Optogenetic activation of cholinergic neurons in the PPT or LDT induces REM sleep

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Rapid eye movement (REM) sleep is tightly regulated, yet the mechanisms that control REM sleep remain incompletely understood. Early pharmacological and unit recording studies suggested that ACh was important for REM sleep regulation (1, 2). For example, injection of cholinergic drugs into the dorsal mesopontine tegmentum reliably induced a state very similar to natural REM sleep in cats (3–6). Unit recordings from the cholinergic areas of the mesopontine tegmentum revealed cells that were active during wakefulness and REM sleep, as well as neurons active only during REM sleep (7–13). Electrical stimulation of the laterodorsal tegmentum (LDT) in cats increased the percentage of time spent in REM sleep (14), and activation of the pedunculopontine tegmentum (PPT) in rats induced wakefulness and REM sleep (15). If cholinergic PPT and LDT neurons are necessary for REM sleep to occur, as the early studies suggest, then lesioning the PPT or LDT should decrease REM sleep. In cats, lesions of the PPT and LDT do disrupt REM sleep (16, 17), but lesions in rodents have had limited effect on REM sleep or increased REM sleep (18–22). Additionally, c-fos studies have found very few cholinergic cells activated under high REM sleep conditions. When c-fos-positive cholinergic neurons in the PPT and LDT are found to correlate with the percentage of REM sleep, they still account for only a few of the total cholinergic cells in the area (23). Juxtacellular recordings of identified cholinergic neurons in the LDT found these cells had wake and REM active firing profiles, with the majority firing the highest during REM sleep (13). These discrepancies have led to alternative theories of REM sleep regulation, where cholinergic neurons do not play a key role (18, 19, 23, 24 and reviewed in 25, 26).

The PPT and LDT are made up of heterogeneous populations of cells, including distinct populations of cholinergic, GABAergic, and glutamatergic neurons (27–29). Many GABAergic neurons are active during REM sleep, as indicated by c-fos (25), and both GABAergic and glutamatergic neurons have been found with maximal firing rates during REM sleep in the LDT and medial PPT (13). To distinguish the differential roles of each cell type in REM sleep regulation, a method that can modulate specific cell types in the behaving animal is needed. Optogenetics now provides this ability to target specific subpopulations of neurons and control them with millisecond temporal resolution (30). Therefore, we aimed to determine the role of cholinergic neurons in the PPT and LDT in REM sleep regulation using optogenetics.

Results

Channelrhodopsin Expression Was Selective to Cholinergic Neurons in the PPT and LDT and Functional in Vitro. Mice expressing channelrhodopsin (ChR2) conjugated to YFP under the choline acetyltransferase (ChAT) promoter and WT littermates were used (six PPT ChAT-ChR2+ mice, five PPT ChAT-ChR2−, five LDT ChAT-ChR2+, and six LDT ChAT-ChR2− mice per group, and one ChAT-ChR2+ patch-clamp mouse) (31). Immunohistochemistry for ChAT confirmed that ChR2 was expressed selectively in cholinergic neurons in the PPT and LDT. Quantification revealed that 96.1% (2,636 of 2,742) and 94.0% (1,992 of 2,119) of ChAT-positive neurons were also positive for ChR2-YFP in the PPT and LDT, respectively. Colocalization of ChAT and ChR2-YFP in this range is consistent with previous reports demonstrating selective expression in the cortex (100%), striatum (100%), globus pallidus (100%), and medial habenula (98.2%) for the same mouse strain (31). No ChR2-YFP–only neurons were found (Fig. 1). Fig. 2A demonstrates that LDT neurons activated with ChR2-YFP lead to a substantial increase in firing rate. As expected, ChR2-negative yFP-labeled ChAT-positive neurons showed no detectable change in firing rate. These data indicate that ChR2-YFP is selectively expressed in cholinergic neurons and will activate these neurons with visible light. PPT neurons were not positive for ChR2-YFP, as expected, and these neurons showed no activation with ChR2-YFP.

Significance

Rapid eye movement (REM) sleep is a critical component of restful sleep, yet the mechanisms that control REM sleep are incompletely understood. Brainstem cholinergic neurons have been implicated in REM sleep regulation, but heterogeneous cell types in the area have made it difficult to determine the specific role of each population, leading to a debate about the importance of cholinergic neurons. Therefore, we selectively activated brainstem cholinergic neurons to determine their role in REM sleep regulation. We found that activation of cholinergic neurons during non-REM sleep increased the number of REM sleep episodes but not REM sleep duration. Our data demonstrate that brainstem cholinergic neurons are important modulators of REM sleep and clarify their role in REM sleep initiation.


Reviews included: H.A.B., University of Tennessee; and H.C.H., Stanford University.

The authors declare no conflict of interest.

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(one shown, n = 2 tested) had reliable rapid-onset action potentials following 5-ms light pulses. Fig. 2B shows the ability of a cell to follow the light pulses at 5 Hz over 2 s. The cell never missed the first light pulse, but the probability of a spike occurring decreased with progressive light pulses. The likelihood of a spike occurring varied as a function of the starting membrane potential. If the cell started near −55 mV, it was more likely to fire and follow the light pulses. If the cell started closer to −60 mV, it missed more light pulse-induced action potentials. The latency from start of the light pulse to the beginning of the action potential ranged from 4.6 ms for the first light pulse to 20 ms for the 10th light pulse in the series.

**Activation of Cholinergic Neurons in the PPT and LDT Increases the Probability of REM Sleep.** Mice expressing ChR2 in cholinergic neurons (ChR2+) and their WT littermates (ChR2−) were implanted with bilateral fiber optics for the PPT or LDT, as well as EEG and electromyogram (EMG) electrodes. REM sleep was classified by high levels of theta (5–9 Hz) in the EEG and no muscle tone. EEG and EMG traces demonstrate that optogenetic activation of cholinergic neurons in the PPT during non-REM (NREM) sleep induced REM sleep (Fig. 3). The probability of REM sleep over time increased between ChR2+ mice (n = 11) and ChR2− mice (n = 11) for all stimulations for both the PPT and LDT (Fig. 4). REM sleep probability was significantly higher [nonoverlapping confidence intervals (CIs)] for 30–60 s beyond the stimulation for ChR2+ PPT and LDT 60-s and 80-s stimulations compared with ChR2− 60-s and 80-s stimulations. REM sleep probability for the PPT 180-s stimulation reached its peak of 0.30 (95% CI: 0.26–0.35) at 2 min and 25 s, and then started to decline. REM sleep probability for the LDT 180-s stimulation significantly increased between 2 and 3 min (nonoverlapping CIs) compared with the ChR2− 180-s stimulation. Group data from 22 mice and an average of 173 stimulations per condition (range: 99–226 stimulations per condition; Table 1) show that optogenetic activation of the PPT or LDT increased REM sleep (Fig. 5 A and D). PPT 180-s stimulation increased REM sleep more than the LDT 180-s stimulation for ChR2+ mice (difference of means = 15.72 s, 99% CI: 3.90–27.51 s). NREM sleep decreased between ChR2+ mice and ChR2− mice for all PPT stimulations and the 60-s LDT stimulation (Fig. 5 B and E). Wakefulness did not change for either PPT or LDT stimulation, except for a small increase in wakefulness for the 60-s PPT stimulation (Fig. 5 C and F). Inferences of the differences between groups were calculated using 99% CIs (details are provided in Table 2 and SI Materials and Methods, Data Analysis).

**Increase in REM Sleep Is Due to More REM Sleep Episodes.** The increase in REM sleep occurred by increasing the number of REM sleep episodes (percentage of stimulations that induced REM sleep; Fig. 6 A and D) but not the duration of REM sleep episodes (Fig. 6 B and E). The induced REM sleep was electrophysiologically similar to natural REM sleep. Power spectral analysis of the EEG during induced REM sleep was not significantly different from the power spectra during natural REM sleep in the same ChR2+ mice (Fig. 6 C and F), as indicated by overlapping 95% CIs.

**Fiber Optics Were Localized to the PPT and LDT.** Fig. 7 summarizes the results of the histological analyses demonstrating that the tips of the fiber optics were localized just above the PPT or LDT bilaterally. Placing the tips of the fibers at the top of the nuclei ensured as complete as possible activation of the entire nuclei based on Yizhar et al.’s (32) calculations of light dissipation in the brain. Stained sections were compared with the mouse brain atlas (33) to identify their final location (Fig. 7A). The average PPT stereotaxic coordinates were 4.80 mm posterior to bregma, 1.14 mm lateral to the midline, and −3.15 mm ventral. The average LDT stereotaxic coordinates were 5.03 mm posterior to bregma, 0.59 mm lateral to the midline, and −3.03 mm ventral.

![Fig. 1](image1)  
**Fig. 1.** ChAT and ChR2-YFP were colocalized in the PPT (A; bregma, −4.84 mm) and LDT (B; bregma, −5.02 mm). Confocal images of coronal brain sections stained for ChAT and ChR2-YFP in the PPT (A, Top) and LDT (B, Top) are shown. (Magnification: 25×) Confocal images show robust colocalization of ChAT to the cell bodies and ChR2-YFP to the cell membrane in the PPT (A, Bottom) and LDT (B, Bottom). (Magnification: 40×.)
Wake and REM Sleep Stimulations. Eighty-second (5 ms at 5 Hz) stimulations during wakefulness caused a small increase in the probability of wakefulness for the 4 min after the beginning of the stimulation [0.92 (95% CI: 0.91–0.93, n = 167) for ChAT-ChR2+ mice compared with 0.90 (95% CI: 0.90–0.91, n = 75) for ChAT-ChR2− mice; difference of the means = 1.6 (95% CI: 0.9–2.4)]. Eighty-second (5 ms at 5 Hz) stimulations during REM sleep did not change the duration of REM sleep between ChAT-ChR2+ mice 73.2-s (95% CI: 62.9–83.8, n = 87), ChAT-ChR2− mice 71.5-s (95% CI: 57.3–86.7, n = 51), and baseline days with no stimulation for 82.8 s in both ChAT-ChR2+ and ChAT-ChR2− mice (95% CI: 77–88.5, n = 333). Lack of a significant difference was calculated using the difference of the means and 98.3% (Bonferroni correction for three comparisons) CIs of the difference.

Table 1. Number of stimulations per condition

<table>
<thead>
<tr>
<th>Stimulation length, s</th>
<th>ChR2+</th>
<th>ChR2−</th>
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<tbody>
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<td>PPT</td>
<td>60</td>
<td>150</td>
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<tr>
<td>80</td>
<td>168</td>
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</tr>
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<td>180</td>
<td>171</td>
<td>99</td>
</tr>
<tr>
<td>LDT</td>
<td>60</td>
<td>196</td>
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<tr>
<td>80</td>
<td>226</td>
<td>219</td>
</tr>
<tr>
<td>180</td>
<td>163</td>
<td>183</td>
</tr>
</tbody>
</table>

Discussion

PPT and LDT Cholinergic Neurons Are Important for REM Sleep Initiation but Not REM Sleep Maintenance. Activation of cholinergic neurons in the PPT or LDT during NREM sleep increased the probability of REM sleep (Fig. 4) and the number of REM sleep episodes (Fig. 5) but not the duration of REM sleep episodes (Fig. 6). REM sleep probability for the PPT 180-s stimulation reached its peak of 0.30 (95% CI: 0.26–0.35) at 2 m and 25 s, and then started to decline (Fig. 4C). As has been suggested before (34), there are likely different mechanisms controlling REM sleep initiation vs. REM sleep maintenance. Activation of cholinergic cells in the basal forebrain during REM sleep was able to increase the duration of REM sleep episodes (35), whereas stimulation of the PPT during REM sleep in this study was not able to prolong REM sleep episodes. The present study suggests that cholinergic PPT and LDT neurons are important modulators of REM sleep initiation but not REM sleep maintenance. PPT 180-s stimulation increased REM sleep more than the LDT 180-s stimulation for ChR2+ mice (difference of means 15.72 s, 99% CI: 3.90–27.51 s). This difference suggests that
noninduced REM sleep in the same ChR2 carrier. The duration of REM sleep episodes that started within the stimulation window was different for PPT-induced (18) and LDT-induced (19) REM sleep compared with noninduced REM sleep in the same ChR2 mice. *Significant differences between groups with 99% confidence (details of the numbers used to generate the inference are provided in Table 2).

although both areas contain cholinergic neurons, the PPT may be better situated in the REM sleep circuitry to control REM sleep initiation.

### Implications for the Role of PPT and LDT Cholinergic Neurons in REM Sleep Modulation

The PPT and LDT contain multiple subpopulations of REM-on neurons, which have made it difficult to distinguish the differential roles of each subtype. C-fos studies show both cholinergic and GABAergic REM-on neurons in rats (23), and juxtacellular recordings have found both GABAergic and glutamatergic REM-on neurons (13). Interestingly, this juxtacellular study did not find any cholinergic REM-on neurons. Instead, the identified cholinergic neurons were active during both wake and REM sleep, with the majority firing the highest during REM sleep (13). The cholinergic agonist carbachol induced prolonged REM sleep by inhibiting presumably cholinergic REM-on neurons and exciting presumably noncholinergic REM-on neurons in the cat pons (10). The present study demonstrated that selective optogenetic activation of cholinergic neurons in the PPT and LDT induced REM sleep in mice.

Monoamines are thought to inhibit REM sleep, and cholinergic neurons in the LDT are inhibited by serotonin in both rat (36) and guinea pig (37) brain slices. The selective 5-hydroxytryptamine 1A (5-HT1A) receptor agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) selectively inhibits presumably cholinergic PPT REM-on neurons but not wake-on/REM-on neurons in cats (8). Therefore, it was surprising that Grace et al. (20) found that local PPT delivery of 8-OH-DPAT increased REM sleep in rats. In theory, this manipulation should inhibit cholinergic REM-on cells, which would have been expected to decrease REM sleep. In light of the multiple cell types that are now known to exhibit REM-on firing patterns in the LDT and PPT (13), it is possible that serotonin inhibited GABAergic or glutamatergic REM-on neurons. In addition, it is possible that serotonin inhibition of cholinergic PPT occurs through other receptor subtypes or that selectivity for the 5-HT1A receptor by 8-OH-DPAT is lost at high concentrations. Also, 5HT1A serotonin receptor mRNA has only been found in GABAergic but not cholinergic neurons in the PPT of the mouse (38). Taken together, these data suggest that it is possible Grace et al. (20) inhibited GABAergic or glutamatergic REM-on neurons to get an increase in REM sleep, whereas we selectively activated cholinergic REM-on neurons to get an increase in REM sleep. Interestingly, both manipulations resulted in a change in the number of REM sleep episodes, and REM sleep episode duration suggests that the PPT is involved in REM sleep initiation but not REM sleep maintenance. Additional studies are needed to tease apart the differential roles of cholinergic, GABAergic, and glutamatergic REM-on cells in the PPT and LDT. There are about 50% fewer cholinergic cells compared with GABAergic cells in the PPT and LDT (29), in addition to intermingled glutamatergic cells. The fact that activation of a small number of cholinergic cells can elicit a strong increase in REM sleep adds to the strength of the evidence that those cholinergic neurons are important for REM sleep initiation.

Basal forebrain cholinergic neurons also contribute to the regulation of sleep and wakefulness. Carbachol injection into the basal forebrain of cats increased wakefulness (39). A recent study optogenetically activated cholinergic neurons in the basal forebrain during wake, NREM sleep, and REM sleep (35). Activation of basal forebrain cholinergic neurons during NREM sleep induced transitions to wake and REM sleep. However, these states were shorter in duration than natural wake and REM sleep episodes, and the stimulation did not influence whether wake or REM sleep was induced. Stimulation of the cholinergic basal forebrain during wakefulness in Han et al. (35) decreased NREM sleep. In the present study, stimulation of cholinergic neurons in the PPT during wakefulness caused a small increase in the probability of wakefulness. Optogenetic stimulation of REM sleep episodes, and the stimulation did not influence whether wake or REM sleep was induced. Stimulation of the cholinergic basal forebrain during wakefulness in Han et al. (35) decreased NREM sleep. In the present study, stimulation of cholinergic neurons in the PPT during wakefulness caused a small increase in the probability of wakefulness. Optogenetic stimulation of

### Table 2. Difference of ChR2 and ChR2– means and 99% CIs

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<th>Upper CI</th>
<th>Significance</th>
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<td></td>
<td></td>
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<tr>
<td>REM</td>
<td>60 4.2 1.5 7.2 Increase</td>
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<tr>
<td>80 8.0 3.0 13.0 Increase</td>
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<td>180 24.5 11.6 37.0 Increase</td>
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<tr>
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<td>180 −30.9 −43.8 −16.2 Decrease</td>
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<tr>
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<tr>
<td>180 6.4 −2.2 13.9 No change</td>
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<tr>
<td>Stimulations that induced</td>
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<tr>
<td>REM</td>
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<td>180 1.2 −26.3 28.8 No change</td>
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Fig. 6. Optogenetic activation of cholinergic neurons in the PPT and LDT increased the number of REM sleep episodes but not REM sleep episode duration. The percentage of stimulations that induced REM sleep per number of REM sleep episodes increased for the PPT (A) and LDT (B). The duration of REM sleep episodes that started within the stimulation window did not change for the PPT (C) or LDT (E). The power spectra were not different for PPT-induced (C) or LDT-induced (F) REM sleep compared with noninduced REM sleep in the same ChR2 mice.
cholinergic basal forebrain neurons during REM sleep increased the duration of REM sleep episodes, whereas stimulation of PPT cholinergic neurons during REM sleep in the present study did not prolong REM sleep. Basal forebrain carbachol injection decreased the amount of pontine carbachol injection-induced REM sleep-like state (39), suggesting that the basal forebrain arousal promoting system interacts with the pontine REM promoting system. Taken together, these findings suggest that there may be a forebrain component to REM sleep maintenance. Han et al. (35) concluded that the basal forebrain cholinergic neurons are responsible for terminating NREM sleep. This interpretation is complementary to our interpretation that brainstem cholinergic neurons are important for initiating REM sleep.

Implications for the Role of PPT and LDT Cholinergic Neurons in Wakefulness. Given the wakefulness and REM sleep firing profile of cholinergic PPT and LDT neurons (7–13), we would have expected a larger effect on wakefulness. Instead, we only found two conditions that slightly increased wakefulness: the 60-s PPT stimulations during NREM sleep and the 80-s PPT stimulations during wakefulness. The PPT and LDT are well positioned to activate the cortex during both wakefulness and REM sleep. However, when activated during NREM sleep, the brainstem cholinergic neurons preferentially shift an animal to REM sleep vs. wakefulness.

Possible Explanations for the Delay in REM Sleep Onset After Stimulation. The probability of REM sleep builds over the time course of the stimulation. A few possibilities exist for why the REM sleep transition is not an immediate switch with short latency: (i) cholinergic tone must build up to a certain level and meet a network threshold for the transition to occur; (ii) incomplete activation of the nucleus due to limited spread of the light out of the fiber optic or nonoptimal placement of the fibers; (iii) only one cholinergic brainstem center was activated at a time due to the small size of the mouse brain and limited space to target both the PPT and LDT simultaneously; and (iv) cholinergic cells in the PPT and LDT are modulators of REM sleep and exert their effect via projections to other REM-on areas, such as the sublaterodorsal nucleus. Slice recordings support cholinergic modulation of the sublaterodorsal nucleus, where carbachol activated spinally projecting sublaterodorsal nucleus neurons (40). Animals with fibers that were optimally positioned over the PPT and had the highest fiber transmittance had the strongest REM induction effect.

Limitations. The transgenic mice used in the present study have been found to express extra copies of the vesicular ACh transporter gene and have increased cholinergic tone (41). Behaviorally, these mice have prolonged motor endurance and impaired attention and memory. These mice were reported to be more active at night than WT mice but had no difference in activity level during the day. In our hands, we recorded sleep during the day and found that the ChR2 mice had 4% REM sleep compared with 3.7% REM sleep in the ChR2 mice, and this difference was not statistically different. All of our experiments were performed during the day, when locomotor activity and percentage of REM sleep were the same between experimental groups; therefore, we think our results are representative of the true effect. The transgenic mice used in the present study expressed ChR2 in cholinergic neurons throughout the brain. Hence, it is possible that activation of cholinergic fibers of passage from other brain regions to the PPT or LDT contributed to REM sleep induction. Future studies that selectively inhibit cholinergic neurons in the PPT and LDT of nonhypercholinergic mice are needed to determine if cholinergic neurons are necessary for REM sleep generation.

Conclusions

The present findings demonstrate that activation of cholinergic neurons in the PPT or LDT during NREM sleep is sufficient to increase REM sleep in mice. The induced REM sleep state closely resembles natural REM sleep. Selectively increasing cholinergic...
tone in the PPT or LDT increases the number of REM sleep episodes but not the duration of REM sleep episodes. Therefore, cholinergic neurons in the PPT and LDT remain potent modulators of REM sleep initiation. This modulation of REM sleep expression may occur via activation of other REM-on neuron populations, such as the pontine reticular formation and sublaterodorsal nucleus.

Materials and Methods

Adult male mice (n = 23) expressing ChR2 under the ChAT promoter (stock no. 014546; The Jackson Laboratory) (31) and their WT littermates were implanted with EEG and EMG electrodes and bilateral fiber optics in the PPT or LDT. All experiments were approved by the Massachusetts Institute of Technology Committee on Animal Care. The mice were placed in a recording chamber while EEGs and EMGs were recorded for 6–8 h. Blue light from a laser was used to stimulate the cholinergic PPT or LDT neurons optogenetically during NREM sleep. Stimulations were 60 s, 80 s, or 180 s long, separated by at least 1 min, and each experiment consisted of ∼25 stimulations. EEGs and EMGs were used to score sleep manually. Variables calculated included the amount of time spent in NREM sleep, REM sleep, and wake with the stimulation; REM sleep episode duration; and percentage of REM sleep occupancy. The probability of REM sleep over the time course of the stimulation was also plotted. Fiber placement and specific expression of ChR2 in cholinergic neurons were confirmed by histology. Patch-clamp of a ChAT-ChR2+ mouse LDT slice was performed to confirm light-induced action potentials. Statistical inferences were determined by calculating 95% CIs for each group. The decision rule was to reject the null hypothesis if zero is not in the 95% CI of the difference between the groups. Details of the experiment procedures are provided in the SI Materials and Methods.

ACKNOWLEDGMENTS. This study was supported by NIH Grant DP1-OD003846 (to E.N.B.), NIH Grant TR01-GM104984 (to E.N.B. and M.A.W.), and NIH Grant T32-HL07901 (to C.J.V.); a Massachusetts General Hospital Executive Committee on Research Fellowship (to C.J.V.); and the Department of Anesthesia, Critical Care, and Pain Medicine, Massachusetts General Hospital.

Supporting Information

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SI Materials and Methods

Animals. Twenty-three adult male transgenic mice expressing ChR2 conjugated to YFP under the ChAT promoter (stock no. 014546; The Jackson Laboratory) and WT littermates were used (six PPT ChAT-ChR2+, five PPT ChAT-ChR2−, five LDT ChAT-ChR2+, and six LDT ChAT-ChR2− mice per group, and one ChAT-ChR2+ patch-clamp mouse). Line 6 of these ChAT-ChR2 mice was characterized for colocalization of ChAT and ChR2-YFP in the cortex (100%), striatum (100%), globus pallidus (100%), and medial habenula (98.2%) by Zhao et al. (1). Mice were bred in-house and genotyped for the YFP-containing transgene by sending ear tissue to Transnetyx. All mice were kept on a 12:12-h light/dark cycle (lights on at 7:00 AM, lights off at 7:00 PM), with ad libitum access to food and water. All animal procedures were reviewed and approved by the Massachusetts Institute of Technology Committee on Animal Care.

Surgery. Mice were anesthetized with 3% (vol/vol) isoflurane anesthesia in oxygen and placed in a stereotaxic frame (David Kopf Instruments). An incision was made in the skin, and cranialotomies were made above the target regions for PPT bregma (−4.72 mm anterior posterior and ±1.25 mm lateral to the midline) and for LDT bregma (−5.02 mm anterior posterior and ±0.5 mm lateral to the midline). Bilateral fiber optics (200-μm inner diameter, 0.22 N.A.; Doric Lenses) coated with 1-dioctadecyl-3,3,3,3′-tetramethylindocarbocyanine perchlorate were lowered into the brain 3.25 mm for the PPT and 3.15 mm for the LDT so that the tip of the fiber was at the top of the PPT or LDT. Cranialotomies were also made for EEG electrodes (0.005-inch stainless steel; A-M Systems) over the prefrontal cortex and somatosensory cortex, as well as for six anchor screws and a ground screw (0.7-mm diameter, 2 mm long; Antrin Miniature Specialties). EMG electrodes (0.002-inch seven-stranded stainless steel; A-M Systems) were placed in the nuchal muscle. EEG and EMG electrodes were connected to an eight-channel electrode interface board (Neuralynx). Anchor screws, electrodes, an electrode interface board, and fibers were adhered to the skull with dental acrylic. Animals were singly housed after surgery and had a minimum of 1 wk to recover before experiments began. Each experiment was separated by at least 3 d.

Optogenetic Stimulation and Sleep Recordings. Animals were conditioned to handling and the recording chamber after surgery. Experiments started at least 1 wk after surgery to allow for recovery, and began at the beginning of the light cycle between 7:00 and 9:00 AM. The mice behaved freely while EEGs (filtered 0.5–500 Hz) and EMGs (filtered 10–500 Hz) were recorded for 6–8 h using a 64-channel Neuralynx Digital recording system at a sampling rate of 254.344 Hz (Neuralynx). A patchcord (Doric Lenses) was connected to the implanted fiber optic with a ceramic sleeve to join the 1.25-mm metal ferrules on each end of the fibers. Blue light was generated with a 200-mW MBL-H 473-nm laser with a PSU-H-FDA power supply (Opto Engine). Stimulation parameters were controlled using the analog output of a Multichannel System stimulus generator STG4000 (ALA Scientific Instruments), and the start and end of each pulse were marked by a transistor–transistor logic (TTL) pulse into the Neuralynx recording system. The laser power output was measured using a PM100D power meter (Thor Labs). The tip of the patch cord measured 30 mW with continuous light. Due to light loss at the connections, the fibers transmitted about 50% of the initial continuous light, so the power at the tip of the fiber was ~15 mW. According to Yizhar et al. (2), you need 1–5 mW/mm² to activate ChR2. We wanted to activate as much of the PPT and LDT as possible, so we targeted the middle of each nucleus in the anterior-to-posterior plane, which spans ~0.7 mm, and placed the fiber at the top of each nucleus, which has a depth of ~0.8 mm. Blue light attenuates in brain tissue at a measured rate and can be calculated using the tool at www.optogenetics.org/calc. With our parameters of 473 mm of light; 200-μm diameter, 0.22 N.A. fiber optics; and 15 mW of power output, we have 477 mW/mm² immediately out of the tip and 9.5 mW/mm² at 0.8 mm below the tip of the fiber, so we should be able to activate the entire depth of the nuclei as well as the anterior-to-posterior span of the nuclei.

To mimic the natural firing rate of these neurons during REM sleep, the stimulation frequency was based on published firing rates (3, 4) for the PPT and LDT across sleep and wakefulness in the cat, where presumably cholinergic REM-on neurons fired at ~5 Hz during REM sleep. In our pilot multiunit in vivo recording studies, light pulses shorter than 5 ms did not induce reliable action potentials. Therefore, the final values used for these studies were 5-ms pulses at 5 Hz. The duration of stimulations used in these experiments was based on pilot behavioral studies, which found that 60-s stimulations increased the percentage of REM sleep but not the duration of REM sleep. We chose 60-s stimulations to start with because based upon the literature, a 60-s stimulation should not cause damage to the brain due to heat (2) and it was a physiologically relevant length of time for REM sleep to occur. To determine if continued activation of cholinergic neurons could prolong the REM sleep episode duration, we next tried 180-s stimulations. These stimulations increased the percentage of REM sleep even more than the 60-s stimulations but still did not increase the REM sleep episode duration. The peak REM sleep probability in the 180-s stimulations occurred at 80 s, so next we tested if 80 s of light stimulation was sufficient to generate the same amount of REM sleep as the 180-s stimulations. Experiments consisted of allowing the animals to adjust to the recording chamber for ~2 h. Then, once the animal naturally went into NREM sleep for at least 60 s, the light pulses were initiated. Each animal had ~25 stimulations per 6- to 8-h session, where each stimulation was separated by at least 1 min. Each stimulation was considered independent because it was separated in time. Stimulations from multiple animals were compiled to yield the group data. Experiment days were separated by at least 3 d. The experiments reported in this study used four optogenetic stimulation conditions in random order: baseline sleep (no stimulation) and 60-, 80-, or 180-s stimulations (5-ms pulses at 5 Hz). EEG, EMG, and videotapes were recorded and used to score sleep.

Wake and REM Stimulations. In a subset of animals, we also stimulated during wake and REM sleep with the same parameters (5-ms pulses at 5 Hz for the middle-duration 80 s) on a separate experiment day from the NREM sleep stimulations. Wake stimulations began when the animal had been awake for at least 60 s. REM sleep stimulations began when the animal had been in REM sleep for 5–10 s. For wake stimulations, the probability of wakefulness was calculated for the 4 min after the beginning of the stimulation between ChAT-ChR2+ PPT mice and ChAT-ChR2− PPT mice. For REM sleep stimulations, REM sleep duration was compared between baseline days where no stimulation occurred, ChAT-ChR2+ PPT mice, and ChAT-ChR2− PPT mice.
**Patch-Clamp Recordings: Acute Slice Preparation.** Mice were anesthetized with isoflurane and decapitated. The brains were removed and immediately immersed in carbogenated (95% O₂, 5% CO₂) ice-cold cutting solution containing 105 mM N-methyl-D-glucamine, 2.5 mM KCl, 1.24 mM Na₂HPO₄, 10 mM MgCl₂, 0.5 mM CaCl₂, 26 mM NaHCO₃, 15 mM glucose, and 1 mM Na-ascorbate at an osmolarity of 300 mOsm. The pH was adjusted to 7.3 with HCl. Brains were then rapidly blocked, and 350-μm coronal slices were cut in the same solution with a vibrating blade microtome (VT1200; Leica). Slices containing the PPT and LDT were transferred to an incubation chamber filled with carbogenated warm (32 °C) cutting solution for 10 min and then 50% cutting solution/50% artificial cerebral spinal fluid (ACSF) (32 °C) for 20 min for recovery. Slices were then stored in a holding chamber filled with carbogenated room temperature (~23 °C) ACSF containing 119 mM NaCl, 2.5 mM KCl, 1.24 mM Na₂HPO₄, 1.3 mM MgCl₂, 2.5 mM CaCl₂, 26 mM NaHCO₃, and 10 mM glucose at an osmolarity of 300 mOsm (pH 7.3) for at least 1 h before being used for patch experiments.

**In Vitro Electrophysiology.** After recovery, slices were transferred to a recording chamber perfused with carbogenated room temperature (~23 °C) ACSF at a flow rate of 2 mL/min. A borosilicate glass pipette (tip resistance of 3–6 MΩ) was filled with internal solutions containing 130 mM K-gluconate, 10 mM KCl, 1 mM MgCl₂, 10 mM Hepes, 4 mM Mg-ATP, and 0.5 mM Na-GTP at an osmolarity of 290 mOsm (pH 7.25). Photocurrents were recorded in voltage-clamp mode with membrane potential held at ~70 mV, and photovoltaged spikes were recorded in current-clamp mode with a holding potential at around ~55 mV. Cholinergic ChR2-YFP-expressing cells were identified by YFP expression, and cells were photostimulated by 470-nm, 5-msec pulses at 5 Hz for 2 s. Data were acquired with an Axon Multiclamp 700B amplifier and a Digidata 1440 digitizer (Molecular Devices). Data were analyzed using Axon Clampfit. Recordings with access resistance greater than 25 MΩ or with changes in access resistance greater than 15% were discarded.

**Histology.** At the end of all experiments, the optical fiber positions were verified by postmortem histological analyses. Animals were perfused transcardially with PBS, followed by 10% (vol/vol) neutral buffered formalin. The brains were postfixed in 10% neutral buffered formalin overnight. Brains were sliced at 50 μm using a Leica VT1000 S vibratome (Leica Microsystems). Confirmation of specific expression in the targeted neuronal population was done with immunohistochemical staining for ChAT (goat anti-ChAT, 1:200 dilution, catalog no. AB144P; Millipore), a marker of cholinergic neurons (Fig. 1), and ChR2-YFP as marked by YFP colocalization (rabbit anti-GFP Alexa Fluor 488 conjugate, 1:400 dilution, catalog no. A21311; Invitrogen). The secondary antibody was donkey anti-goat conjugated to Alexa 568 (1:200 dilution, catalog no. A21432; Invitrogen). High-resolution images were taken with a Zeiss 710 laser scanning confocal fluorescent microscope (Keck Imaging Facility, Whitehead Institute, Massachusetts Institute of Technology). The expression of ChR2-YFP in cholinergic neurons in the ChAT-ChR2-YFP line 6 strain has been well characterized in many other brain regions and is consistent between animals (1). To document expression of ChR2-YFP in cholinergic neurons of the PPT and LDT of this mouse strain, we counted multiple 50-μm sections spanning the entire PPT and LDT of three animals using the cell counter plug-in of Fiji/ImageJ (Image Processing and Analysis in Java) (5). To identify fiber placement, images were taken with a Zeiss Axio Imager M2 fluorescence microscope. Confirmation of optical fiber placement in the correct brain region was done by comparing images with the mouse brain atlas (6).

**Data Analysis.**

**Sleep scoring.** Wakefulness, NREM sleep, and REM sleep were scored visually in 2-s epochs using Spike2 (CED), following standard mouse sleep scoring parameters (7), by the same experienced nonblinded scorer. Briefly, wake was classified by activated EEG and muscle tone. NREM sleep was classified by high delta power (0.5–4 Hz) in the EEG and decreased muscle tone. REM sleep was classified by high theta (5–9 Hz) in the EEG and no muscle tone. A videotape was used to help classify any epochs that were not clear from the EEG and EMG.

**Making inferences by constructing 99% bootstrap CIs.** To assess the significance of the differences between groups, we used a bootstrap procedure to construct CI data. The bootstrap is especially useful in situations like ours, in which empirical distributions can be easily constructed and used to draw random (bootstrap) samples. The bootstrap procedure is carried out by drawing repeated random samples from the empirical distribution, estimating the quantity of interest from each sample, and thus constructing its bootstrap distribution, which is the Monte Carlo estimate of the probability density of the quantity of interest. Statistical inferences can be made about this quantity of interest because the bootstrap distribution is an estimate of the true distribution of the quantity of interest.

For example, if the quantity of interest is the difference in REM sleep time between the ChAT-ChR2⁺ and ChAT-ChR2⁻ mice, the bootstrap distribution provides an estimate of the probability density of this difference. The 99% CI for the true value of this difference can be constructed by taking the 0.5th and the 99.5th percentiles of the bootstrap distribution. We report 99% CIs to conduct our inferences because, unlike P values, the CIs estimate the quantity of interest and its associated uncertainty. In contrast, the P value only provides an assessment of how likely the observed quantity of interest is to have occurred by chance if the assumed null hypothesis is true. As such, the P value answers a binary question. It is well known that a 95% CI can be used to conduct a test of the null hypothesis against a two-sided alternative at the 5% level of significance. The decision rule is to reject the null hypothesis if zero is not in the 95% CI of the difference or with the Bonferroni correction 99% (1 to 0.05/5) based on comparing five variables: NREM sleep, REM sleep, and wake time within the stimulation; REM sleep episode duration; and percentage of REM sleep induced. Furthermore, if zero lies outside the CI, the magnitude of the difference between the two groups can be appreciated by assessing the distance between zero and the bounds of the CIs; the greater the distance, the greater is the effect.

Table 2 shows the difference of the means, CI of the differences, and resulting significance for all data with statistical inferences.

**Bootstrap procedure.**

i) Draw n samples with replacement from X_{ChAT⁺} (1), . . . , X_{ChAT⁺}(n).

ii) Draw m samples with replacement from X_{ChAT⁻} (1), . . . , X_{ChAT⁻}(m).

iii) Compute the mean of n values (ξ) and the mean of m values (η).

iv) Save ξ in the bootstrap vector ̃B_{ChAT⁺}, and save η in the bootstrap vector ̃B_{ChAT⁻}.

v) Compute B_{ChAT⁺} − ̃B_{ChAT⁻} = d_{ChAT⁺} − ̃d_{ChAT⁻}.

vi) Repeat steps 1 through 5 for a total of 10,000 times.

vii) Sort in ascending order the bootstrap vectors ̃B_{ChAT⁺}, ̃B_{ChAT⁻}, and d_{ChAT⁺} − ̃d_{ChAT⁻}.

viii) Index the 50th and 9,950th values of each group to get the lower 99% confidence bound and upper 99% confidence bound, respectively.

**Spectrogram.** The spectrogram of the EEG was calculated using the mtspecgram function of Chronux, an open-source MATLAB.
(MathWorks) software package for analyzing neural data (chronux.org) (8), using a moving 2.0-s window and a 0.1-s step size. A time-frequency spectrum was generated with a half-bandwidth of 1 Hz, a frequency band from 0 to 25 Hz, and three tapers. The spectrum values were converted to decibels.

**Probability of REM sleep.** The probability of REM sleep was calculated by marking each 2-s state epoch with a 1 for REM sleep or a 0 for NREM sleep and wake. Two hundred forty seconds (120 epochs) starting at the beginning of the stimulation were analyzed. This binary vector was arrayed in an $n$ by 120 matrix, where 120 was the number of 2-s epochs and $n$ was the total number of stimulations. The binary data from each epoch (column) were pooled from all $n$ stimulations to create a binary time series. The probability of REM sleep was calculated by finding the mean and lower and upper 95% confidence bounds using a hidden Markov model state-space smoothing algorithm (9). This method has the advantage of using the entire dataset to calculate CIs so that there are not multiple comparisons. The probability of REM sleep is significantly different if the 95% CIs do not overlap.

**Power spectra.** EEG values were normalized using the standard score to compute $z$ values, which are a dimensionless quantity. $z = (x - \bar{x}) / SD$, where $x$ is an EEG value, $\bar{x}$ is the mean of the population, and $SD$ is the standard deviation of the population. The normalized EEG was used to compute the power spectra densities for natural ChR2$^+$ mouse REM sleep and stimulation-induced ChR2$^+$ REM mouse sleep using the Chronux toolbox in MATLAB (8). The multitaper method was used with 20 tapers and a bandwidth of 5 Hz. The Jackknife method in Chronux was used to construct 95% CIs around the power spectra.