in theta/delta ratio following C21 delivery into the basal forebrain of rats expressing only mCherry in cholinergic neurons (control group) [$P = .17$, CI: −0.020 – 0.10]. The electroencephalographic activation was followed by behavioral arousal (Supplemental Digital Content, Video 1). The arousal score following C21 administration was significantly higher [mean ± sd: 3.1 ± 1.3, $P < .001$, CI: 1.80 – 4.35] than that observed after C21 administration in the control rats lacking hM3D(Gq) receptors [mean ± sd: 0.0 ± 0.0] (Figure 3B). Out of thirteen rats, two regained righting reflex, four attempted righting, one showed coordinated body movements, five showed isolated body movements, and one rat showed orofacial movements and eye blinks. The mean time to electroencephalographic activation following the start of C21 delivery was longer for female rats [mean ± sd: 34.8 ± 17.9 minutes] than for male rats [mean ± sd: 18.4 ± 16.9 minutes]. Behavioral arousal after C21 administration in rats with hM3D(Gq) receptors was accompanied by significant increase in respiration rate [mean ± sd: from 56 ± 9.0 breaths/minute to 97 ± 33 breaths/minute, $P < .001$, CI: 22.81 – 58.78] and heart rate [mean ± sd: from 325 ± 25 beats/minute to 386 ± 50 beats/minute, $P < .001$, CI: 36.19 – 85.32] (Figure 3C, 3E). There was no significant change in heart and respiration rate after C21 delivery in the control rats (Figure 3D, 3F).

Sevoflurane anesthesia reduced prefrontal acetylcholine levels to below waking levels [$P < .001$, CI: 0.13– 0.54] (Figure 3G), which is consistent with previous reports from our and other laboratories.[1,7] EEG activation and behavioral arousal induced by C21 delivery into basal forebrain were accompanied by significant increase in prefrontal acetylcholine levels [$P < .001$, CI: 1.73 – 7.46, as compared to sevoflurane], which was not significantly different than that observed during baseline wake state [$P = 1$, CI: 0.45– 1.97] (Figure 3G). Acetylcholine level during post-sevoflurane recovery wake state remained significantly higher than that observed during sevoflurane anesthesia [$P < .001$, CI: 2.05 – 8.85] (Figure 3G) but was not significantly different as compared to that observed during baseline wake state [$P = .97$, CI: 0.54 – 2.32] or C21-induced behavioral arousal [$P = .92$, CI: 0.57 – 2.46] (Figure 3G).

In the control group, C21 delivery into basal forebrain did not produce any change in EEG in four out of five rats; one female rat showed transient activation of EEG. Sevoflurane anesthesia in control rats decreased prefrontal acetylcholine levels as compared to that observed during wake state [$P = .001$, CI: 0.13 – 0.58] (Figure 3H). The delivery of C21 into basal forebrain during sevoflurane anesthesia did not have any significant effect on

prefrontal acetylcholine levels [P = .80, CI: 0.58 – 2.59, as compared to sevoflurane], which remained significantly below that observed during baseline wake state [P = .005, CI: 0.16 – 0.71] (Figure 3H). After cessation of sevoflurane administration, prefrontal acetylcholine levels returned to waking levels [P = .85, CI: 0.38 – 1.72], and were significantly higher than that observed during sevoflurane anesthesia [P = .005, CI: 1.40 – 6.23] or C21 delivery [P = .019, CI: 1.15 – 5.10] (Figure 3H).

A separate group of ChAT-Cre rats (n = 5 male) received intravenous bolus of C21 (5 mg/kg) under similar conditions as described above for the rats that received C21 via dialysis into basal forebrain. These rats showed electroencephalographic activation and behavioral arousal (Supplemental Digital Content, Figure S1). Surprisingly, intravenous C21 (5 mg/kg) in wild type Long-Evans rats that lacked hM3D(Gq) receptors (n = 2 male) also showed similar electroencephalographic activation and behavioral arousal, thus demonstrating non-specific off-target effects of systemic C21 as has been reported recently[24] (Supplemental Digital Content, Figure S1).

Bilateral electrical stimulation of basal forebrain during sevoflurane anesthesia caused electroencephalographic and physiologic activation, and produced behavioral arousal

Histological analysis confirmed the electrical stimulation sites to be within basal forebrain (Figure 4A–B). Stimulation of basal forebrain (**Session 1**) during sevoflurane anesthesia produced clear behavioral arousal and statistically significant increase in arousal score: one rat fully regained the righting reflex, two attempted righting, four showed coordinated body movements, and one showed orofacial movements and eye blinks (Figure 5A; Supplemental Digital Content, Video 2). In contrast, bilateral stimulation of piriform cortex, a site adjacent to basal forebrain, did not produce behavioral arousal in any but one female rat, in which isolated hindlimb movement was observed. The arousal score after piriform cortex stimulation remained significantly lower than that observed after basal forebrain stimulation in session 1 [mean ± sd: 0.3 ± 0.8 for piriform cortex vs. 3.3 ± 1.2 for basal forebrain, P < .001, CI: 1.85 – 4.08] (Figure 5B). Behavioral arousal after bilateral electrical stimulation of basal forebrain was accompanied by increase in respiration rate [mean ± sd: from 57 ± 4.7 breaths/minute to 95 ± 17 breaths/minute, P < .001, CI: 24.15 – 53.82] but did not produce a statistically significant increase in heart rate (Figure 5C–D); there was a statistically significant increase in theta/delta ratio [P = .020, CI: 0.019 – 0.22] (Figure 5E). The respiration and heart rates were significantly elevated during the post-stimulation period [mean ± sd: 72 ± 11 breaths/minute, P = .038, CI: 0.88 – 30.55; 377 ± 64 beats/minute; P = .018, CI: 9.38 – 98.04] (Figure 5C–D).

Inactivation of prefrontal cortex attenuated the electroencephalographic, physiologic, and behavioral arousal induced by bilateral electrical stimulation of basal forebrain during sevoflurane anesthesia

The sites of TTX microinjection into prefrontal cortex and electrical stimulation sites in basal forebrain were histologically confirmed (Figure 4). Basal forebrain stimulation during sevoflurane anesthesia, in the presence of TTX in prefrontal cortex, attenuated electroencephalographic, physiologic, and behavioral arousal as was observed in session 1 (without TTX) (Figure 5A, 5F–H; Supplemental Digital Content, Video 2). The arousal

score was significantly reduced [mean ± sd: 1.3 ± 1.5, $P < .001$, CI: −2.98 – −1.02] as compared to that observed after electrical stimulation of basal forebrain without concurrent prefrontal inactivation in session 1 (Figure 5A). There was a significant increase in respiration rate following electrical stimulation [mean ± sd: from 59 ± 7.1 breaths/minute to 80 ± 16 breaths/minute, $P < .001$, CI: 11.50 – 31.82] (Figure 5F) but no significant effect on heart rate was observed [mean ± sd: from 301 ± 26 beats/minute to 324 ± 49 beats/minute, $P = .063$, CI: −1.19 – 47.35] (Figure 5G). Moreover, TTX administration decreased theta/delta ratio as compared to during sevoflurane anesthesia alone in the same experiment [$P = .002$, CI: −0.073 – −0.018] (Figure 5H). After about a week, in session 3, we repeated session 1 experiments in these rats to ensure that the lack of arousal phenotype in session 2 (i.e., stimulation with TTX in prefrontal cortex) was not because of any potential damage to basal forebrain during the session 1. Repeating the electrical stimulation protocol without TTX in prefrontal cortex (session 3) produced behavioral arousal: two rats regained the righting reflex, two attempted righting, two showed coordinated body movements, one showed isolated body movements, and one showed orofacial movements and eye blinks. The arousal score [mean ± sd: 3.4 ± 1.4] was not significantly different from that observed in session 1 [$P = .94$, CI: −0.86 – 1.11] (Figure 5A). Along with behavioral arousal, there was an increase in respiration rate [mean ± sd: from 59 ± 6.3 breaths/minute to 111 ± 21 breaths/minute, $P < .001$, CI: 31.77 – 72.00], heart rate [mean ± sd: from 311 ± 19 beats/minute to 375 ± 51 beats/minute, $P < .001$, CI: 30.98 – 97.85], and theta/delta ratio [$P < .001$, CI: 0.087 – 0.25] (Figure 5I–K).

Bilateral stimulation of piriform cortex failed to produce any statistical change in heart rate [$P = .57$, CI: −7.49 – 16.92], respiration rate [$P = .53$, CI: −2.48 – 5.98], or theta/delta ratio [$P = .26$, CI: −0.064 – 0.015] (Figure 5L–M).

Discussion

We demonstrate that stimulation of basal forebrain, including the specific chemogenetic excitation of cholinergic neurons, is sufficient to induce behavioral arousal in sevoflurane-anesthetized rats. Furthermore, concurrent inactivation of prefrontal cortex attenuated the arousal-promoting effect of basal forebrain stimulation. The results related to stimulation of cholinergic neurons are consistent with two murine studies published over the last year. Luo and colleagues[18] showed that genetic lesions of basal forebrain cholinergic neurons delayed the emergence from isoflurane and propofol anesthesia while chemogenetic stimulation of basal forebrain cholinergic neurons had an opposite effect. In a similar and more recent study, Wang and colleagues[19] demonstrated that optogenetic stimulation of cholinergic and glutamatergic neurons in basal forebrain accelerated the emergence from propofol anesthesia. Although the results from these two studies and our findings from the current study converge, it is important to note that, unlike facilitating passive emergence from anesthesia as was done in past studies, we were able to actively reverse the state of general anesthesia (in the continued presence of sevoflurane) and provide the most robust evidence for a direct role of basal forebrain cholinergic neurons in promoting behavioral arousal and emergence from anesthesia. Our data comparing intravenous and localized dialysis delivery of C21 suggest that administration of chemogenetic agonists directly into a brain site represents a powerful new approach to chemogenetic study design. Furthermore,

we used C21, which demonstrates a lower EC_{-50} at hM3D(Gq) receptors (i.e., is more potent), less activity at endogenous hM3 receptors, and higher brain concentrations upon administration compared to clozapine-N-oxide.[21–23] These considerations may underlie the marked arousal phenotype observed in our study. The systemic off-target effects reported previously[24] and as observed after intravenous C21 in this study, further emphasize the need for appropriate controls and suggest that none of the chemogenetic ligands (e.g., clozapine-N-oxide, clozapine, C21) offer complete target selectivity.

Studies from multiple laboratories have revealed several subcortical sites that could be manipulated to alter behavioral arousal. For example, electrical stimulation of ventral tegmental area as well as the selective activation of ventral tegmental dopaminergic neurons have been shown to facilitate passive emergence and/or active reversal of the state of general anesthesia.[26,33,34] Similarly, activation of neurons in parabrachial nucleus has been shown to both facilitate passive recovery from general anesthesia[35] as well as induce active emergence in the continued presence of general anesthetic.[25] Despite these remarkable insights into subcortical sites, relatively few studies have attempted to map the neural circuits involving the cortex that may play a role in behavioral arousal. Our results show that the chemogenetic stimulation of basal forebrain cholinergic neurons produced behavioral arousal and simultaneously increased acetylcholine levels in prefrontal cortex. There are abundant data to show a close association between cortical acetylcholine and behavioral arousal,[4–10] and given that basal forebrain is the primary source of prefrontal acetylcholine,[12,13] we tested the hypothesis that the prefrontal cortex gates the arousal-promoting effect of basal forebrain stimulation. We show that bilateral electrical stimulation of basal forebrain during sevoflurane anesthesia was sufficient to induce behavioral arousal, and concurrent inactivation of prefrontal cortex attenuated these arousal-promoting effects.

Although dialysis delivery of C21 directly into basal forebrain allowed us to circumvent any systemic effects, combining this approach with simultaneous TTX-mediated inactivation of prefrontal cortex would have required multiple insertions of microdialysis probes into basal forebrain (three times, once for each experimental session over a span of 3 weeks). The scar tissue formed due to the probe insertion can prevent efficient diffusion of C21 molecules from the probe membrane into the surrounding tissue, which can affect the response to C21, as was observed in our pilot studies (data not shown). In our study, it was critical to demonstrate that the lack of arousal phenotype after TTX-mediated inactivation of prefrontal cortex was not caused by damage to neural circuitry. Therefore, we decided to use electrical stimulation, which allows repeated experimental manipulations without the need for multiple insertions of microdialysis probes and multiple drug administrations. A major drawback of electrical stimulation is that it is not specific to neuronal phenotype and can excite the passing fibers. Therefore, it is not possible to rule out that the effect of electrical stimulation in our study could have been mediated through additional circuits. However, it is highly unlikely that electrical stimulation excited the passing fibers while completely sparing the cholinergic neurons. Furthermore, given the similarity between the effects of basal forebrain electrical stimulation and chemogenetic stimulation of cholinergic neurons, it is likely that the behavioral arousal after basal forebrain electrical stimulation is at least partially mediated via local cholinergic neurons. Further studies combining cholinesterase delivery to prefrontal cortex during basal forebrain stimulation may provide

more definitive data on the role of cholinergic pathway from basal forebrain to prefrontal cortex in behavioral arousal.

Of note, despite the clear behavioral arousal induced by chemogenetic and electrical stimulation of basal forebrain, only two out of thirteen rats in the chemogenetic cohort and two out of eight rats in the electrical stimulation cohort regained righting reflex. This demonstrates the stringent experimental conditions presented by continued exposure to the anesthetic and also precluded us from statistically comparing the return of righting reflex between experimental and control groups, which is a limitation in our study.

Although data from this study and previous reports[18,19] provide compelling evidence that the basal forebrain is an arousal-promoting area, we cannot discount the possibility that —rather than being a source of arousal, *per se*—the basal forebrain may be a point of convergence for the arousal-promoting influence of other subcortical nuclei and/or that the basal forebrain may be activating other brain regions to promote behavioral arousal. For example, selective activation of orexinergic terminals in basal forebrain was shown to accelerate the emergence from isoflurane anesthesia[36] and orexin administration in basal forebrain has been shown to increase cortical acetylcholine and facilitate recovery from anesthesia.[37] Similarly, disinhibition of medullary glutamatergic neurons in anterior nucleus gigantocellularis induced emergence from isoflurane anesthesia and increased the activity of several subcortical nuclei, including basal forebrain.[30]

In summary, we report a novel, reverse-dialysis approach to chemogenetic activation that circumvents off-target effects and that demonstrated a direct causal role for basal forebrain cholinergic neurons in behavioral arousal during general anesthesia. We also provide evidence that supports the role of the prefrontal cortex as a cortical mediator of basal forebrain-regulated arousal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

The authors would like to thank Chris Andrews, Ph.D. (Consultant, Consulting for Statistics, Computing & Analytics Research, University of Michigan, 3550 Rackham, 915 East Washington Street, Ann Arbor, MI 48109-1070) for help with statistical analysis.

Funding:

This work was supported by the National Institutes of Health (Bethesda, MD, USA) grant (R01 GM111293) to GAM and DP, and funding from the Department of Anesthesiology, University of Michigan Medical School, Ann Arbor.

Glossary

C21	Compound 21
ChAT	Choline acetyl transferase
EEG	Electroencephalogram

TTX	Tetrodotoxin

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Key Points:

Question:

Is the arousal-promoting effect of basal forebrain mediated through prefrontal cortex?

Findings:

Inactivation of prefrontal cortex attenuates basal forebrain-mediated arousal.

Meaning:

This provides further evidence that the prefrontal cortex is part of the arousal circuitry.

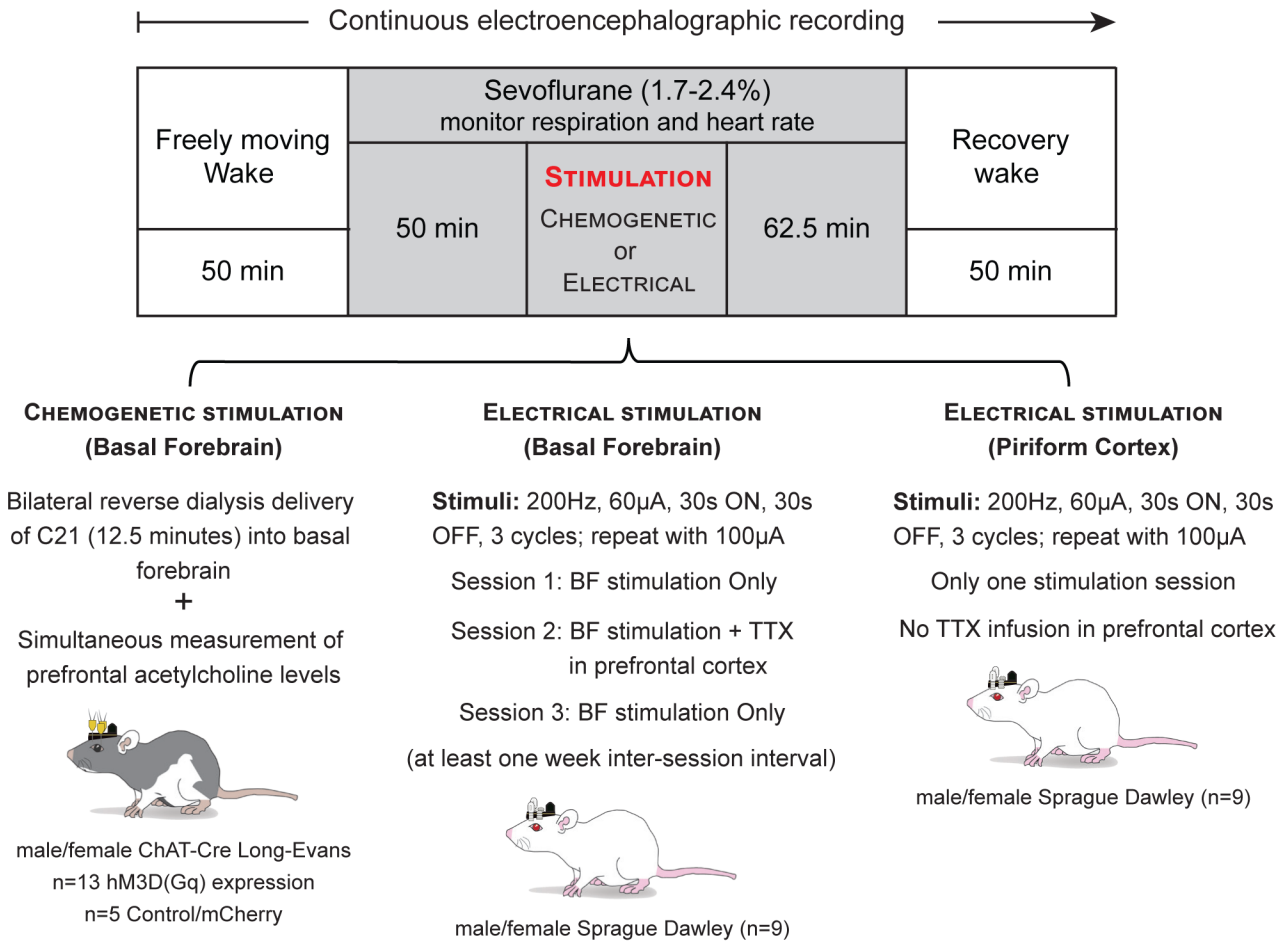
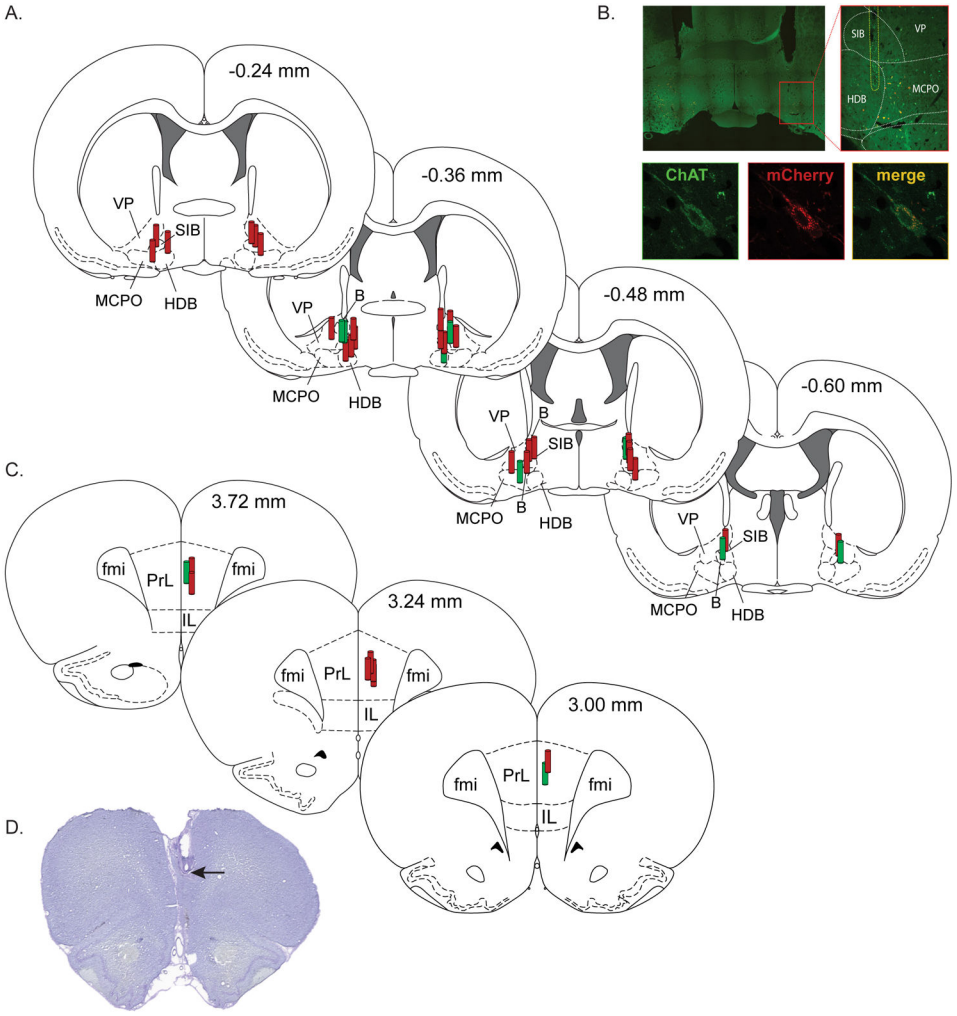


Figure 1.

Schematic to show the experimental design and timeline. The chemogenetic experiments were conducted in adult male and female *Long-Evans-Tg(ChAT-Cre)5.1 Deis* rats; referred herein as ChAT-Cre rats. The EEG data were recorded continuously while the microdialysis samples from prefrontal cortex were collected every 12.5 minutes. Baseline wake data were collected for 50 minutes after which the recording chamber was sealed and sevoflurane adminstration (1.7–2.4%) started. Respiration and heart rate were then monitored using a rodent pulse oximeter until the end of sevoflurane exposure. After 50 minutes of sevoflurane anesthesia, Compound 21 (C21), the agonist for hM3D(Gq) receptors, was reverse dialyzed for 12.5 minutes into the basal forebrain of ChAT-Cre rats. Female rats received 0.5 mM C21 while the male rats received 1 mM C21. At the completion of C21 delivery, the rats were maintained at the same sevoflurane concentration until the post-sevoflurane recovery epoch. Post-sevoflurane recovery data were collected for 50 minutes. Adult male and female Sprague Dawley rats were used for bilateral electrical stimulation of basal forebrain; electrical stimulation of piriform cortex was conducted to confirm the specificity of response

to electrical stimulation of basal forebrain. The experimental design was similar to that followed for chemogenetic experiments except that instead of C21 administration, the rats received electrical stimuli in basal forebrain with or without bilateral infusion of 500 nL of 156 μM tetrodotoxin (TTX) into prefrontal cortex.



B - basal nucleus (Meynert), fmi - forceps minor of the corpus callosum, HDB - nucleus of the horizontal limb of the diagonal band, IL - infralimbic area, MCPO - magnocellular preoptic nucleus, PrL - prelimbic cortex, SIB - substantia innominata, VP - ventral pallidum

Figure 2.

Histological verification of the sites of dialysis delivery of Compound 21 (C21) into basal forebrain, and microdialysis sites in prefrontal cortex for quantification of acetylcholine levels. **A,** Stereotaxic maps from rat brain atlas show the location of microdialysis probes (red and green cylinders) in basal forebrain for C21 delivery. **B,** Top left panel shows a representative coronal brain section (30 μm) through basal forebrain immunostained for choline acetyl transferase (ChAT) and mCherry, the fluorescent tag for hM3D(Gq) receptors. The inset encompasses the basal forebrain area and is enlarged in the panel on the right to show outline of dialysis probe (white vertical broken lines). The bottom panels show a close-up of an individual neuron stained for ChAT (green), mCherry (red), and overlay of both (yellow) to show colocalization. **C,** Stereotaxic maps from rat brain atlas show the location of microdialysis probes in prefrontal cortex. **D,** Cresyl violet-stained representative coronal brain section (30 μm) through prefrontal cortex; arrow indicates the tip of the microdialysis

probe. In (**A**) and (**C**), red cylinders represent the microdialysis probe locations in rats with hM3D(Gq) receptors expressed in cholinergic neurons (experimental group) while the green cylinders show probe locations in rats that expressed only mCherry (control group). The numbers on the top right of stereotaxic maps show the anteroposterior distance from Bregma: positive numbers are anterior to Bregma while the negative numbers are posterior to Bregma. B – basal nucleus (Meynert), fmi – forceps minor of the corpus callosum, HDB – nucleus of the horizontal limb of the diagonal band, IL – infralimbic area, MCPO – magnocellular preoptic nucleus, PrL – prelimbic cortex, SIB – substantia innominata, VP – ventral pallidum.

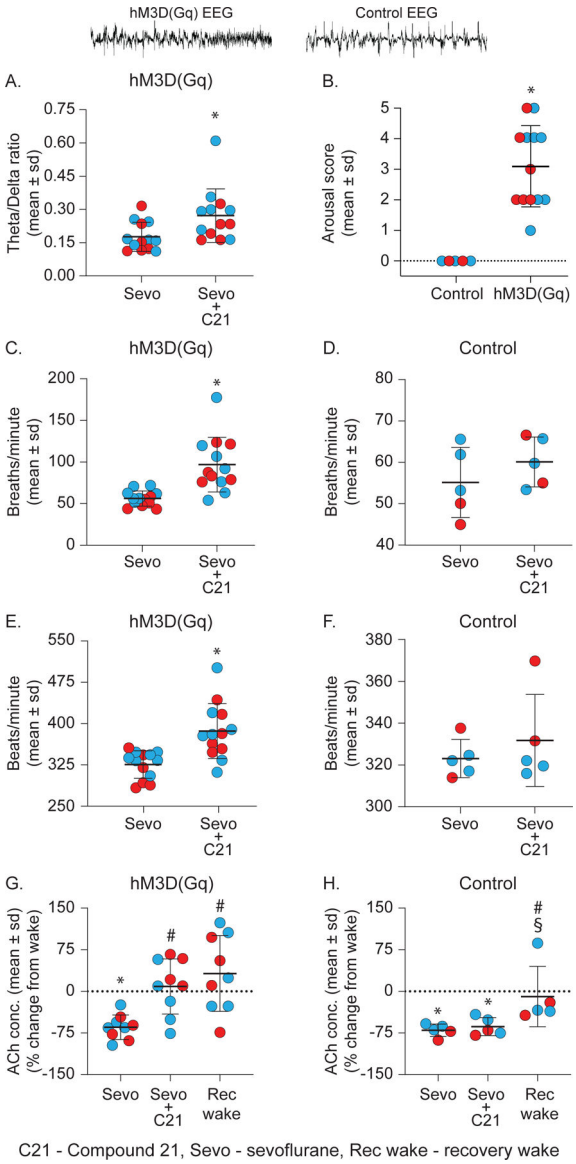
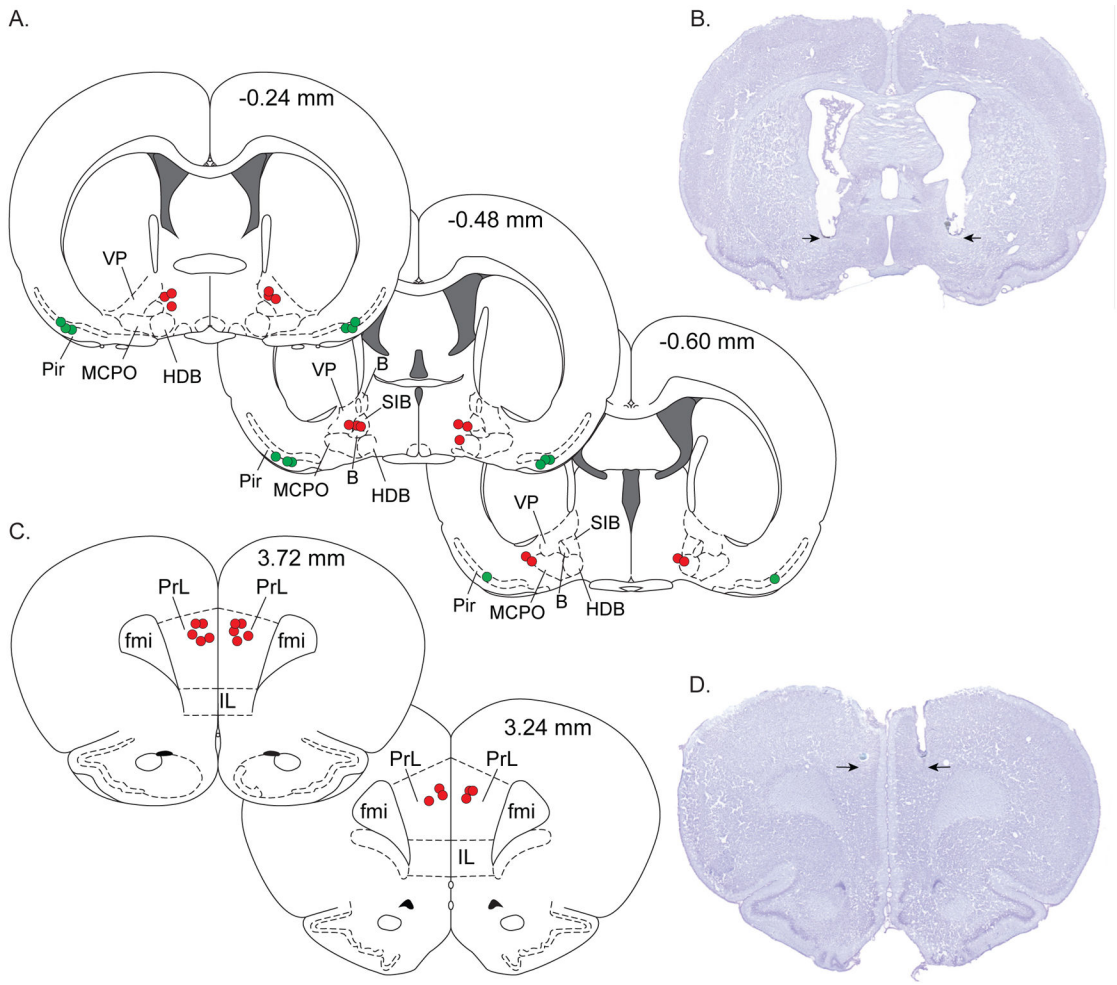


Figure 3.

Chemogenetic stimulation of basal forebrain cholinergic neurons during sevoflurane anesthesia caused electroencephalographic and physiologic activation, increased prefrontal acetylcholine, and produced behavioral arousal. The EEG traces on top are representative frontal EEG segments to show activation in ChAT-Cre rats expressing hM3D(Gq) receptors (left) and the lack of activation in ChAT-Cre rats (Control) without hM3D(Gq) receptors (right). Each red (female) and blue (male) dot represent data from one rat. Male rats received 1.0 mM C21 while female rats received 0.5 mM C21. Dialysis delivery of Compound 21 (C21) into basal forebrain of sevoflurane-anesthetized ChAT-Cre rats expressing hM3D(Gq) receptors significantly increased **A**), theta/delta ratio, and **B**) behavioral arousal score. **C** and **E**, show the respiration rate and heart rate 300 s before (sevoflurane - Sevo), and 300 s after (Sevo + C21) C21-induced EEG changes in ChAT-Cre rats expressing hM3D(Gq) receptors. Dialysis delivery of C21 into basal forebrain significantly increased respiration (**C**) and heart

(**E**) rate. **D** and **F**, show that C21 delivery into basal forebrain of ChAT-Cre rats expressing only mCherry (Control group) failed to elicit significant changes in respiration or heart rate. **G** and **H**, show the changes in acetylcholine (ACh) levels in prefrontal cortex (% change from baseline wake state) during Sevo, Sevo + C21, and recovery wake (Rec wake) epochs. The C21 delivery into basal forebrain during sevoflurane anesthesia caused a significant increase in ACh levels to baseline wake levels in ChAT-Cre rats expressing hM3D(Gq) receptors (**G**) but no such increase was observed in ACh levels in the control group (**H**). A linear mixed model controlling for sex was used for within-rat statistical comparisons while a linear regression controlling for sex was used for between-rat (**B**) statistical comparisons. Post-hoc comparisons were Tukey corrected. For **B, C, E:** *compared to Control/Sevo. For **G-H**: *compared to baseline wake state, #compared to Sevo, § compared to Sevo + C21. The *P* values are shown at < .05 but the actual *P* values are reported in the main text and in the Supplemental Digital Content, Tables S1–S3. A video showing representative behavior after chemogenetic stimulation of basal forebrain cholinergic neurons in ChAT-Cre rats is provided in the Supplemental Digital Content, Video 1.



B - basal nucleus (Meynert), fmi - forceps minor of the corpus callosum, HDB – nucleus of the horizontal limb of the diagonal band, IL - infralimbic area, MCPO – magnocellular preoptic nucleus, Pir - piriform cortex, PrL - prelimbic cortex, SIB – substantia innominata, VP – ventral pallidum

Figure 4.

Histological verification of the sites of electrical stimulation in basal forebrain and piriform cortex, and tetrodotoxin microinjection sites in prefrontal cortex. **A**, Stereotaxic maps from rat brain atlas show the sites of electrical stimulation in basal forebrain (red dots) and piriform cortex (green dots). **B**, Cresyl violet-stained representative coronal brain section (30 μm) through basal forebrain shows the site of bilateral electrical stimulation (arrows). **C**, Stereotaxic maps from rat brain atlas show the sites of tetrodotoxin microinjection into prefrontal cortex (red dots). **D**, Cresyl violet-stained representative coronal brain section (30 μm) through prefrontal cortex shows the site of bilateral tetrodotoxin microinjection (arrows). The numbers on the top right of stereotaxic maps show the anteroposterior distance from Bregma: positive numbers are anterior to Bregma while the negative numbers are posterior to Bregma. B – basal nucleus (Meynert), fmi – forceps minor of the corpus

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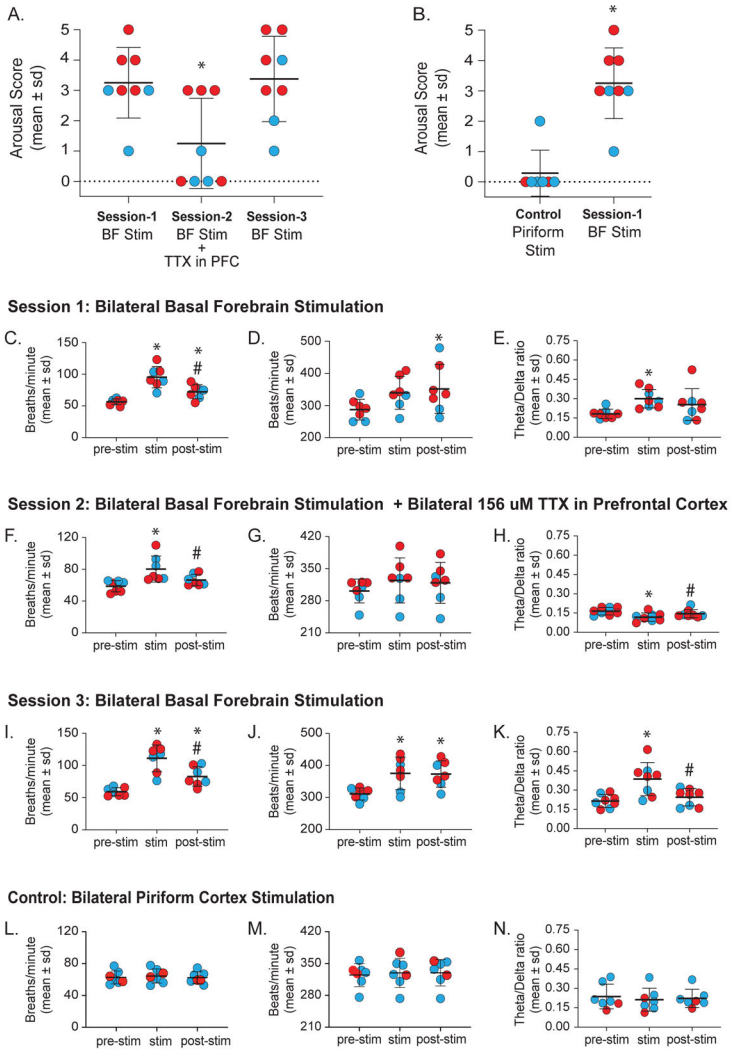

BF - basal forebrain, PFC - prefrontal cortex, Piriform - piriform cortex, pre-stim - 300 seconds prior to stimulation, stim - stimulation period, post-stim - 300 seconds following stimulation period, TTX -tetrodotoxin

Figure 5.

Inactivation of prefrontal cortex attenuates electroencephalographic, physiologic, and behavioral arousal induced by bilateral electrical stimulation of basal forebrain during sevoflurane anesthesia. Each red (female) and blue (male) dots show the individual rat data. **A**, Comparison of behavioral arousal score after bilateral electrical stimulation of basal forebrain with or without concurrent inactivation of prefrontal cortex. As compared to the bilateral electrical stimulation of basal forebrain without prefrontal inactivation (session 1), bilateral electrical stimulation of basal forebrain with concurrent prefrontal inactivation (session 2) caused a significant decrease in the arousal score. Repeat of session 1 (i.e., session 3) showed the arousal score to be significantly higher than that observed in session 2 and reached the levels observed during session 1. **B**, Behavioral arousal score after bilateral electrical stimulation of basal forebrain (session 1) were significantly higher than the arousal score after bilateral electrical stimulation of piriform cortex (anatomical control). Bilateral

electrical stimulation of piriform cortex did not produce behavioral arousal as in session 1 rats. **C-E**, Session 1 - bilateral electrical stimulation of basal forebrain in sevoflurane-anesthetized rats increased respiration rate (**C**), heart rate (**D**) and theta/delta ratio (**E**). The pre-stimulation data were quantified 300 s prior to stimulation (pre-stim) and the post-stimulation data were quantified 300 s after the stimulation (post-stim). **F-H**, Session 2 - bilateral basal forebrain stimulation in the presence of tetrodotoxin (TTX) in prefrontal cortex produced a significant increase in respiration rate (**F**), but no significant change was observed in heart rate (**G**). Theta/delta ratio showed a significant decrease during electrical stimulation (**H**). **I-K**, Session 3 - bilateral electrical stimulation of basal forebrain in the same rats used in sessions 1 and 2 produced increase in respiration rate (**I**), heart rate (**J**), and theta/delta ratio (**K**). A video showing representative behavior after stimulation of 1) basal forebrain, and 2) after stimulation of basal forebrain along with concurrent inactivation of prefrontal cortex is provided in the Supplemental Digital Content, Video 2. **L-N**, Bilateral electrical stimulation of piriform cortex during sevoflurane anesthesia did not produce any statistical change in respiration rate (**L**), heart rate (**M**), or theta/delta ratio (**N**). A linear mixed model controlling for sex was used for within-rat statistical comparisons while a linear regression controlling for sex was used for between-rat (**B**) statistical comparisons. Post-hoc comparisons were Tukey corrected. The *P* values are shown at < .05 but the actual *P* values are reported in the main text and in the Supplemental Digital Content, Tables S4–S8. For panel **A**: *Compared to session 1. For panel **B**: *Compared to Control. For panels **C-N**: *Compared to pre-stimulation, #compared to stimulation.

Table 1.

Quantification of behavioral arousal.

Arousal score	Criteria
0	Complete absence of behavioral arousal
1	Orofacial movements or eye blinks
2	Isolated body movements, i.e. movement of only one set of limbs (either front or hind limbs) or head or tail
3	Coordinated body movements, i.e., simultaneous movement of one or more set of limbs along with head and/or tail movements
4	Attempt at regaining righting reflex
5	Return of righting reflex