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 brainstem 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 \( \alpha_1 \)-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;61:00-00.)

Key Words: asphyxia ■ chemoreception ■ Phox2b ■ sleep apnea ■ sympathetic nervous system
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-eGFP 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
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, 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. 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.

**$\alpha$-1-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 RVL photostimulation, we administered the selective $\alpha$-1-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), but had no significant effect on heart rate ($P=0.09$; Figure 6A and 6B), and reduced the increase in MV by 43.8% ($P<0.01$), owing primarily to a 46.5% reduction in the respiratory frequency response ($P<0.05$; 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 $\alpha$-1-adrenoreceptor blockade attenuates the

---

**Figure 3.** The respiratory effect of C1/retrotrapezoid 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 hydroxylase–immunoreactive neurons in the ipsilateral RVLM of counted sections (range, 28.3%–80.5%). The number of ChR2+ neurons was weakly correlated with the change in MV caused by photostimulation during NREMS (Pearson r=0.37; P=0.034), but there was no correlation between the number of ChR2+ neurons and the probability of arousal during NREMS (Pearson r=0.01; P=0.63) or REMS (Pearson r=0.01; P=0.76). The fiber optic tip was typically located within 500 μm of the ventral surface of the brain and dorsal to the bulk of the ChR2--mCherry neurons (Figure 7B).

**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 excitation 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.

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.13,17 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.

**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.2 Acute changes in AP cause arousal from sleep in lambs and humans,18,19 which can be eliminated by sinoaortic denervation,18 implicating the baroreceptors or the carotid bodies in this effect. In the present study, arousal elicited by photostimulating ChR2+ C1/RTN neurons was not significantly reduced by eliminating the AP rise with prazosin suggesting that baroreceptor activation contributed minimally to the arousal. Furthermore, the persistence of the arousal in prazosin-treated rats suggests that this effect does not rely principally on the release of catecholamines, unlike the arousal caused by selective stimulation of the locus ceruleus.20

The correlation between the intensity of the breathing response and the arousal caused by photostimulation during NREMS suggests that sensory afferent activation secondary to increased breathing effort could be responsible for arousal during NREMS. However, direct CNS connections between C1/RTN neurons and wake-promoting networks would provide an equally satisfactory explanation for the correlation between arousal and changes in breathing during stimulation. In support of a CNS mechanism, prazosin reduced the respiratory effects of photostimulation by half without significantly changing arousal probability during NREMS, and photostimulation during REMS produced arousal, despite greatly attenuated respiratory stimulation. This evidence is suggestive, but insufficient, to parse out the relative contribution of peripheral respiratory feedback versus CNS mechanisms in the arousal elicited by activating C1/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,
Abbott et al. Stimulating C1 and RTN Neurons Causes Arousal

Autonomic and respiratory activity, and motor atonia. \textsuperscript{21} During REMS, the arousal response to hypoxia, hypercapnia, and upper-airway stimulation is attenuated in dogs. \textsuperscript{22–24} The arousal response to hypoxia, hypercapnia, and upper-airway stimulation is not consistently blunted during REMS in healthy humans, \textsuperscript{25–27} but obstructive events are more common and oxygen desaturation more severe during REMS in obstructive sleep apnea patients. \textsuperscript{28,29} This has been attributed to an increase in arousal threshold during REMS relative to NREMS. \textsuperscript{2,30} 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, \textsuperscript{31} 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. \textsuperscript{2,32} 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. \textsuperscript{33,34} Motor atonia during REMS disproportionately affects nondiaphragmatic respiratory

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{The cardiovascular effect of C1/retrotrapezoid nucleus neuron photostimulation is sleep state dependent. \textbf{A}, Effects of 3-s photostimulation trials (20 Hz, 5 ms) on arterial pressure (AP) and heart rate (HR) during each arousal state in ChR2\textsuperscript{+} rats. y axis: Flow, 50 mL/s; electroencephalograph (EEG) and electromyograph (EMG), 0.5 mV. \textbf{B}, Normalized time course changes in mean AP (MAP) and HR during a 3-s stimulus trains during each arousal state in ChR2\textsuperscript{+} rats (n=6). NREMS indicates nonrapid eye movement sleep; and REMS, rapid eye movement sleep.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{\textalpha1-Adrenoreceptor antagonism does not prevent arousal (nonrapid eye movement sleep), but attenuates the cardiorespiratory activation during photostimulation. \textbf{A}, Effects of photostimulation before (left) and after (right) administration of prazosin (1 mg/kg, IP). y axis: Flow, 35 mL/s, electroencephalograph (EEG) and electromyograph (EMG), 0.25 mV. \textbf{B–D}, Average cardiovascular (B), respiratory (C), and arousal response to photostimulation before (open columns) and after prazosin treatment (closed columns; n=6, *P<0.05, **P<0.01, ***P<0.001, paired Student t test). HR indicates heart rate; MAP, mean arterial pressure; and MV, minute volume.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{ChR2\texttextsuperscript{mCherry} is preferentially expressed by C1 and retrotrapezoid nucleus neurons. \textbf{A}, Rostro-caudal distribution of neurons expressing ChR2\texttextsuperscript{mCherry}, tyrosine hydroxylase (TH), or both in the rostral ventrolateral medulla (n=22 ChR2\textsuperscript{+} rats). \textbf{B}, Location of the fiber placement in all experiments (n=22 ChR2\textsuperscript{+} and 8 control rats). Scale bar, 0.5 mm.}
\end{figure}
muscle groups, such as cranial motor neurons, and motor nerves 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 outflow changes predictably during REMS; muscle sympathetic nerve activity is elevated, whereas the splanchnic, 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 aonomic 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 (11POST7190001).

Disclosures

None.

References

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.