Supporting Information

Carter et al. 10.1073/pnas.1202526109

SI Methods

Immunohistochemistry. For brightfield immunohistochemistry experiments (Fig. 1 *B* and *C*), free-floating sections were treated with 1% (vol/vol) hydrogen peroxide H_2O_2 in PBS to inactivate endogenous peroxidase activity. Nonspecific antigens were blocked by incubating sections in PBS containing 4% BSA and 0.1% Triton X-100 for 2 h at room temperature. Sections were then incubated overnight at 4 °C with either rabbit anti-orexin A (C-19) antibody (1:500; sc-8070; Santa Cruz Biotechnology) or chicken anti-tyrosine hydroxylase antibody [1:1,000; tyrosine hydroxylase (TH), batch TH1205; Aves Laboratories]. After 4 × 10-min washes in PBS with 0.1% Triton X-100 (PBST), sections were incubated for 2 h in blocking buffer with biotinylated anti-goat or anti-chicken IgG secondary antibodies (1:200 dilution with ABC Vectastain Elite kit; Vector Laboratories), respectively. After 4 × 10-min washes in PBST, sections were incubated in an avidin–biotin–horseradish peroxidase solution (ABC Vectastain Elite kit; Vector Laboratories) for 1 h. Hcrt-immunoreactive fibers were dyed in black/gray with the 3,3′-diaminobenzidine (DAB)-nickel enhanced technique: Sections were incubated in Tris-buffered solution (TBS, pH 7.5) containing 0.04% DAB (Sigma) and 0.2% ammonium nickel sulfate (Sigma) supplemented with H_2O_2 every 4 min to obtain increasing H_2O_2 concentrations (0.00015%, 0.0003%, 0.0006%, 0.0012%, 0.0024%, and 0.0048%). TH-immunoreactive neurons were dyed in brown by incubations in TBS with 0.04% DAB and increasing concentrations of H_2O_2 as before. All DAB reactions were stopped by three 10-min rinses in Tris·HCl (0.125 M, pH 7.5) followed with 3 × 10-min washes in TBS. Sections were mounted onto SuperFrost slides (Fischer Scientific). After dehydration in increasing concentrations of ethanol, slides were cleared in xylene and coverslipped with DPX mounting medium (BDH Chemicals).

For double-labeled immunofluorescent experiments (Fig. 1*D* and Fig. S2*A*) brain sections were washed in PBST for 10 min at room temperature and incubated in block solution for 1 h at room temperature. For primary antibody exposure, sections were incubated in chicken anti-tyrosine hydroxylase (1:2,000; TH; Aves Laboratories) and rabbit anti-c-Fos (1:5,000; PC05; Calbiochem) (Fig. 1*D* and Fig. S2*A*) in block solution at 4 °C for ∼16 h. After 3 × 10-min washes in PBST, sections were incubated in Alexa Fluor 568 goat anti-chicken IgG (1:500; Molecular Probes; A-11041) and either Alexa Fluor 488 goat anti-rabbit IgG (1:500; Molecular Probes; A-11008) (Fig. 1*D*) or Alexa Fluor 350 goat anti-rabbit IgG (1:500; Molecular Probes; A21068) (Fig. S2*A*) in block solution for 1 h at room temperature. Sections were washed three times in PBS, mounted onto glass slides, and coverslipped with Vectashield with DAPI Mounting Media (Vector Laboratories; H-1200).

Quantification of colocalization (Fig. 1*F* and Fig. S2*B*) was performed on alternating sections from approximately bregma –5.20 to –5.80 (exactly 21 sections per mouse). TH-positive cells were scored for the presence of c-Fos by an investigator (M.E.C.) blind to the side of stimulation and identity of the virally transduced mouse (*Hcrt::ChR2-mCherry* or *Hcrt::mCherry*). c-Fos expression was recorded as being coincident with TH expression only if c-Fos immunofluorescence was expressed in the nucleus of an immunofluorescent TH neuron.

Microscopy. Images were collected on a Carl Zeiss fluorescent microscope, using fluorescent reflected light. Digital images were minimally processed using Adobe Photoshop CS5 (Adobe Systems) in the same way between experimental conditions to avoid artificial manipulation between different datasets.

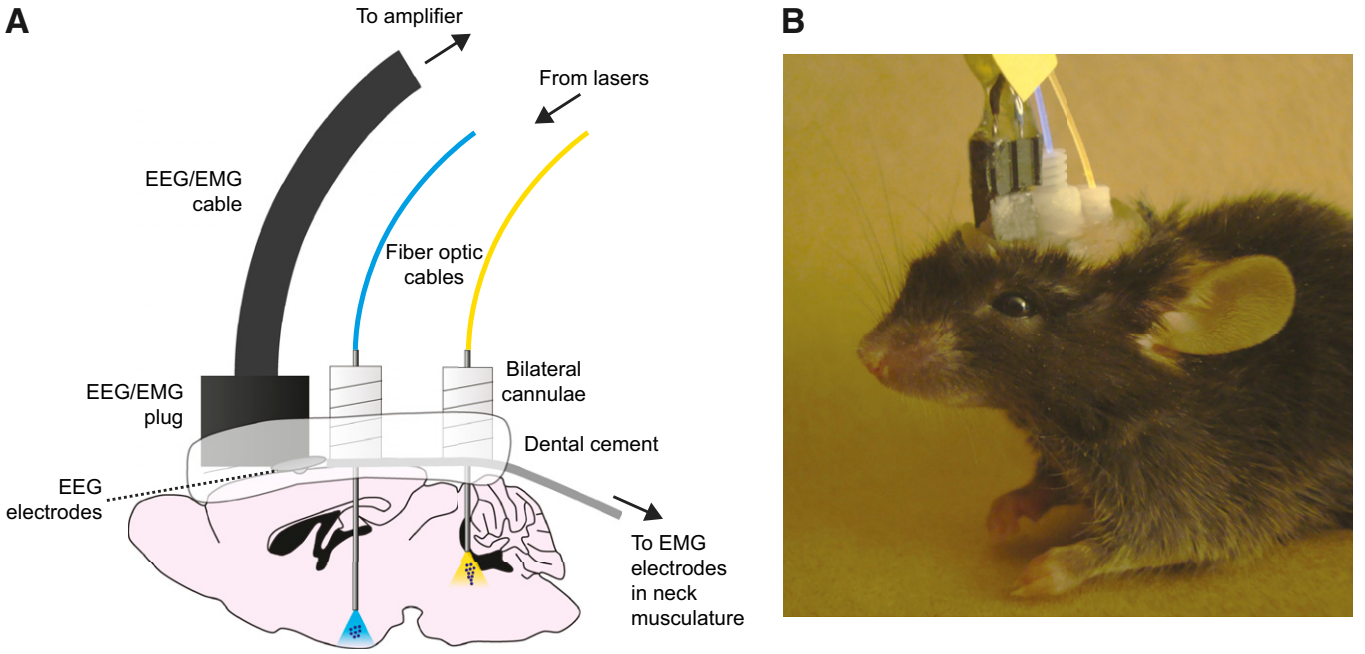


Fig. S1. Surgical implantation of cannulae and electroencephalographic/electromyographic (EEG/EMG) electrodes for in vivo light delivery and recordings. (*A*) Diagram depicting surgical implants. A bilateral cannula was placed above the locus coeruleus (LC) [anteroposterior (AP), −5.45 mm; mediolateral (ML), ±1.0 mm; dorsoventral (DV), 3.0 mm]. A second bilateral cannula was placed above the Hypocretin (Hcrt) field (AP, −1.6 mm; ML, ±0.75 mm; DV, 4.5 mm). EEG electrodes were placed on the skull above the frontal lobes, and EMG electrodes were placed within the neck musculature. (*B*) Photograph of a typical individual following surgery and implantation of fiber-optic cables.

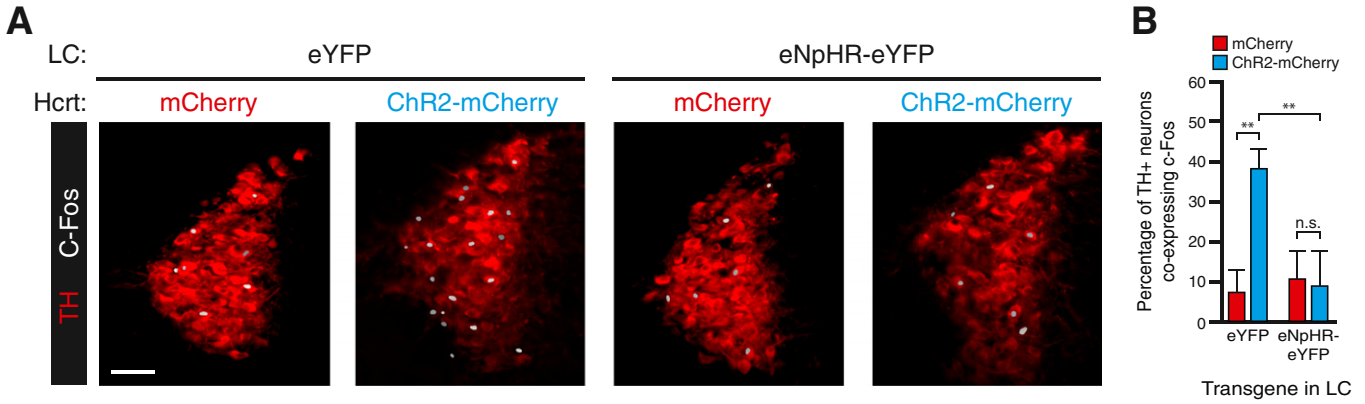
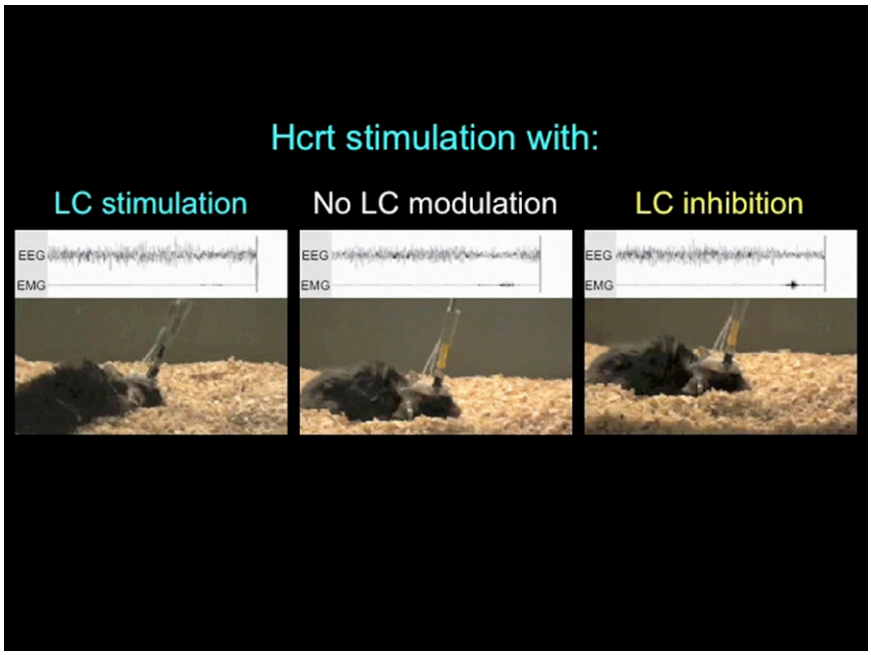
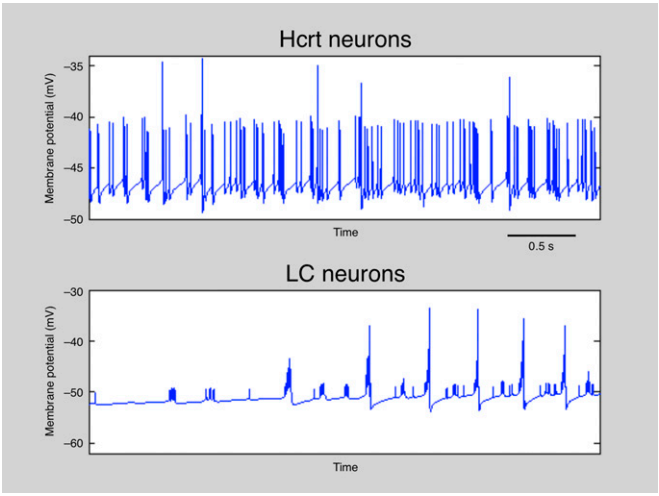


Fig. S2. Simultaneous inhibition of LC neurons blocks the increase in c-Fos immunoreactivity in the LC following unilateral stimulation of Hcrt neurons. (*A*) Representative images of the ipsilateral LC costained for TH (red) and c-Fos (white). (Scale bar, 25 μm.) (*B*) Quantification of the percentage of neurons showing TH immunoreactivity that also express c-Fos. Data represent the mean ± SD. $^{**}P < 0.001$, two-way ANOVA followed by Tukey's posthoc test.



Movie S1. Representative movie showing Hcrt stimulation with simultaneous LC modulation. All movies show *Hcrt::ChR2-mCherry* animals stimulated with 10 s blue light (15-ms pulses at 10 Hz) starting 15 s after the onset of NREM sleep. Leftmost movie shows LC stimulation with a single 10-ms pulse of blue light delivered to *ChR2(C128S)-eYFP*–transduced LC neurons 5 s before Hcrt stimulation (representative of Fig. 5*B*). Center movie shows no LC modulation. Rightmost movie shows bilateral LC inhibition with 10 s constant yellow light concurrent with Hcrt stimulation in *eNpHR(3.0)-eYFP*–transduced LC neurons (representative of Fig. 2*B*).

Movie S1



Movie S2. Model of the effect of Hcrt stimulation on LC membrane potential. Shown is computational simulation of the average activity of Hcrt and LC neurons before, during, and after 10-s stimulation of Hcrt neurons with ChR2. The LC neurons follow Hcrt neurons due to the slow depolarization caused by Hcrt release. The model presents the activity of 10 Hcrt neurons and 30 LC neurons and represents the same simulation as Fig. 6*B*.

Movie S2