



Optogenetic stimulation of cholinergic brainstem neurons during focal limbic seizures: Effects on cortical physiology

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SUMMARY

Focal temporal lobe seizures often cause impaired cortical function and loss of consciousness. Recent work suggests that the mechanism for depressed cortical function during focal seizures may depend on decreased subcortical cholinergic arousal, which leads to a sleep-like state of cortical slow-wave activity. To test this hypothesis, we sought to directly activate subcortical cholinergic neurons during focal limbic seizures to determine the effects on cortical function. Here we used an optogenetic approach to selectively stimulate cholinergic brainstem neurons in the pedunculopontine tegmental nucleus during focal limbic seizures induced in a lightly anesthetized rat model. We found an increase in cortical gamma activity and a decrease in delta activity in response to cholinergic stimulation. These findings support the mechanistic role of reduced subcortical cholinergic arousal in causing cortical dysfunction during seizures. Through further work, electrical or optogenetic stimulation of subcortical arousal networks may ultimately lead to new treatments aimed at preventing cortical dysfunction during seizures.

KEY WORDS: Epilepsy, Consciousness, Optogenetics, Neurostimulation, Acetylcholine, Sudden unexpected death in epilepsy (SUDEP).

Temporal lobe epilepsy (TLE) is the most common epilepsy of adulthood, with seizures originating from limbic structures, particularly the hippocampus.¹ Consistent with the role of the limbic system in memory and emotions, temporal lobe seizures typically cause transient amnesia and emotional changes. However, an additional, puzzling fea-

ture is loss of consciousness, occurring even when seizures remain confined to the temporal lobe.² Why would focal impairment of the temporal lobe cause loss of consciousness? This longstanding mystery is underscored by the fact that even bilateral removal of the temporal lobes does not impair alertness and behavioral responsiveness.

Imaging and intracranial recordings in patients, as well as previous animal model studies, have led to the “network inhibition” hypothesis, in which temporal lobe seizures impair consciousness via inhibition of subcortical arousal circuits.³ Inhibited arousal, in turn, causes a transition of remote brain areas crucial for maintaining consciousness, particularly frontal and parietal cortices, into a sleep-like mode. Consistent with this hypothesis, focal hippocampal seizures cause slow-wave activity on cortical electroencephalography (EEG) resembling deep sleep.^{2,3} Recent data have further shown that subcortical arousal neurons, including cholinergic neurons in the upper brainstem, are suppressed during focal hippocampal seizures.⁴ Despite these compelling findings, direct evidence for a mechanistic link

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between inhibited arousal and cortical dysfunction during seizures has been lacking. Crucially, the network inhibition hypothesis predicts that stimulating arousal circuits during focal hippocampal seizures would reverse cortical slowing and restore fast cortical activity. Here, we used recent advances in optogenetics^{5,6} to test this prediction, through selective optostimulation of cholinergic neurons of the pedunculopontine tegmental nucleus (PPT).

METHODS

Animals

All experimental protocols for this study were approved by the institutional animal care and use committee and conducted in accordance with the U.S. Public Health Service's Policy on Humane Care and Use of Laboratory Animals. Male or female rats were used and were 4–16 weeks old at the time of viral injections. Rats expressing the P1 bacteriophage gene produce causing recombination (Cre)-recombinase specifically in cholinergic neurons⁵ were bred by mating choline-acetyltransferase (ChAT)-Cre positive breeders to adult wild-type, Long-Evans rats (Charles River Laboratories). Pups were genotyped using genomic polymerase chain reaction (PCR; Transnetyx, Inc.).

Channelrhodopsin-2 (ChR2) vector injections

To generate cell-type specific expression of ChR2 in cholinergic neurons, we used a Cre-lox approach, by injecting a Cre-inducible recombinant adeno-associated virus (AAV) vector containing ChR2 (pAAV-Ef1 α -DIO-hChR2 (H134R)-EYFP-WPRE-pA) into rats expressing Cre-recombinase in ChAT-positive neurons (Fig. 1A).⁵ This viral construct carries an inverted ChR2 fused to the fluorescent marker enhanced yellow fluorescent protein (EYFP), flanked by a pair of canonical loxP sites (loxP) and a pair of mutated loxP sites (lox2272). In the presence of Cre, ChR2-EYFP is inverted into the sense direction and expressed from the EF1- α promoter.^{6,7} AAV particles of serotype 5 were produced by the Vector Core Facility at The University of North Carolina at Chapel Hill.

Rats were deeply anesthetized using ketamine/xylazine (90/15 mg/kg, i.m.), and virus was delivered with a Quintessential Stereotaxic Injector (53311; Stoelting) via a pulled glass micropipette (21-175B; Fisher Scientific) lowered through a small durotomy to 6.2 mm below the cortical surface. A bolus of 0.8 μ l of virus (4×10^{12} viral molecules per ml) was injected into the pedunculopontine tegmental nucleus (PPT; coordinates below) at 0.1 μ l/min. The pipette was held in place for 5 min after the injection before being removed. Animals were maintained for 2–4 weeks to allow ChR2 expression prior to experiments.

Electrophysiology

Animals were anesthetized with ketamine/xylazine (90/15 mg/kg, i.m. for surgical procedures, lightened to 40/

7 mg/kg for seizure induction) and recordings of multiunit activity (MUA) and local field potentials (LFPs) were performed as described previously.^{4,8,9} Briefly, a bipolar LFP electrode was placed in the dorsal hippocampus (anteroposterior [AP] -3.8 ; mediolateral [ML] 2.5 ; superior-inferior [SI] 2.6) and a high-impedance MUA/LFP microelectrode placed in orbitofrontal cortex (AP 4.2 ; ML 2.2 ; SI 3.7) or in PPT (AP -7.8 ; ML 2.0 ; SI 7.0) (coordinates in mm relative to Bregma¹⁰). Recordings were filtered to obtain LFP (0.1 – 100 Hz) and MUA (0.4 – 10 kHz), digitized at 1 kHz for LFP and 20 kHz for MUA. For respiratory measurements, a nasal thermistor (BAT-12 Microprobe Thermistor; Physitemp) with a type T thermocouple copper-constantan sensor (RET-3; Physitemp) was placed into the rat's nose.¹¹ Temperature fluctuations associated with inspiration and expiration were used to determine breathing frequency.

Seizures were induced by a 60 Hz, 2 s train of square-wave biphasic pulses (1 msec/phase) to the hippocampus, titrated by approximately 200 μ A steps to the lowest intensity (range 200 – $1,000$ μ A) producing a seizure >30 s in duration.^{4,8,9} Animals were perfused and brains collected to histologically verify all electrode locations as previously described.¹²

Light stimulation

Light stimulation was delivered by a 473 nm laser (OptoEngine, MBL-III-473) with computer-controlled pulses; 20 – 80 mW/mm² was delivered via a 200 μ m diameter unjacketed optical fiber (M51L01, CFM12L10; Thorlabs) placed in the PPT (AP -7.8 ; ML 2.0 ; SI 7.0). For initial calibration experiments (Fig. S1), light pulse duration and frequency varied. For seizure experiments, light pulses were given at 40 msec at 10 Hz, with 10 s train duration, and stimulation started 10 s after seizure onset.

Immunohistochemistry

One hundred micrometer sections (Leica VT1000S Vibratome) were incubated overnight at room temperature in 5% donkey serum and phosphate-buffered saline (PBS) with 0.3% Triton X-100 with anti-Chat primary antibody (AP144, $1:250$; Millipore) and anti-YFP (A21311, $1:1,000$; Life Technologies), washed and then incubated for 2 h at room temperature in secondary antibody (A11058, $1:500$; Life Technologies), and washed and then mounted on glass slides with Vectashield with the nuclear stain 4',6-diamidino-2-phenylindole (DAPI) (H-1200; Vector Laboratories). ChR2-yellow fluorescent protein (YFP) expression was scored by hand through examination of every 100 μ m coronal section ($n = 5$ rats) for quantification of co-labeling of ChR2-YFP and ChAT using a Zeiss AxioImager Z2 microscope (Carl Zeiss).

Data and statistical analysis

LFP power was analyzed in 10 s epochs before and after effects of optogenetic stimulation. Analysis windows were

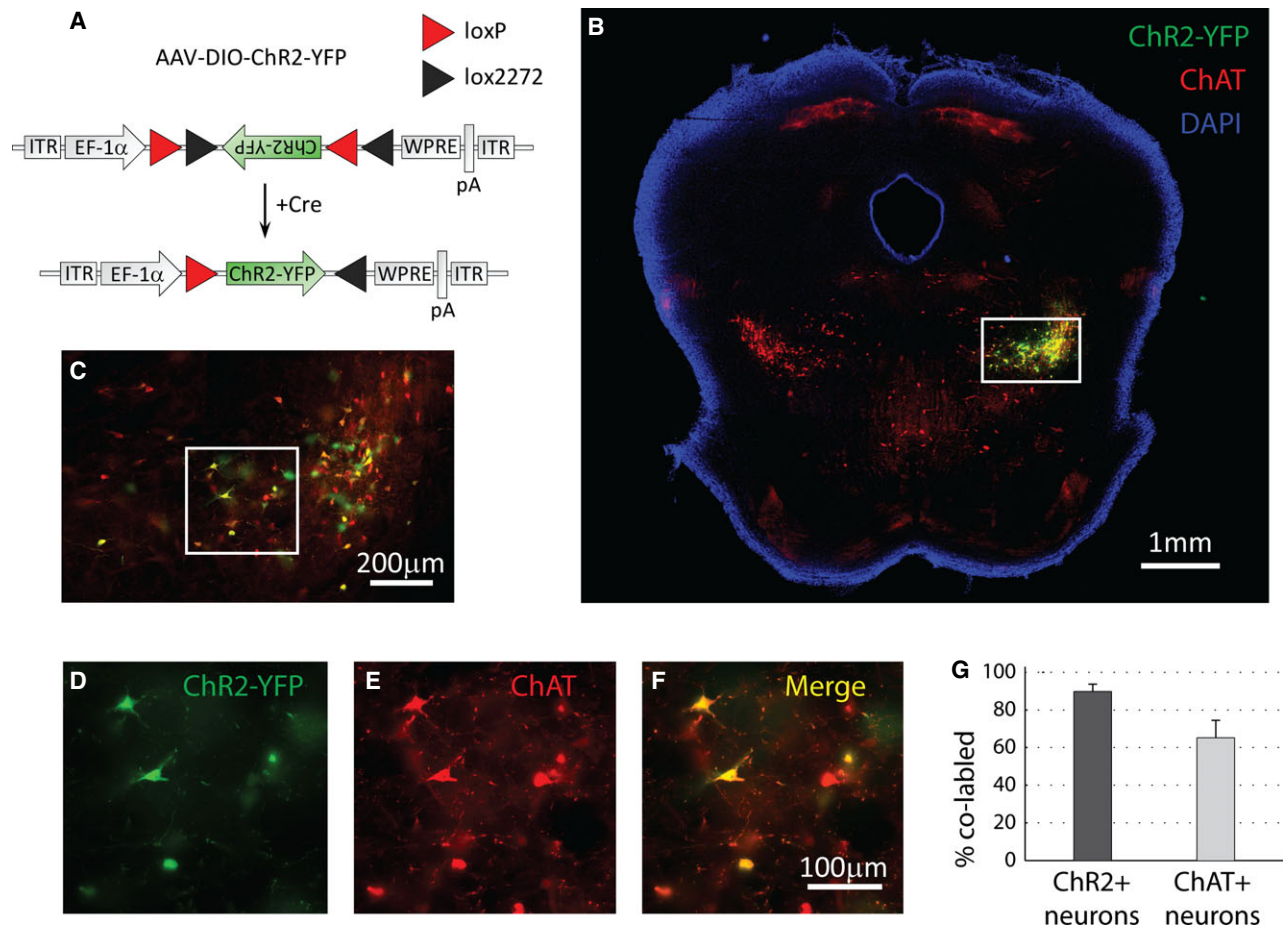


Figure 1.

Selective expression of channelrhodopsin-2 (ChR2) in cholinergic brainstem neurons. **(A)** We used a Cre-loxP technology to deliver the light-activated protein ChR2 into the pedunculopontine tegmental nucleus (PPT) of rats. We injected an adeno-associated virus (AAV) containing ChR2 and yellow-fluorescent protein (YFP) into ChAT-Cre transgenic rats expressing Cre-recombinase specifically in cholinergic neurons. In the presence of Cre-recombinase, ChR2 and YFP are flipped from their antisense encoding into the functional right-sense direction and then expressed under the control of the strong promoter EF-1 α . ITR, inverted terminal repeat; pA, poly(A); WPRE, woodchuck hepatitis B virus posttranscriptional element; DIO, double-floxed inverted open reading frame. **(B–F)** Coronal brainstem slice (approximately -7.8 mm relative to bregma) 21 days after targeted virus injection into the right-side PPT. Box in **B** shows area enlarged for **C**, and box in **C** shows area enlarged for **D–F**. Correct targeting of ChR2-YFP is confirmed by the overlap (yellow) between virus expression (ChR2-YFP, green) and immunoreactivity for choline-acetyltransferase (ChAT, red), which delineates the PPT and marks cholinergic neurons. The nuclear stain DAPI was used to highlight the slice margins (**B**). **(G)** Quantification at a cellular level indicates that ChR2-YFP was selectively expressed in ChAT+ neurons ($89.7 \pm 3.9\%$ of ChR2-YFP neurons colabeled with ChAT, $n = 5$). In addition, a large fraction of ChAT+ neurons in the PPT on the injected side of the brain expressed ChR2-YFP ($65.0 \pm 9.5\%$, $n = 5$).

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selected based on the slight delay in the effects of optogenetic stimulation (see Figs. S2 and S3), so that both the baseline and stimulated analysis window were shifted forward in time. Thus we used 5–15 s after seizure onset as baseline, and 18–28 s after seizure onset (8–18 s after optogenetic stimulus onset) as the optogenetic stimulation analysis window. Any seizures exhibiting secondary generalization based on polyspike discharges in the frontal cortical LFP⁹ during the analysis epochs were excluded. Data are presented as mean $\pm$ standard error of the mean (SEM). Changes in LFP power were analyzed in Spike2 and MATLAB with a two-tailed *t*-test significance threshold $p < 0.05$.

RESULTS

Histologic analysis confirmed selective expression of ChR2 in cholinergic neurons of the PPT (Fig. 1). To test the functionality of ChR2 in the PPT, we conducted in vivo recordings from the PPT during optical stimulation and optimized stimulation parameters to maximize ChR2-mediated firing of PPT neurons (Fig. S1). These measurements led us to use a light pulse duration of 40 msec and stimulation at 10 Hz for subsequent experiments. It is notable that we never observed changes in PPT neuronal firing in control optical stimulation experiments with the same

parameters in ChAT-Cre rats, which had been injected with an AAV construct containing YFP but not ChR2 ($n = 4$, data not shown).

Next, we applied our fine-tuned stimulation paradigm to the question at hand, namely, the role of subcortical arousal networks in controlling the cortical state and slow-wave activity during focal limbic seizures induced in an established rat model under light anesthesia.^{4,8,9} We found that

optogenetic stimulation of cholinergic PPT neurons led to a marked reduction in frontal cortical slow-wave activity during limbic seizures (Fig. 2A,B). Specifically, frontal cortical delta power (0.5–4 Hz) was reduced by $18.4 \pm 2.7\%$ in ChR2-YFP experiments, compared to an increase of $12.0 \pm 7.52\%$ in YFP control experiments ($p < 0.001$; Fig. 2C) ($n = 41$ seizures in eight animals for ChR2-YFP experiments; $n = 26$ seizures in four animals for YFP con-

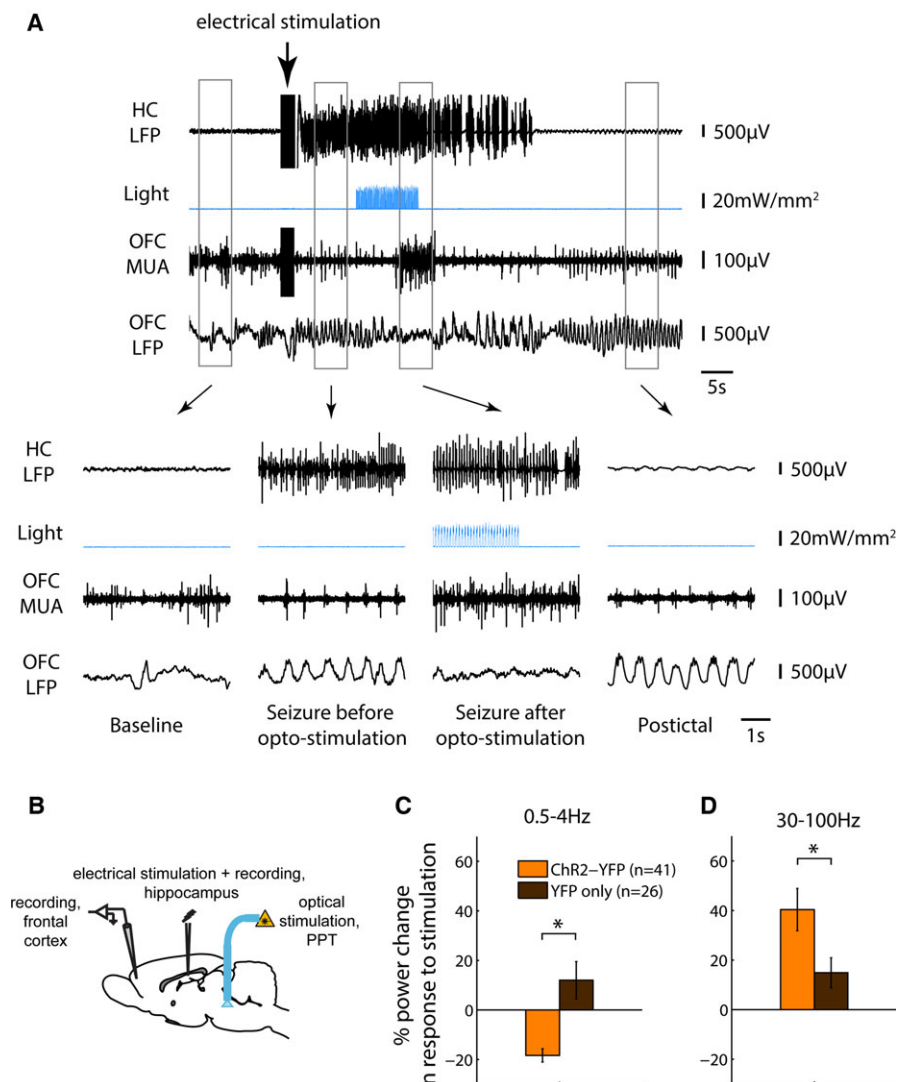


Figure 2.

Optogenetic stimulation of the PPT reduces cortical slow wave activity during seizures. We induced focal seizures by injecting a brief (2 s) electric current via a bipolar electrode implanted into the hippocampus (see part **B** for schematic). The same electrode was used for recording hippocampal local field potential (LFP). Ten seconds after seizure onset, we delivered a 10 s-long train of blue light pulses (473 nm; pulse duration 40 msec, frequency 10 Hz) into the PPT. We also recorded LFP and multiunit activity from the orbitofrontal cortex (OFC). In control seizures (not shown), in which YFP but no ChR2 had been injected into the PPT, cortical slow wave activity persisted throughout the seizure as described previously.^{8,9} (**A**) Optogenetic stimulation of the PPT following ChR2 injection resulted in a transition of the cortical LFP from slow waves to low-voltage fast activity. (**B**) Schematic of seizure experimental setup. (**C, D**) Changes in cortical LFP delta (0.5–4 Hz) and gamma (30–100 Hz) power in response to optogenetic stimulation. LFP power was quantified shortly after seizure onset and following optogenetic stimulation (5–15 and 18–28 s after seizure onset, respectively), and the percent change in power between the two time windows was averaged across seizures. * $p < 0.05$.

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trols). At the same time, frontal cortical gamma power (30–100 Hz), a marker of cortical arousal¹³ was enhanced by $40.4 \pm 8.52\%$ in ChR2-YFP experiments compared to $14.8 \pm 6.1\%$ in YFP control experiments ($p < 0.033$; Fig. 2D). Changes in cortical physiology occurred with a slight delay after onset of optogenetic stimulation in PPT (Fig. 2A,B; see also Figs. S2 and S3). Of interest breathing frequency, which may provide another marker for increased arousal, also increased in response to optogenetic stimulation in ChR2-injected animals ($9.0 \pm 2.4\%$) compared to YFP-injected controls ($0.2 \pm 1.9\%$; $p = 0.01$).

DISCUSSION

Impaired consciousness during focal temporal lobe seizures has long puzzled clinicians and basic researchers, given that the temporal lobe per se is not required for maintaining consciousness. Physiologically, impaired consciousness is characterized by prominent slow-wave activity in the frontoparietal cortical network.² Herein we demonstrate that optogenetic stimulation of cholinergic neurons in the upper brainstem during focal hippocampal seizures in rats reduces cortical slow-wave activity. These findings provide the first direct mechanistic link between suppressed subcortical arousal during seizures and neocortical dysfunction as observed both in patients and in animal models of TLE.³

We focused our experiments on the cholinergic system because of the following: (1) its well established role in arousal¹⁴; (2) previous data from our lab specifically implicating the cholinergic system during seizures⁴; and (3) the availability of genetic-molecular tools to manipulate cholinergic neurons in the rat.⁵ It is important to note that, many other neurotransmitter systems play essential roles in arousal¹⁵ and are likely modulated during seizures as well. The relative contribution of these neurotransmitter systems to impaired behavioral responsiveness during seizures remains to be determined.

Loss of consciousness increases the risk of injury in patients with epilepsy, and limits their ability to engage in normal activities such as driving. In cases when seizures cannot be fully controlled by medication or surgery, treatments to reduce the severity of seizures and to prevent loss of consciousness may offer a promising avenue to ameliorate the quality of life of patients. Whereas optogenetic stimulation remains purely a research tool for investigating brain circuits in animal models, other clinical treatments, particularly electrical deep brain stimulation, may be applied for restoring consciousness during seizures based on better understanding of the underlying networks.

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DISCLOSURE

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

REFERENCES

1. Williamson PD, Thadani VM, French JA, et al. Medial temporal lobe epilepsy: videotape analysis of objective clinical seizure characteristics. *Epilepsia* 1998;39:1182–1188.
2. Englot DJ, Yang L, Hamid H, et al. Impaired consciousness in temporal lobe seizures: role of cortical slow activity. *Brain* 2010;133:3764–3777.
3. Blumenfeld H. Impaired consciousness in epilepsy. *Lancet Neurol* 2012;11:814–826.
4. Motelow JE, Li W, Zhan Q, et al. Decreased subcortical cholinergic arousal in focal seizures. *Neuron* 2015;85:561–572.
5. Witten IB, Steinberg EE, Lee SY, et al. Recombinase-driver rat lines: tools, techniques, and optogenetic application to dopamine-mediated reinforcement. *Neuron* 2011;72:721–733.
6. Cardin JA, Carlen M, Meletis K, et al. Driving fast-spiking cells induces gamma rhythm and controls sensory responses. *Nature* 2009;459:663–667.
7. Atasoy D, Aponte Y, Su HH, et al. A FLEX switch targets Channelrhodopsin-2 to multiple cell types for imaging and long-range circuit mapping. *J Neurosci* 2008;28:7025–7030.
8. Englot DJ, Modi B, Mishra AM, et al. Cortical deactivation induced by subcortical network dysfunction in limbic seizures. *J Neurosci* 2009;29:13006–13018.
9. Englot DJ, Mishra AM, Mansuripur PK, et al. Remote effects of focal hippocampal seizures on the rat neocortex. *J Neurosci* 2008;28:9066–9081.
10. Paxinos G, Watson C. *The rat brain in stereotaxic coordinates*. San Diego, CA: Academic Press; 1998.
11. Wesson DW. Sniffing behavior communicates social hierarchy. *Curr Biol* 2013;23:575–580.
12. Gummadavelli A, Motelow JE, Smith N, et al. Thalamic stimulation to improve level of consciousness after seizures: evaluation of electrophysiology and behavior. *Epilepsia* 2015;56:114–124.
13. Cape EG, Jones BE. Differential modulation of high-frequency gamma-electroencephalogram activity and sleep-wake state by noradrenaline and serotonin microinjections into the region of cholinergic basal ganglia neurons. *J Neurosci* 1998;18:2653–2666.
14. Jones BE. Modulation of cortical activation and behavioral arousal by cholinergic and orexinergic systems. *Ann N Y Acad Sci* 2008;1129:26–34.
15. Saper CB, Scammell TE, Lu J. Hypothalamic regulation of sleep and circadian rhythms. *Nature* 2005;437:1257–1263.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Optimization of neural responses in the PPT to light-pulse trains of varying frequency and pulse duration.

Figure S2. Peak changes in cortical LFP power occur with a delay after onset of optogenetic stimulation of the PPT.

Figure S3. Spectrograms showing contrasting changes in 0.5–4 and 30–100 Hz frequency bands of cortical LFP power after optogenetic stimulation of the PPT.