Optogenetic Stimulation of C1 and Retrotrapezoid Nucleus Neurons Causes Sleep State–Dependent Cardiorespiratory Stimulation and Arousal in Rats

Stephen B.G. Abbott, Melissa B. Coates, Ruth L. Stornetta, Patrice G. Guyenet

Abstract—C1 catecholaminergic neurons and neurons of the retrotrapezoid nucleus are integrative nodes within the brain stem network regulating cardiorespiratory reflexes elicited by hypoxia and hypercapnia, stimuli that also produce arousal from sleep. In the present study, Channelrhodopsin-2 was selectively introduced into these neurons with a lentiviral vector to determine whether their selective activation also produces arousal in sleeping rats. Sleep stages were identified from electroencephalographic and neck muscle electromyographic recordings. Breathing was measured using unrestrained whole body plethysmography and blood pressure by telemetry. During nonrapid eye movement sleep, unilateral photostimulation of the C1 region caused arousal in 83.0±14.7% of trials and immediate and intense cardiorespiratory activation. Arousal during photostimulation was also observed during rapid eye movement sleep (41.9±5.6% of trials), but less reliably than during nonrapid eye movement sleep. The cardiorespiratory responses elicited by photostimulation were dramatically smaller during rapid eye movement sleep than nonrapid eye movement sleep or wakefulness. Systemic α1-adrenoreceptor blockade reduced the cardiorespiratory effects of photostimulation but had no effect on the arousal caused by photostimulation during nonrapid eye movement sleep. Postmortem histology showed that neurons expressing Channelrhodopsin 2–mCherry were predominantly catecholaminergic (81%). These results show that selective activation of C1 and retrotrapezoid nucleus neurons produces state-dependent arousal and cardiorespiratory stimulation. These neurons, which are powerfully activated by chemoreceptor stimulation, may contribute to the sleep disruption associated with obstructive sleep apnea. (Hypertension. 2013;61:835-841.)

Key Words: asphyxia ■ chemoreception ■ Phox2b ■ sleep apnea ■ sympathetic nervous system

Hypoxia, hypercapnia, and asphyxia during sleep produce both cardiorespiratory stimulation and arousal. The respiratory stimulation and arousal are life-saving in case of airway obstruction, but the sleep fragmentation and intermittent hypoxia associated with chronic obstructive sleep apnea create a cohort of acute and chronic cardiovascular and other health problems.¹ The mechanisms responsible for the cardiorespiratory stimulation cause by hypoxia and hypercapnia are reasonably well understood, but the neural mechanisms of arousal are still obscure.²

The C1 adrenergic neurons and the retrotrapezoid nucleus (RTN) located in the rostral ventrolateral medulla (RVLM) play a pivotal role in the cardiorespiratory responses to hypoxia and hypercapnia.³,⁴ C1 neurons regulate sympathetic vasomotor tone and are excited by hypoxia and to a lesser extent by hypercapnia.⁵ C1 neurons have extensive central nervous system (CNS) projections⁶ to regions of the brain that regulate sleep and arousal⁷ and therefore could contribute to hypoxia-induced arousal. RTN neurons are putative central chemoreceptors⁸ that innervate respiratory centers⁹ and mediate ≈60% of the hypercapnic respiratory chemoreflex in conscious rats.⁹ RTN neurons are also activated by hypoxic stimulation of the carotid bodies.¹⁰

Combined optogenetic stimulation of C1/RTN neurons elicits a rapid, robust cardiorespiratory response in awake rats¹¹ that is similar to the response elicited by asphyxia in humans.¹² The present study seeks to determine whether such stimulations also produce arousal from sleep. The second objective is to test whether the cardiorespiratory responses elicited by activating these neurons are sleep state dependent.

Methods

All experiments were conducted using male Sprague-Dawley rats (n=30; 364±7 g at the time of experimentation, Taconic, USA) in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Virginia Animal Care and Use Committee. An expanded Methods section is available in the online-only Data Supplement.

Results

Photostimulation of ChR2⁺ C1/RTN Neurons During NREMS Causes Arousal

Unilateral photostimulation (20 Hz, 20-s trains, 5- to 10-ms pulses) of C1/RTN neurons in ChR2⁺ rats (injected with PRSx8-ChR2-mCherry¹³ into the RVLM) during nonrapid eye

Received December 13, 2012; first decision January 30, 2013; revision accepted February 1, 2013.
From the Department of Pharmacology, University of Virginia, Charlottesville, VA.
The online-only Data Supplement is available with this article at http://hyper.ahajournals.org/lookup/doi/10.1161/HYPERTENSIONAHA.111.00860
Correspondence to Patrice G. Guyenet, University of Virginia Health System, PO Box 800735, 1300 Jefferson Park Ave, Charlottesville, VA 22908-0735. E-mail pgg@virginia.edu
© 2013 American Heart Association, Inc.
Hypertension is available at http://hyper.ahajournals.org
DOI: 10.1161/HYPERTENSIONAHA.111.00860

Downloaded from http://hyper.ahajournals.org/ by guest on May 3, 2016
movement sleep (NREMS) produced immediate respiratory stimulation followed by arousal (Figure 1A). Arousal events consisted of an abrupt and sustained decrease in electroencephalographic (EEG) slow wave activity and an increase in high-frequency oscillations (Figure 1A and Figure S1A in the online-only Data Supplement). Neck electromyographic (EMG) activation was not always detected, but in many cases either brief EMG bursts associated with large augmented inspiratory efforts (ie, sighs) and persistent increases in EMG tone were observed. In control rats (injected with saline or PRSx8-AllatoR-eGFP13 into the RVLM), respiration was unaffected by RVLM photostimulation during NREMS (Figure S1B). The arousal probability during photostimulation was significantly greater in ChR2+ rats than controls for frequencies of ≥10 Hz (P<0.001 for the interaction between the presence of ChR2+ and stimulation frequency, n=17 ChR2+ and 8 controls; Figure 1B). At a stimulus frequency of 20 Hz, 83.0±14.7% of trials in ChR2+ rats resulted in arousals within the 20-s stimulus in contrast to 23.1±14.3% of trials in control rats (P<0.001; Figure 1B). The earliest signs of cortical desynchronization could be observed within 1 s of the stimulus onset in some trials, with 68.6±2.8% of arousal events occurring in the first 10 s of 20-Hz photostimulation trials (Figure 1C). In control rats, arousal events were evenly distributed across the photostimulation period (Figure 1C), implying that these arousals were spontaneous (ie, unrelated to the photostimulation).

**Photostimulation of ChR2+ C1/RTN Neurons During REMS Causes Arousal**

Photostimulation during REMS produced arousal in ChR2+ rats, but less reliably than during NREMS. Arousal events during REMS trials consisted of abrupt EEG desynchronization, increases in neck EMG tone and EMG bursts associated with movement, and in some cases a delayed sigh (Figure 2A and Figure S1C). Arousal events during photostimulation in REMS were rare in control rats (Figure S1D). At a stimulus frequency of 20 Hz, 41.9±5.6% of photostimulation trials in ChR2+ rats resulted in arousal from REMS within the 20-s stimulus (versus 16.3±2.8% in control rats; P<0.01, n=13 ChR2+ and 8 control rats; Figure 2B). However, the effectiveness of high-frequency photostimulation in evoking arousal in ChR2+ rats was significantly less during REMS than NREMS (P<0.001 for the interaction effect between sleep state and stimulation frequency in ChR2+; compare Figure 1B and 1C with Figure 2B and 2C). Unexpectedly, there was no correlation between the probability of arousal during photostimulation in NREMS and REMS in

---

**Figure 1.** Photostimulation of C1/retrotrapezoid nucleus neurons during nonrapid eye movement sleep (NREMS) causes arousal. **A,** Photostimulation trial during NREMS in a ChR2+ rat. Open arrowheads indicate first signs of cortical desynchronization. **Inset,** Expanded view of the initial respiratory effects and cortical desynchronization in A. Flow indicates barometric plethysmographic recording of breathing; EEG, electroencephalograph; EMG, neck electromyograph. y axis: Flow, 50 mL/s; EEG and EMG, 0.5 mV. **B,** The probability of arousal during photostimulation in NREMS in ChR2+ and control rats (n=17 ChR2+ and 8 control rats, **P<0.01, ***P<0.001). **C,** Cumulative frequency of arousals during photostimulation in NREMS (1-s bins, n=12 ChR2+ and 8 control rats).

**Figure 2.** Photostimulation of C1/retrotrapezoid nucleus neurons during rapid eye movement sleep (REMS) causes arousal. **A,** As in Figure 1A except during REMS. **B,** The probability of an arousal during photostimulation in REMS in ChR2+ and control rats (n=13 ChR2+ and 8 control rats, **P<0.01). **C,** Cumulative frequency of arousals during photostimulation in REMS (1-s bins, n=12 ChR2+ and 8 control rats). EEG indicates electroencephalograph; and EMG, electromyograph.
ChR2+ rats (Pearson \( r=0.08, P=0.12 \)), raising the possibility that different arousal mechanisms or pathways are engaged by photostimulation during NREMS versus REMS.

In quietly resting awake rats, photostimulation did not produce overt behavior or detectable changes in EEG or EMG activity. Specifically, photostimulation in awake rats produced no change in total EEG power \( (P=0.68) \) or within any spectral band evaluated \( (P=0.99) \).

**Respiratory Stimulation Produced by Activating ChR2+ C1/RTN Neurons Is Sleep State Dependent**

Consistent with other reports,14,15 we observed significant sleep state differences in respiratory frequency, estimates of tidal volume, and minute volume (MV) measured using whole body unrestrained plethysmography (Table S1). Photostimulation in ChR2+ rats during quiet wakefulness and NREMS increased respiratory frequency, tidal volume, and MV to a similar extent (episode 1 [NREMS] and episode 5 [quiet wakefulness] in Figure 3A and Figure S2A), but these effects were greatly suppressed during REMS (episodes 2 and 3 in Figure 3A and Figure S2A; group data in Figure 3B and Figure S2B). On average, photostimulation at 20 Hz increased MV by 132±50% of resting values during quiet wakefulness and 124±40% during NREMS, but only by 32±29% during REMS \( (P<0.0001 \) for REMS versus NREMS and quiet wakefulness at 20 Hz between sleep state and stimulation frequency; Figure 3B). Interestingly, stimulus-evoked respiratory activation instantly reappeared with the first signs of cortical desynchronization during REMS trials resulting in arousal (inset of Figure 2A). This observation shows that the absence of the respiratory response to photostimulation of ChR2+ neurons is temporally linked with the cortical indications of REMS.

**Arousal From NREMS Is Correlated With the Changes in Ventilation**

Previous studies have shown a high degree of correlation between increased inspiratory efforts and arousal from sleep.2 Therefore, we determined whether the probability of arousal during photostimulation of ChR2+ C1/RTN neurons was correlated with degree of respiratory activation. These two variables were correlated during NREMS (Pearson \( r=0.78; P<0.0001 \), but not during REMS (Pearson \( r=0.24; P=0.21 \); Figure 4A and 4B).

**Cardiovascular Effects of ChR2+ C1/RTN Neuron Photostimulation Are Sleep State Dependent**

These studies were performed in 6 fully instrumented ChR2+ rats in which arterial pressure (AP) was recorded from the femoral artery with an implanted telemetry device. We applied 3-s high-frequency stimulus trains at 0.01 Hz to compare the AP and heart rate response elicited during waking, NREMS, and REMS. These trains produced biphasic reciprocal changes in mean AP and heart rate (Figure 5A). The initial pressor effect of photostimulation was significantly smaller during REMS than during NREMS \( (P<0.01 \) and quiet wakefulness \( (P<0.001 \); Figure 5B, Figure S3A). All other features of the mean AP and heart rate response were similar between arousal states. This evidence shows that the cardiovascular effects elicited by photostimulating ChR2+ C1/RTN neurons are depressed during REMS in a similar fashion to the breathing effects.

**Photostimulation Produces Sighs Associated With Signs of Arousal**

Details are available in the online-only Data Supplement.

**α1-Adrenoreceptor Blockade Does Not Prevent Arousal, but Attenuates the Cardiorespiratory Activation During Photostimulation**

To test whether the rise in AP might contribute to arousal during RVLM photostimulation, we administered the selective α1-antagonist prazosin (1 mg/kg; IP; Figure 6 and Table S1A). Administration of prazosin blocked the stimulus-evoked increase in mean AP \( (P<0.0001 \), Figure 6A and 6B), had no significant effect on heart rate \( (P=0.09 \); Figure 6A and 6B), and reduced the increase in MV by 43.8% \( (P<0.01 \), Figure 6A and 6C). Treatment with prazosin did not significantly affect the occurrence of arousals during photostimulation (predrug versus postdrug: 92.1±5.0% versus 76.9±10.4% of trials; \( P=0.27 \); Figure 6D). Thus central and peripheral α1-adrenoreceptor blockade attenuates the

---

**Figure 3.** The respiratory effect of C1/retrotropeazoid nucleus neuron photostimulation is sleep state dependent. A. Photostimulation trials (20 Hz) spanning 3 arousal states (1. nonrapid eye movement sleep [NREMS] to wake, 2. REMS, 3. REMS, 4. REMS to wake, 5. wake). Note that the ventilatory response is attenuated during REMS trials. y axis: Flow, 50 mL/s, electroencephalograph (EEG) and electromyograph (EMG), 0.5 mV. B. Changes in minute volume (MV) during photostimulation in waking, NREMS, and REMS in ChR2+ and control rats \( (P<0.05, **P<0.01, ***P<0.001 \), repeated measures 2-way ANOVA comparing sleep state and stimulus frequency in ChR2+ rats. ###P<0.001, 2-way ANOVA comparing the presence of ChR2 and sleep state at a stimulus frequency of 20 Hz.
cardiorespiratory effects, but not the arousal effects of ChR2+ C1/RTN neuron activation.

**Histological Results**

The number and location of ChR2+ neurons was mapped in each rat by identifying ChR2–mCherry by immunohistochemistry (Figure 7A and Figure S5). On average, mCherry was detected in 132±10 neurons counted in a 1:6 coronal series (n=22, 792 neurons per rat without correction). Of the neurons expressing ChR2–mCherry, 80.7±2.1% had detectable tyrosine hydroxylase–immunoreactivity (range, 57.0%–94.6%) accounting for 59.8±2.5% of tyrosine hydroxy-

**Discussion**

The key novel observation is that selective stimulation of C1/RTN neurons is sufficient to produce cortical desynchronization in sleeping rats in addition to cardiorespiratory activation. Both effects (arousal and cardiorespiratory stimulation) were attenuated during REMS.

**Limitations**

In this study, arousal was defined as an all-or-none event according to established criteria, providing a proof of principle that C1/RTN neuron activation interrupts sleep in rodents. Activating C1/RTN neurons may also evoke graded activation of subcortical and cortical regions that would not have been reflected in gross EEG recordings. Variations in sleep history (eg, sleep deprivation), sleep-epoch duration, and response habituation attributed to repeated photostimulation trials could also influence the probability of arousal during photostimulation.

**Mechanisms of Arousal During Activation of C1/RTN Neurons**

The arousal-promoting effects of ChR2+ C1/RTN neuron photostimulation could be attributed to 2 classes of mechanisms, singly or in combination: sensory feedback (eg, baroreceptors, airway and lung afferents, or chest proprioceptors) or direct activation of CNS mechanisms promoting arousal. Acute changes in AP cause arousal from sleep in lambs and humans, implying the baroreceptors or the carotid bodies in this effect. In the present study, arousal elicited by photostim-

**Figure 4.** Arousal during C1/retrotrapezoid nucleus neuron photostimulation is correlated with changes in ventilation during nonrapid eye movement sleep (NREMS), but not rapid eye movement sleep (REMS). A, B, x-y scatter plots of the change in minute volume (MV) and the probability of arousal during 2-, 10-, and 20-Hz photostimulation trials in ChR2+ rats during NREMS (A) and REMS (B). (n=12 for NREMS and 10 for REMS). Correlation determined using Pearson correlation calculation.

ChR2-based optogenetics allows reproducible and precisely timed activation of subsets of genetically coded neurons in conscious rats, which could not be accomplished with previous technology, especially in the lower brain stem. However, this approach has limitations. For example, excitation imposed by optogenetic stimulation may not precisely replicate the effect of a naturalistic stimulus, for example, hypoxia. Also, it is possible that a change in the excitability of C1/RTN neurons between sleep states modulates ChR2-mediated excitation. Furthermore, our ChR2-targeting strategy relies on the established selectivity of the PRSx8 promoter for Phox2-expressing neurons. Most ChR2+ neurons were tyrosine hydroxylase–immunoreactive (80.6%); therefore C1 neurons. Tyrosine hydroxylase–negative neurons were presumed to be RTN cells based on location and appearance. We found no correlation between the relative number of each population expressing ChR2–mCherry and the effects of photostimulation. Thus, the physiological effects observed in this study could be driven by activation of either C1 or RTN neurons.

**Arousal and Cardiorespiratory Activation Evoked by C1/RTN Neuron Stimulation Is Sleep State Dependent**

REMS is generated by reciprocally connected pontomedullary neurons and is characterized by distinctive cortical.
autonomic and respiratory activity, and motor atonia. During REMS, the arousal response to hypoxia, hypercapnia, and upper-airway stimulation is attenuated in dogs. The arousal response to hypoxia, hypercapnia, and upper-airway stimulation is not consistently blunted during REMS in healthy humans, but obstructive events are more common and oxygen desaturation more severe during REMS in obstructive sleep apnea patients. This has been attributed to an increase in arousal threshold during REMS relative to NREMS. Here, we show that direct activation of C1/RTN neurons is a less effective arousal stimulus during REMS. The reduced arousal during photostimulation in REMS may be related to a reduction in the activity of arousal-promoting networks, for example, serotonergic neurons, or the absence of somatic feedback related to the blunted cardiorespiratory stimulation during REMS.

The notion that central cardiovascular and respiratory networks are less excitable during sleep is based on evidence that chemo- or somatic reflexes are state dependent. Our study provides direct evidence that the excitability of central cardiorespiratory networks is markedly attenuated during REMS by directly monitoring the consequences of activating specific neurons involved in cardiorespiratory regulation.

During REMS, decreased excitability of respiratory and somatic motor neurons is caused by reduced monoaminergic tone and active inhibition. Motor atonia during REMS disproportionately affects nondiaphragmatic respiratory...
muscle groups, such as cranial motor neurons, and motor neurons controlling chest compliance, upper-airway tone, and active expiration. Reductions in motor neuron excitability may explain the attenuated effects of photostimulation on tidal volume during REMS. However, the markedly reduced effect of photostimulation on breathing frequency suggests that the respiratory rhythm generator is also less excitable during REMS.

The pattern of sympathetic output changes predictably during REMS; muscle sympathetic nerve activity is elevated, whereas the splanchic, renal, and cardiac sympathetic nerve activity is reduced. Muscle sympathetic nerve activity is recruited during obstructive events in obstructive sleep apnea patients; however, studies in lambs indicate that the hypertension caused by hypercapnia is reduced during REM-like sleep states. Consistent with the latter, the pressor effect of photostimulation was attenuated during REMS. As stated earlier, these changes may reflect a reduction in the excitability of critical pathways in the effects of photostimulation or C1/RTN neurons themselves. Changes in monoaminergic tone at the level of the sympathetic preganglionic neurons during REMS may also explain the reduced hypertensive effects of photostimulation during this sleep state.

Perspectives

C1 and RTN neurons mediate a major portion of the autonomic and respiratory responses to hypoxia and hypercapnia in anesthetized rodents. We suggest here that the same neurons may also cause arousal, an effect that facilitates circulatory and respiratory responses to hypoxia and hypercapnia. By extension, we propose that C1/RTN neurons may be important in the cardiorespiratory effects and sleep fragmentation caused by sleep apnea in man. Furthermore, the blunted effects of C1/RTN neuron stimulation during REMS imply that the integration of excitatory drive in central cardiorespiratory networks is attenuated during REMS. A similar mechanism may explain the increased severity of apneic events during REMS in man.

Acknowledgments

We thank Dr Robert Darnall for helpful comments during preparation of the article.

Sources of Funding

This work is supported by grants to P.G. Guyenet from the National Institutes of Health (HL028785) and a postdoctoral fellowship to S.B.G. Abbott from the American Heart Association (11post7170001).

Disclosures

None.

References

Abbott et al  

Stimulating C1 and RTN Neurons Causes Arousal


---

**Novelty and Significance**

**What Is New?**

- Selective optogenetic activation of C1 and retrotrapezoid nucleus neurons cause both cardiorespiratory stimulation and arousal in sleeping rats. The study also shows the reduced excitability of lower brain stem cardiorespiratory network during rapid eye movement sleep.

**What Is Relevant?**

- Obstructive sleep apnea causes hypoxia and hypercapnia resulting in cardiorespiratory activation and sleep disruption. Also, the duration of apnea and oxygen desaturation is both greater when apneas occur during rapid eye movement sleep. The present study presents a brain stem pathway that may underlie the cardiorespiratory and sleep disruption associated with obstructive sleep apnea.

**Summary**

Selective optogenetic activation of C1 and retrotrapezoid nucleus neurons causes arousal and cardiorespiratory activation in rats. Both responses were attenuated during rapid eye movement sleep. Arousals were correlated with the degree of respiratory activation during nonrapid eye movement sleep, and arousal persisted after pharmacological blockade of the blood pressure effects of stimulation.
Optogenetic Stimulation of C1 and Retrotrapezoid Nucleus Neurons Causes Sleep State-Dependent Cardiorespiratory Stimulation and Arousal in Rats

Hypertension. 2013;61:835-841; originally published online February 25, 2013; doi: 10.1161/HYPERTENSIONAHA.111.00860

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/61/4/835

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2013/02/25/HYPERTENSIONAHA.111.00860.DC1.html

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
ONLINE SUPPLEMENT

OPTOGENETIC STIMULATION OF C1 AND RETROTRAPEZOID NUCLEUS NEURONS CAUSES SLEEP STATE DEPENDENT CARDIORESPIRATORY STIMULATION AND AROUSAL IN RATS


Department of Pharmacology, University of Virginia, Charlottesville, VA 22908

Short title: Stimulating C1 and RTN neurons causes arousal

Address correspondence to:
Patrice G. Guyenet, PhD
University of Virginia Health System
P.O. Box 800735, 1300 Jefferson Park Avenue
Charlottesville, VA 22908-0735.
Tel: (434) 924-9974; Fax: (434) 982-3878; E-mail: pgg@virginia.edu
Supplemental Methods

All experiments were conducted using male Sprague-Dawley rats (N=30; 364 ± 7 gm at the time of experimentation, Taconic, USA) in accordance with NIH Guide for the Care and Use of Laboratory Animals and approved by the University of Virginia Animal Care and Use Committee.

Injection of virus and instrumentation

We injected previously described lentiviral vectors or saline into the left rostral ventrolateral medulla (RVLM) of 275-300 gm rats anaesthetized with an intramuscular bolus of ketamine (75 mg/kg), xylazine (5 mg/kg) and acepromazine (1 mg/kg). Control rats received injections into the RVLM of saline (N=5) or a lentiviral vector (N=3) encoding a non-photoactivable protein, the drosophila receptor allatostatin, and eGFP, under control of the Phox2-binding promoter PRSx8 (PRSx8-AllatoR-eGFP)\(^1\)\(^-\)\(^2\). Experimental rats (N=22) received injections of a lentiviral vector encoding the photoactivatable cation channel channelrhodopsin-2 (ChR2, H134R) fused to mCherry under the control of PRSx8 (PRSx8-ChR2-mCherry)\(^2\)\(^-\)\(^3\). A total volume of 600 nL was injected unilaterally into RVLM using previously described methods \(^4\) (approximate stereotaxic coordinates: 1.9 mm lateral, 2.7-3.1 mm caudal to lambda and 8.5-9.0 mm ventral from the surface of the brain). Electrodes and connectors designed to record the electroencephalogram (EEG) and the neck electromyogram (EMG) were implanted during the same surgery. For EEG recordings, stainless steel jeweler screws (Plastics One) were implanted extra-durally (1mm anterior, 1 mm lateral to bregma and 3 mm posterior, 2 mm lateral to bregma above the contralateral hemisphere). An additional screw was implanted 2 mm lateral and 2 mm posterior to bregma for structural support for the head stage. Teflon-coated braided stainless steel wire (A-M systems) was stripped at the tip and wrapped around the implanted screws. Two additional wires were stripped at the tips and implanted in the superficial muscles of the neck for EMG recordings of postural activity. Both recordings were grounded through a screw implanted in the middle of the occipital bone. All wires were crimped to amphenol pins (A-M systems) and inserted into a plastic headstage (Plastics One). To allow photostimulation of ChR2-expressing (ChR2\(^+\)) neurons, rats were implanted with either a guide cannula or an implantable optical fiber during instrumentation. In 10 rats (7 injected with PRSx8-ChR2-mCherry and 3 injected with PRSx8-AllatoR-eGFP), a 5 mm guide cannula was implanted into the brain at coordinates above the virus injection site \(^5\). A dummy internal cannula was used to maintain patency of the guide cannula during recovery and between experiments. In 20 rats (15 ChR2\(^+\) rats and 5 saline injected controls), an implantable optical fiber (230µm, numerical aperture-0.39; Thorlabs) \(^6\) was lowered to within 0.5 mm of the virus injection site. The head-stage and guide cannula or ferrule was secured to the skull using a 2-part epoxy (Loctite). Rats were treated with ampicillin (100 mg/kg, I.M.) and ketorolac (0.6 mg/kg, I.P.) post-operatively and daily for two consecutive days thereafter. Rats recovered for 3-6 weeks before physiological experiments in the University of Virginia vivarium. A subset of rats was implanted with radio-telemetry probes (PA-C10, Datasciences International) one week before the commencement of experiments to record blood pressure from descending aorta via the right femoral artery.

Experimental design
One day prior to the experiment, rats were moved from the vivarium to the study location and habituated to the experimental conditions. Rats were tested in visually-isolated and low-ambient noise conditions in either a homecage or Buxco unrestrained plethysmography chamber modified to allow tethered EEG/EMG recordings and optical stimulation. Rats were habituated to the experimental conditions for 1 day and were housed overnight in the laboratory to reduce commuting stress. On the day of the experiment, rats were lightly anesthetized with isoflurane (induction with 5% maintenance on 1.5% in pure oxygen for <1 min) to reduce stress during insertion of the fiber optic, or connection to the ferrule and EEG/EMG recording assembly. A minimum of 1 hr was allowed for recovery from anesthesia and the emergence of stable sleep patterns. Recordings were made between 12 pm-5 pm. During this period, rats spent 29.9 ± 3.2% of time in wakefulness, 57.3 ± 2.3% of time in non-rapid eye movement sleep (NREMS), and 13.4 ± 1.3% of time in rapid eye movement sleep (REMS) (N=5). Rats implanted with a guide cannula were evaluated on 2 occasions, once in a modified home cage environment and once in the plethysmography chamber, whereas rats implanted with ferrules were tested on multiple days in the plethysmography chamber. After the completion of testing, all rats were deeply anesthetized and transcardially perfused for histological processing.

Unrestrained whole body plethysmography

The ventilatory effects of photostimulation were assessed using barometric, unrestrained whole-body plethysmography (EMKA technologies). The plethysmography chamber was continuously flush at 1-2 L/min of 21% O₂ balanced with N₂ regulated by servo-controlled mass flow controllers for O₂, N₂ and CO₂ (Alicat). Temperature and humidity within the plethysmography chamber was very stable within experiments (± 0.5° C, ± 10 % relative humidity), but varied from 23-25 ° C and 30-60 % relative humidity between experiments. Respiratory frequency (f₉, breaths/min) and tidal volume (V₉, area under the curve during the inspiratory period based on waveforms generated by injecting 5 mL of dry air from a syringe during the experiment, expressed in mL per 100g body weight (BW)) were calculated using Spike software v7.3 (CED). These values were used to calculated minute ventilation (MV= f₉ * V₉, expressed as mL/100g BW/min).

During quiet waking, EEG activity consisted of very low amplitude, high frequency oscillations. EMG tone was present in this state and bouts of movement were reflected by large EMG discharges. During NREMS, EEG spectra were dominated by delta activity and there was reduced high frequency activity, with little or no EMG activity, and a stable breathing pattern. REMS was characterized by stable theta oscillations (7.5-8 Hz), neck muscle atonia, and an irregular breathing pattern.

Photostimulation

Photostimulation of the ventrolateral medulla was performed using a diode-pumped solid state blue laser (473 nm, CrystaLaser) controlled by TTL-pulses from a Grass model S88 stimulator (AstroMed Inc.) delivered using a 200 µm-thick multimode optical fiber (Thor labs). In rats implanted with a guide cannula (Plastics one), the optical fiber was inserted into the brainstem as previously described 5 after measuring the light output at the tip of the fiber using a light meter (Thorlabs). For rats implanted with an implantable optical fiber, a 200 µm-thick multimode optical fiber was terminated with a ferrule and mated to the implanted ferrule with a zirconia sleeve 6. Optical matching gel (Fiber Instrument Sales) was applied at the ferrule junction to
reduce light loss. The transmission efficiency of each implantable optical fiber was tested prior to implantation with a light meter (Thorlabs). Based on this test, the power of the laser was adjusted so that the final output at the tip of the implanted fiber was estimated between 8-9mW in vivo.

Photostimulation trials consisted of 20 s of repeating pulses delivered during established (>10 s) NREMS or REMS at frequencies between 2-20 Hz. Pulse width was varied between 5 and 10 ms to yield the maximal cardiorespiratory effect. Between 3-15 stimulations trials per stimulus frequency were conducted in each rat during NREMS and REMS. We have previously shown that unit discharge of C1 and retrotrapezoid nucleus (RTN) neurons reliably follows 20 Hz trains of 5-10 ms light pulses, and this activation ceases immediately upon termination of the photostimulus train. The resting discharge rate of C1 or RTN neurons in conscious rats is unknown. Under anesthesia C1 neurons can fire at more than 30 Hz when challenged by hypoxia and RTN neurons fire up to 12 Hz during hypercapnia, but have higher rates of activity when they are facilitated by hypothalamic inputs. Therefore the discharge frequencies that we presumably imposed on C1 and RTN neurons in the present study (10-20 Hz) are within a physiological range.

Data acquisition and analysis

Physiological signals were acquired and processed using Spike v7.03 software (CED). EEG and EMG were amplified and band pass filtered (EEG: 0.1-100 Hz, x1000. EMG: 100-3000 Hz, x1000) and acquired at a sampling frequency of 1 KHz. The signal generated by the differential pressure transducer connected to the plethysmography chamber was amplified and band pass filtered (0.1-15 Hz, x100) and acquired at a sampling frequency of 1 KHz. The signal from the radio telemetry probe was acquired at a sampling frequency of 0.2 KHz. Systolic, diastolic, mean arterial pressure and heart rate were extracted from pulsatile blood pressure recordings from the descending aorta based on values calibrated prior to implantation of the telemetry probe. Periods of waking, NREMS, and REMS were classified on the basis of EEG, EMG activity and the pattern of breathing where available. The power (mV^2) of delta (0.5-3.9 Hz), theta (4.4-7.8 Hz), alpha (8.3-13.7 Hz) and beta (14.2-35.6 Hz) spectral frequencies were generated using Fourier–like transformations (Hanning window, 2048 Hz, 2.05 s window) and expressed relative to total spectral power (i.e. Sum of EEG band power/sum of total EEG power in the range of 0.5-35.6 Hz).

Arousal from NREMS was defined as greater than 3 s of cortical desynchronization, characterized by the absence of slow-wave activity and the appearance of high frequency oscillations, with or without EMG activation. Arousal from REMS was defined as greater than 3 s of suppressed theta activity and the activation of EMG. Sighs were identified based on the profile presented in supplemental figure 4. The probability of arousal and sighs was calculated by expressing the number of trials at 3 stimulus frequencies containing an arousal or a sigh relative to the number of trials. We focused on the outcomes of trials at 2, 10 and 20 Hz, with each probability representing the result of 5-15 trials per frequency per animal. The effects of stimulation at 10 Hz during REMS were recorded in ChR2^+ but not control rats because there were insufficient REMS trials at 10 Hz in controls for reliable values for arousal and sighs. For this reason, the probability of arousal and the probability of sighs during 10 Hz trials in REMS were excluded from the analysis presented in figure 2B and supplemental figure 4A and B. However, the outcome of 10 Hz stimulation trials during REMS in ChR2^+ rats are show in X-Y
plots presenting the relationship between the probability of arousal during REMS in ChR2⁺ rats and the other effects of photostimulation during this state (i.e. changes in MV; Figure 4A, B; and probability of sighs.

Average breathing values were extracted from trials in NREMS and REMS that were not contaminated by body movements, as indicated by EMG activity. Baseline and recovery values for all parameters were calculated from the 10-20 s preceding and following the photostimulation period, respectively. Values for correlation analysis (Pearson’s parametric) constitute the probability of arousals and sighs, based on greater than 5 stimulus trials at 3 stimulus frequencies (i.e. 2, 10 and 20 Hz) and the corresponding average of the initial increase (first 5 s of stimulus trial) in MV expressed as % change from baseline. To test the effects of alpha1-adrenoreceptor blockade, we compared the cardiorespiratory effects and probability of arousal during stimulation based on 10 trials at 20 Hz before and after treatment with prazosin (1 mg/kg, I.P., Sigma) sonicated in dH₂O (1 mg/ml). Following tests for normality, differences within and between groups were determined using either a Student’s t-test or a Mann–Whitney–Wilcoxon test, one- or two-way ANOVA with Bonferroni multiple comparisons. Pearson correlation was used to determine the relationship between dependent variables. All values are expressed as mean ± SEM, unless otherwise noted. P<0.05 was considered significant.

**Light Microscopy Histology**

After completion of testing, rats were overdosed with sodium pentobarbital (Nembutal; 100mg/kg, I.P.) and perfused with a heparin solution followed by a saline rinse and buffered 4% paraformaldehyde, pH 7.4. Brains were processed for immunohistochemical detection of mCherry (marker for ChR2⁺ neurons), tyrosine hydroxylase (TH) using previously established methods and antibodies 4,10. Cell counts were taken from a 1 in 6 series of sections using a computer-assisted plotting program (Neurolucida, v.10, MBF Bioscience) allowing tracing of each coronal section and precise placement of cell profiles and the tip of the fiber. Only profiles containing a nucleus were counted. Tracings were exported as vector drawings to Canvas software (v. 10, ACD Systems) for final assembly and labeling.

**Supplemental Results**

**Photostimulation during NREMS and REMS causes spectral shifts in EEG**

Photostimulation during NREMS in ChR2⁺ rats caused a 68.7 ± 4.5% reduction in total EEG power from pre stimulus levels (vs. 4.6 ± 7.2% reduction in controls, P<0.01, N=4 for both). In addition, photostimulation during NREMS in ChR2⁺ rats caused a shift in EEG spectra away from delta activity (decreased by 12.5 ± 2.5%) and towards theta activity (increased by 9.9 ± 4.2%) (Controls, -1.5 ± 1.1% in delta activity, P<0.01; +1.5 ± 1.6 % in theta activity, P<0.05) (Figure S1A).

Photostimulation during REMS in ChR2⁺ rats caused a 44.9 ± 11.8% reduction in total EEG power from pre stimulus levels (vs. 8.8 ± 5.8% reduction from baseline in controls, P<0.05, N=4 for both). In addition, photostimulation during REMS in ChR2⁺ rats caused a shift in EEG spectra away from theta activity (decreased by 23.6 ± 2.8%) and towards delta activity (increased by 21.3 ± 2.5%) (Controls, 4.0 ± 4.3% change in theta power, P<0.001; -0.1 ± 2.1% change in delta power, P<0.001, N=4 for both) (Figure S2C).
Photostimulation produces sighs associated with signs of arousal

Photostimulation of ChR2+ C1/RTN neurons commonly produced sighs, or augmented breaths (Figure S4). Sighs mostly occurred as single events and consisted of an initially normal inspiratory effort, followed by a sharp augmented inspiration-expiration sequence and a variable period of respiratory quiescence (Figure S4A). Spontaneous sighs and the sighs occurring during photostimulation were qualitatively similar except that the average duration of the respiratory quiescence that followed the augmented breath was greatly shortened during photostimulation (photostimulation vs. spontaneous, 0.50 ± 0.04 s vs. 2.50 ± 0.37 s from the peak of expiration to the next inspiratory effort, P<0.01, N=5) (Figure S4A). During NREMS, there was a significant interaction effect between the presence of ChR2+ and the stimulation frequency during photostimulation (P<0.001), with a significantly higher proportion of 10 (P<0.05) and 20 Hz (P<0.01) stimulation trials producing sighs in ChR2+ rats compared to controls (Figure S4B). Photostimulation during quiet wakefulness produced sighs in 42.1 ± 7.3% of trials in ChR2+ rats (controls, 6.0 ± 3.7% of trials, N=12 ChR2+ and 5 control rats, P<0.01). In contrast, photostimulation during REMS in ChR2+ rats did not evoke sighs reliably (P= 0.22) (Figure S4B). We found a significant correlation between the increase in MV during photostimulation and the probability of a sigh during NREMS (Pearson’s r =0.54, P<0.0001), but not REMS (Pearson’s r =0.20, P=0.50). This evidence suggests that increased respiratory drive during photostimulation promotes sighs.

Sighs were correlated with arousals during photostimulation of ChR2+ C1/RTN neurons in both sleep states in ChR2+ rats (NREMS: Pearson’s r=0.60, P<0.0001, REMS: Pearson’s r =0.53, P<0.0001). During 20 Hz photostimulation trials in NREMS, 62.0 ± 7.4 % of arousals were associated with a sigh event, compared to 20.0 ± 9.0% of arousals recorded in control rats (P<0.05, N=12 and 5) (Figure S4C). There was no difference in the proportion of arousals containing a sigh event during REMS in ChR2+ and control rats (P=0.71).

Sighs were always coupled with some degree of cortical desynchronization; sighs commonly coincided with the first signs of cortical desynchronization or followed arousal by several seconds (Figure S4D). The average delay between arousal-sigh sequences during photostimulation in ChR2+ rats during NREMS (4.2 ± 0.6, N=12) and REMS (4.0 ± 0.7, N=11) was not different from the delay between arousal-sigh sequences in control rats during NREMS (6.2 ± 0.6s, N=5, P=0.061) and REMS (6.3 ± 1.2s, N=5, P=0.10), respectively. Furthermore, the delay between arousals and sighs during photostimulation of the RVLM was similar between NREMS and REMS in ChR2+ rats (P=0.85). Together, this evidence indicates a close link between arousal and the incidence of sighs during photostimulation of ChR2+ C1/RTN neurons.

Supplemental Discussion

Physiological significance of sighs

Sighs are observed in most mammals and serve to improve pulmonary ventilation by increasing surfactant turnover, preventing atelectasis and hypoxemia. Sighs are facilitated by hypoxia in
rats and cats and reduced by destruction of the vagus and carotid sinus nerve\textsuperscript{11, 12}, showing the importance of afferent signaling in the genesis of sighs.

We show that high frequency photostimulation of C1/RTN neurons facilitates sighs in association with arousal, which is consistent with the role of these neurons in the cardiorespiratory effects of asphyxia. The motor output of sighs requires the core components of the respiratory pattern generator, but their occurrence is sensitive to excitatory neuromodulators, such as substance P and acetylcholine\textsuperscript{13, 14}, and neuropeptides linked with arousal, such as orexin\textsuperscript{15}. Similar to our observations, spontaneous arousals are associated with sighs in humans\textsuperscript{16}, but can occur in the absence of changes in electroencephalographic activity in infants\textsuperscript{17}, suggesting that cortical arousal is not a prerequisite for sighs. Together, the evidence indicates that subcortical wake-promoting neural structures are critical in the genesis of sighs and supports the notion that C1/RTN neurons contribute to the activation of these structures during sleep to produce a stereotypic arousal response to asphyxia.

Reference List


(12) Glogowska M, Richardson PS, Widdicombe JG, Winning AJ. The role of the vagus nerves, peripheral chemoreceptors and other afferent pathways in the genesis of augmented breaths in cats and rabbits. *Respir Physiol* 1972;16(2):179-96.


Table S1. Cardiovascular and respiratory parameters during different sleep-wake states and following prazosin administration.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Wake</th>
<th>NREMS</th>
<th>REMS</th>
<th>NREMS + Prazosin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP mmHg</td>
<td>102.6 ± 2.2</td>
<td>99.1 ± 2.8 ‡</td>
<td>103.7 ± 2.7 †</td>
<td>96.3 ± 1.4</td>
</tr>
<tr>
<td>HR bpm</td>
<td>324.1 ± 10.1</td>
<td>297.6 ± 13.8 ‡‡</td>
<td>298.5 ± 12.7 † †</td>
<td>372.3 ± 15.1 §§</td>
</tr>
<tr>
<td>VT mL/100gBW</td>
<td>0.44 ± 0.02</td>
<td>0.36 ± 0.01</td>
<td>0.33 ± 0.02</td>
<td>0.4 ± 0.01</td>
</tr>
<tr>
<td>fR breaths/min</td>
<td>76.4 ± 2.7</td>
<td>80.3 ± 3.0</td>
<td>103.4 ± 5.0 † †</td>
<td>79.7 ± 5.1</td>
</tr>
<tr>
<td>MV mL/100gBW/min</td>
<td>33.3 ± 1.0</td>
<td>28.8 ± 1.0</td>
<td>33.7 ± 2.0 † †</td>
<td>30.0 ± 1.4</td>
</tr>
</tbody>
</table>

Significant figures - One symbol P<0.05, two symbols P<0.01. Wake vs REMS * , NREMS vs REMS † , NREMS vs Wake ‡ by two-way ANOVA with multiple comparisons, NREMS vs NREMS post-prazosin § by Student’s paired t-test. N=12 for VT, fR, and MV values, N=6 for MAP and HR values.
Figure S1. Photostimulation of ChR2+ C1/RTN neurons during non-rapid eye movement sleep (NREMS) and rapid eye movement sleep (REMS) causes arousal.
A, Relative power of EEG (0.5-35.2 Hz) before and during a 20 s photostimulation trial during NREMS in ChR2+ (upper panel) and control (lower panel) rats (N=4 for both). B, Photostimulation trial during NREMS in a control rat (injected with saline in this case) Y-axis for B and D: Flow- 50ml/s, EEG and EMG- 0.5 mV. C, Relative power of EEG (0.5-35.2 Hz) before and during a 20 s photostimulation trial during REMS in ChR2+ (upper panel) and control (lower panel) rats (N=4 for both). D, Photostimulation trial during REMS in a control rat (injected with saline in this case).
Figure S2. The respiratory effects of ChR2+ C1/RTN neuron photostimulation are sleep state dependent.

A, Expanded traces taken from figure 3A showing trials occurring during NREMS, REMS and wakefulness respectively from left to right. Note that the number in each panel references the trial in figure 3A. B, Time course of the changes in breathing frequency (top panel), tidal volume (middle panel), and minute volume (bottom panel) during 20 s photostimulation trials (20 Hz) in ChR2+ rats during wakefulness, and during NREMS and REMS trials when arousal did not occur. BW- body weight (N=12, repeated-measures two-way ANOVA. Significant figures: one symbol- P<0.05, two symbols- P<0.01, three symbols- P<0.001. REMS vs. wake *, NREMS vs. wake †, NREMS vs. REMS ‡).
Figure S3. The cardiovascular effects of ChR2+ C1/RTN neuron photostimulation are state dependent.

A, Average biphasic change in mean arterial pressure (MAP; upper) and heart rate (HR; lower) following a 3 s stimulus train during each arousal state (N=6, *P<0.05, ***P<0.001, one-way ANOVA between arousal states).
Figure S4. Photostimulation produces sighs associated with arousal.

A, Example of a spontaneous sigh (laser off; left) and a sigh occurring during photostimulation (20 Hz; right) in a ChR2+ rat. B, Probability of sighs during photostimulation in NREMS and REMS in ChR2+ and control rats (N= 12 ChR2+ and 5 control rats, *P<0.05, **P<0.01 ChR2+ vs. control, two-way ANOVA comparing presence of ChR2+ and stimulation frequency for NREMS and REMS conditions separately). C, Proportion of arousals with sigh during photostimulation in NREMS and REMS (N= 12 ChR2+ and 5 control rats respectively, *P<0.05 ChR2+ vs. control, two-way ANOVA comparing the presence of ChR2+ and stimulation frequency for NREMS and REMS conditions separately). Note that values for controls at stimulation frequencies less than 20 Hz (2 and 10 Hz for NREMS trials and 2 Hz for REMS trials) are omitted from B and C, but were included in the statistical analysis. D-E, X-Y scatter plots of the latency of sighs relative to the latency of arousal from the onset of photostimulation (20 Hz) during NREMS (D) and REMS (E) in ChR2+ and control rats.
Figure S5. ChR2-mCherry is preferentially expressed by C1 and RTN neurons. 

A, Micrograph of a coronal brainstem section (approximate bregma level -11.8mm) showing the distribution of ChR2-mCherry expressing neurons and the tract left by a chronically implanted 200μm optical fiber. Scale bar: 200 μm. B, Micrograph of the cluster of ChR2-mCherry expressing neurons in A (in red) showing co-expression with tyrosine hydroxylase (TH, in green). Double labeled neurons appear orange. Tip of the fiber optic tract is visible at the top of the image (dotted line). Scale bar: 50 μm.