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 craniotomies 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. Craniotomies 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 Systems 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/mm^2 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/mm^2 immediately out of the tip and 9.5 mW/mm^2 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-s, 80-s, 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% O_2, 5% CO_2) ice-cold cutting solution containing 105 mM *N*-methyl-D-glucamine, 2.5 mM KCl, 1.24 mM NaH_2PO_4, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 26 mM $NaHCO_3$, 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 NaH_2PO_4, 1.3 mM $MgCl_2$, 2.5 mM $CaCl_2$, 26 mM $NaHCO_3$, 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 $MgCl_2$, 10 mM Hepes, 0.2 mM EGTA, 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 photoevoked 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-ms light 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 fluorescent 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 ($\bar{x}$) and the mean of m values ($\bar{y}$).
iv) Save $\bar{x}$ in the bootstrap vector B_{ChAT+}, and save $\bar{y}$ in the bootstrap vector B_{ChAT-}.
v) Compute $B_{ChAT+} - B_{ChAT-} = d_{ChAT+} - {}_{ChAT-}$.
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 $d_{ChAT+} - {}_{ChAT-}$.
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 mtspecgramc 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.

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