Hypothalamic feedforward inhibition of thalamocortical network controls arousal and consciousness

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During non-rapid eye movement (NREM) sleep, synchronous synaptic activity in the thalamocortical network generates predominantly low-frequency oscillations (<4 Hz) that are modulated by inhibitory inputs from the thalamic reticular nucleus (TRN). Whether TRN cells integrate sleep-wake signals from subcortical circuits remains unclear. We found that GABA neurons from the lateral hypothalamus (LHGABA) exert a strong inhibitory control over TRN GABA neurons (TRNGABA). We found that optogenetic activation of this circuit recapitulated state-dependent changes of TRN neuron activity in behaving mice and induced rapid arousal during NREM, but not REM, sleep. During deep anesthesia, activation of this circuit induced sustained cortical arousal. In contrast, optogenetic silencing of LHGABA–TRNGABA transmission increased the duration of NREM sleep and amplitude of delta (1–4 Hz) oscillations. Collectively, these results demonstrate that TRN cells integrate subcortical arousal inputs selectively during NREM sleep and may participate in sleep intensity.

In mammals, during NREM sleep, electroencephalogram (EEG) activity shows typical signs of brain activity that include a predominant slow wave (<1 Hz) associated with delta oscillations (1–4 Hz) and spindles (11–15 Hz)1–3. Slow-wave oscillations reflect the slow variation of the resting membrane potential of cortical neurons that switches between depolarized (UP) and hyperpolarized (DOWN) states4. During the UP state, thalamic (TC) and corticothalamic (CT) cells show intense synaptic activity and burst firing, whereas, during the DOWN state, their progressive hyperpolarization induces a period of relative quiescence. Slow-wave oscillations have a cortical origin and, presumably, coordinate other sleep rhythms, including spindles and delta oscillations, into a coherent rhythmic sequence of cortico-thalamo-cortical rhythm1,4,6.

Changes in thalamocortical cell firing are largely controlled by inhibitory TRN cells and correlate with spindles, delta waves and spindles during sleep. Inhibitory postsynaptic currents (IPSCs) and synaptic activity drive the transition between UP and DOWN states4. During the UP state, thalamocortical (TC) and corticothalamic (CT) cells show intense synaptic activity and burst firing, whereas, during the DOWN state, their progressive hyperpolarization induces a period of relative quiescence. Slow-wave oscillations have a cortical origin and, presumably, coordinate other sleep rhythms, including spindles and delta oscillations, into a coherent rhythmic sequence of cortico-thalamo-cortical rhythm1,4,6.

RESULTS

Activation of LHGABA neurons induces rapid wakefulness

First, we investigated the role of LHGABA neurons in sleep-wake control in light of their heterogeneous activity and discharge profile during those states. Behavioral consequences of activation of LHGABA neurons during sleep states were studied using optogenetics combined with electrophysiological recordings in freely moving animals (Online Methods). To genetically target GABA neurons in the LH area, we stereotactically injected an adeno-associated virus (AAV) carrying a Cre-inducible vector encoding either the light-activatable channelrhodopsin-2 variant ChETA in-frame fused to enhanced yellow fluorescent protein (ChETA-EYFP) or EYFP (control) in the

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LH of Tg(VGAT)::IRES-Cre transgenic mice (mice expressing the Cre recombinase in cells expressing the vesicular GABA transporter (VGat, also known as Slc32a1) gene) (Fig. 1a). Consistent with other studies3,33, we found that this method allowed selective targeting of LHGABA neuron cell bodies (97.4 ± 0.5% of GAD+/YFP+; 2.2 ± 0.5% of YFP+/GAD– cells (N = 3 sections per animal, 12 animals, F(2,49) = 99.89 P = 0.0001) and REM (right) (1 Hz, N = 8 ChETA, N = 10 YFP, t = 0.213, df = 6, P = 0.173; 20 Hz, N = 8 ChETA, N = 8 YFP, t = 4.36, df = 4.04, P = 0.039; 1 s, N = 3 ChETA, N = 5 YFP, t = 0.0838, df = 8, P = 0.8384). Data analysis was based on >15 stimulations per frequency and per animal (N). **P < 0.001, ***P < 0.0001, unpaired two-tailed Student’s t test.

We found that firing rates and variations of ISI of LHGABA cells did not differ between NREM sleep and wakefulness epochs (6.71 ± 1.17 Hz and 5.78 ± 0.93 Hz, respectively, P = 0.20; coefficient of variation, CV: 0.83 ± 0.05 versus 1.00 ± 0.07; P = 0.10; N = 22 cells, 3 mice). However, LHGABA cells showed a transient increase in firing rate during the first 3 s after NREM sleep-to-wake transitions (11 of 13 cells, 3 mice, P = 0.02, binomial test; Supplementary Fig. 3c), but not during subsequent waking epochs, suggesting that the increase of LHGABA cell firing is transient and limited to the behavioral transition. In accordance with this finding, we found that optical stimulation during NREM sleep induced a rapid (<3 s) transition to wakefulness in ChETA compared with control mice, as revealed by the rapid change in cortical activity and muscle tone (Fig. 1h,i). The latency of behavioral transitions was highly dependent on the frequency of stimulation, with 20-Hz and 1-s continuous illumination inducing the fastest transitions (20-Hz YFP:6.38 ± 6.3 s, N = 11; 20-Hz ChETA: 2.94 ± 0.4 s, N = 9; t = 8.91, df = 18, P = 0.0001; 1-s YFP: 61.0 ± 4.4 s, N = 4; 1-s ChETA: 3.6 ± 0.30 s, N = 3; t = 11, df = 5, P = 0.0001; Fig. 1i). Notably, the behavioral response was selective for NREM sleep state, as activation of LHGABA neurons during REM sleep had no effect (20-Hz YFP: 61.3 ± 3.9 s, N = 8; 20-Hz ChETA: 67.7 ± 4.5 s, N = 8; t = 1.06, df = 14, P = 0.3093; 1-s YFP: 69.0 ± 5.02 s, N = 3; 1-s ChETA: 71.4 ± 12.8 s, N = 3; t = 0.213, df = 6, P = 0.8384, unpaired two-tailed Student’s t test; Fig. 1i). Sustained activation of LHGABA cells using a semi-chronic optical stimulation procedure (5-ms light pulses delivered at 20 Hz for 10 s, every minute for 1 h; Supplementary Fig. 4a,b) of LHGABA neurons systematically increased the number of arousal events.
In an effort to identify a functional subset of LH GABA cells mediating this arousal effect, we optogenetically stimulated LH GABA cells that express the long form of the leptin receptor b (LepRb)34. This experimentally identified a distinct subset of LH GABA cells induces rapid arousal from NREM sleep and causes a prolonged period of wakefulness.

**TRN cells receive monosynaptic inputs from LHGABA neurons**

To determine the downstream circuitries mediating this rapid arousal response, we mapped LHGABA efferents utilizing Chr2-assisted circuit mapping after stereotactic delivery of ChETA-EYFP AAV in the LH of Tg(VGAT)::IRES-Cre mice. We found that LHGABA neurons send projections to various hypothalamic nuclei and long-range projections to sleep-relevant brain nuclei, including the TRN, dorsomedial thalamus, locus coeruleus, neocortex and septum (Fig. 2a and Supplementary Fig. 1a). To our surprise, LHGABA cells projected monosynaptically to the TRN. Representative images are from repeated experiment (>15 sections from N = 4 transduced mice). Scale bars represent 500 µm (left) and 20 µm (right). Genetic targeting was confirmed in animals including in the behavioral study using immunohistochemistry.

Activation of LHGABA-TRN recapitulates arousal transition

LHGABA include a heterogenous population of both wake- and REM sleep–active neurons31. Thus, we hypothesized that the transient activity of LHGABA-TRN circuit supports a direct hypothalamic control over TRN cells, and indirectly, over thalamocortical network during sleep–wake transitions. To test this, we chronically implanted transduced animals with moveable silicon probes and optical fibers directly above TRN for both simultaneous recordings and optogenetic manipulation of LHGABA terminals (Online Methods, Fig. 3a,b and Supplementary Fig. 7a,b). Consistent with previous studies36, cells recorded in the TRN showed a typical narrow spike waveform (half-trough LT-HSV-EGFP lentivirus (Supplementary Fig. 6a–d) restricted to the TRN area, both of which showed a retrograde labeling of GABA and non–GABA cell bodies in the LH. We then used optogenetics to functionally characterize this LHGABA-TRN circuit. We found that optogenetic activation of ChETA-EYFP–expressing LHGABA terminals faithfully evoked postsynaptic currents (IPSCs, 61.7 ± 12 pA peak amplitude, 111.6 ± 5.7 ms decay time, values represent mean ± s.e.m.) in ~80% of TRN cells recorded (Fig. 2c–f). These currents were bicusculine sensitive (~5.16 ± 0.5-pA peak amplitude), suggesting a major role for GABAergic control from LHGABA neurons and the absence of synaptic short-term plastic changes following optogenetic stimulation.
Optogenetic stimulation of LHGABA-TRN circuit induces rapid arousal. (a) Schematic of the in vivo recording setup. (b) Representative traces showing EEG, EMG and TRN unit recording during the spontaneous NREM sleep-to-wake transition in a freely moving mouse. Note the transient period of TRN cell quiescence preceding behavioral transition (shaded box). EEG, EMG traces and TRN units were recorded during 19 experiments, N = 3 mice. (c) Representative signal trace of a TRN unit (wide band) before and after the onset of optical activation of LHGABA terminals (top). Rastergrams of five representative TRN cells (N = 2 mice, altogether 70 recorded TRN cells, N = 3 mice, middle) and their firing probability (bottom) before and after the optical stimulation. Blue bars show the optical stimulation (473 nm). (d) Average firing rates ± s.e.m. of TRN neurons during baseline and following optical stimulation of LHGABA-TRN projections at different frequencies (1 Hz, N = 17; 20 Hz, N = 57; 2 s continuous illumination, N = 17). Optical stimulation led to a frequency-dependent (P = 9.4 × 10−16; F3,31 = 3.28, one-way ANOVA followed by Bonferroni post hoc test) decrease in the firing rate. *P < 0.05, ***P < 0.0001, Student t tests with α adjusted for multiple comparisons. (e) Coefficient of ISI variation (CV) of TRN cells across NREM sleep and wake states (Kruskal Wallis test, P = 3.52 × 10−4) and following optogenetic activation of LHGABA-TRN circuit at 20 Hz (P = 0.0082). (f) Mean latencies of NREM (left) and REM (right) sleep-to-wake transitions following optogenetic stimulation at 1 Hz (ChETA N = 7, YFP N = 5, t = 5.21, df = 10, P = 0.0004, unpaired two-tailed Student’s t test), 20 Hz (ChETA N = 7, YFP N = 5, t = 13.2, df = 10, P = 0.00001, unpaired two-tailed Student’s t test) or 1-s continuous (ChETA N = 7, YFP N = 5, t = 19.1, df = 10, P = 0.0001, unpaired two-tailed Student’s t test) illumination in control (white) and ChETA-EYFP (black) mice. Data analysis is based on more than ten stimulations per frequency and per animal (N). **P < 0.01, ***P < 0.0001. (g) Mean duration ± s.e.m. of optogenetically induced wake episode following optical stimulation at 1 Hz (ChETA, N = 7, YFP, N = 6, t = 0.253, df = 11, P = 0.804), 20 Hz (ChETA, N = 7, YFP, N = 6, t = 0.827, df = 11, P = 0.4259) and 1-s continuous illumination (N = 7, YFP, N = 6, t = 0.568, df = 11, P = 0.5815 using unpaired two-tailed Student’s t test). n.s., not significant. Error bars represent s.e.m. (h) Mean latencies ± s.e.m. of REM sleep to wake transition following optical at 1-Hz stimulation (ChETA N = 4, YFP N = 5, t = 0.139, df = 10, P = 0.891), 20 Hz (ChETA N = 4, YFP, N = 5, t = 2.03, df = 10, P = 0.069) and 1-s continuous illumination (ChETA, N = 4, YFP, N = 5, t = 1.93, df = 10, P = 0.0786) in control (white) and ChETA-EYFP (black) mice, respectively. Data analysis is based on more than ten stimulations per frequency and per animal (N). **P < 0.01, ***P < 0.0001.

Figure 3: Arousal induced by in vivo optical stimulation of LHGABA-TRN circuit. (f) Representative traces showing EEG, EMG and TRN unit recording during the spontaneous NREM sleep-to-wake transition in a freely moving mouse. (g) Average firing rates ± s.e.m. of TRN neurons during baseline and following optical stimulation of LHGABA-TRN projections at different frequencies (1 Hz, N = 17; 20 Hz, N = 57; 2 s continuous illumination, N = 17). Optical stimulation led to a frequency-dependent (P = 9.4 × 10−16; F3,31 = 3.28, one-way ANOVA followed by Bonferroni post hoc test) decrease in the firing rate. *P < 0.05, ***P < 0.0001, Student t tests with α adjusted for multiple comparisons. (f) Coefficient of ISI variation (CV) of TRN cells across NREM sleep and wake states (Kruskal Wallis test, P = 3.52 × 10−4) and following optogenetic activation of LHGABA-TRN circuit at 20 Hz (P = 0.0082). (f) Mean latencies of NREM (left) and REM (right) sleep-to-wake transitions following optogenetic stimulation at 1 Hz (ChETA N = 7, YFP N = 5, t = 5.21, df = 10, P = 0.0004, unpaired two-tailed Student’s t test), 20 Hz (ChETA N = 7, YFP N = 5, t = 13.2, df = 10, P = 0.00001, unpaired two-tailed Student’s t test) or 1-s continuous (ChETA N = 7, YFP N = 5, t = 19.1, df = 10, P = 0.0001, unpaired two-tailed Student’s t test) illumination in control (white) and ChETA-EYFP (black) mice. Data analysis is based on more than ten stimulations per frequency and per animal (N). **P < 0.01, ***P < 0.0001.

To test whether inhibition of TRN cells affect NREM sleep-to-wake transitions, we quantified the latency of arousal transitions from NREM

Time, <0.2 ms; N = 70 cells, 3 mice; Supplementary Fig. 7c-f). Optical activation of LHGABA terminals in the TRN area exerted a strong frequency-dependent inhibitory action on TRN cell activity (N = 70 cells, 3 animals, F(3,3) = 38.2831, P = 9.42 × 10−16, one-way ANOVA followed by Bonferroni post hoc test; Fig. 3c,d). The firing rate of TRN cells progressively decreased with increasing optical stimulation frequency or duration (20 Hz: 57 of 70 cells, 3 mice, P = 1.62 × 10−10, Wilcoxon signed rank test; 2-s continuous illumination: 17 of 17 cells, 1 animal, τ(32,32) = 5.89, P = 0.0000014, un-paired two-tailed Student’s t test); both fast- and slow-firing cells reduced their firing rate following optical stimulation of LHGABA terminals (Supplementary Fig. 7f). Furthermore, we found that the majority of TRN cells displayed state-dependent activity (Fig. 3b, e): the variability of their ISIs (CV) was lower (that is, cells fired more regularly) during spontaneous waking than during NREM sleep (46 of 70 cells, 3 animals, P = 3.523 × 10−9, Wilcoxon signed rank test; Fig. 3e). The CV changed across states independently of changes in firing rate (Pearson correlation, P = 0.26). Cells with and without state-dependent changes of CV displayed similar firing rates (NREM sleep: 6.19 ± 0.71 and 4.86 ± 1.08 Hz, respectively, P = 0.17; waking: 7.58 ± 0.81 and 5.54 ± 0.92 Hz, respectively, P = 0.22) and mean ISI (275.0 ± 44.83 versus 406.99 ± 77.98 ms, respectively, P = 0.2). Optical stimulation of LHGABA-TRN projections replicated state-dependent changes of CV in TRN neurons (20 Hz: N = 46 cells, 3 animals, P = 0.0086, Wilcoxon signed rank test; 2-s continuous illumination: N = 10 cells, 1 animal, P = 0.00195, Wilcoxon signed-rank test; Fig. 3e and Supplementary Fig. 7f). These results strongly support the hypothesis that inhibition of TRN cells controls NREM sleep-to-wake transitions.
and REM sleep following optogenetic activation of LHGABA terminals in the TRN in ChETA-YFP and control animals (Online Methods). Optical stimuli were delivered at the onset of NREM or REM sleep episodes, as detected by real-time EEG and electromyography (EMG) analysis in freely moving mice (Fig. 1g,h). We found that bilateral optogenetic stimulation of LHGABA terminals in the anterior part of the TRN during NREM sleep induced a rapid (<2 s) transition to wakefulness in ChETA compared with control mice (Fig. 3f). The speed of this transition was directly dependent to the frequency of stimulation and the number of light pulses, whereas brief continuous illumination significantly induced the shortest latency of wake onset (ChETA, 2.77 ± 0.299 s, N = 7; control, 63.7 ± 3.84 s, N = 5; t = 19.1, df = 10, P = 0.0001, unpaired two-tailed Student’s t-test; Fig. 3f). Optically induced wakefulness was transient and equal in duration to circadian-matched awake episodes in control animals (on condition: ChETA, 18.1 ± 3.92 s, N = 7; control, 15.5 ± 3.17 s, N = 5, t = 0.56, df = 11, P = 0.58, unpaired t-test).

Figure 5 LHGABA-TRN induced arousal is a result of direct inhibition of TRN cells. (a) Schematic (left) of the genetic targeting of TRN_GABA neurons. AAV2-Dio-ArchT-YFP or AAV2-Dio-eYFP were infused into the TRN of VGAT::IRES-Cre animals. Representative photomicrograph (right) of a coronal hemisection showing ArchT-eYFP–expressing TRN cells in the anterior TRN. Arrow indicates the injection tract. Scale bar represents 500 µm. (b) Representative photomicrograph magnification of the red box in a. Scale bar represents 500 µm. White arrows on the photomicrographs indicate ArchT-eYFP expressing TRN cells in the anterior TRN. Arrow indicates the injection tract. Scale bar represents 500 µm. (c) Representative pharmacological targeting of the experimental timeline. (d) Representative EEG and EMG traces and the corresponding heat map EEG power spectrum illustrating behavioral response to optogenetic silencing (bilateral 5-s continuous illumination, indicated by horizontal yellow bars; 593 nm) during NREM sleep. Note the decrease of the amplitude of low frequency (<4 Hz) oscillations and the EMG tone at NREM-to-wake transition. (e,f) Mean latency ± s.e.m. wake transitions during NREM (e; ArchT N = 6, YFP N = 4, t = 8.54, df = 8, P = 0.0001) and REM sleep (f; ArchT N = 6, YFP N = 5, t = 1.79, df = 9, P = 0.1073) after a single 5-s continuous illumination. Data analysis is based on more than ten stimulations per frequency, per state and per animal (N). ***P < 0.0001, n.s. indicates not significant (P = 0.184), a two-tailed Student’s t-test.
Figure 6 Inhibition LH GABA projections to the anterior TRN during NREM sleep increase in total length of NREM sleep compared to control conditions. (a) Schematic of experimental preparation (left). AAV2-DIO-ArchT-EYFP and AAV2-DIO-EYFP (control) were infused in the LH (AP: −1.45 mm) of Tg(VGAT);IRES-Cre animals. Bilateral fibers were implanted at the level of the anterior TRN (AP: −0.85 mm). Right, photomicrographs of a coronal brain hemisphere showing EYFP labeling of LH cell bodies targeting and projections to the TRN. Scale bar represents 200 μm. (b) Schematic of the experimental timeline. Light was delivered continuously 10 s after the onset of a stable NREM sleep and terminated at the next transition (REM or wake). Representative images from repeated experiment (>10 sections from n = 4 transduced mice in each group). (c,d) Representative EEG/EMG traces and EEG heat map of control (EYFP, c) and ArchT-EYFP (d) animals showing changes in the EEG (top) delta (0.5–4 Hz) oscillation (middle), EMG and correspondent heat map EEG power spectrum (bottom) in response to local optical silencing (yellow bar, 593 nm) of LH GABA-TRN circuit during NREM sleep. (e) Mean duration ± s.e.m. of NREM sleep episodes during baseline (left, gray bar), and during optogenetic inhibition in control (white) and ArchT-EYFP (black) mice (N = 4 per group). Data analysis is based on more than ten stimulations per frequency and per animal during NREM sleep episode (P3,12 = 21.45, P = 0.00001). **P < 0.01, one-way multiple ANOVA followed by Bonferroni post hoc test. (f) Mean ± s.e.m. average of normalized spectral power distribution of relative cortical EEG power density in control animals (gray trace) and ArchT-EYFP (black trace) animals (n = 5 NREM episodes per animal) (n = 4 animals per group, t = 0.239, df = 484, P = 0.811) following optical silencing. *P < 0.05, unpaired two-tailed Student’s t test between control and ArchT animals. (g,h) Mean ± s.e.m. average of normalized spectral power of the relative cortical EEG power density (n = 4 mice per condition, N = 5 NREM episodes per animal, t = 4.42, P = 0.0052, unpaired two-tailed Student’s t test, **P < 0.05, g) and spindle frequencies following optogenetic silencing of EYFP and ArchT-EYFP animals during NREM sleep (N = 4 mice per condition, N = 5 NREM episodes per animal, t = 1.16, P = 0.28, h). n.s. indicates not significant (P > 0.28), unpaired two-tailed Student’s t test. Error bars represent ± s.e.m.

two-tailed Student’s t test; Fig. 3g). Note that stimulation of the postero-r part of the TRN showed slightly longer latencies, presumably as a result of a smaller number of terminals being reached by the optical beam (Supplementary Fig. 5a–c). Notably, we did not observe any behavioral changes with optogenetic stimulation of LH GABA-TRN circuit during REM sleep states (on condition: ChETA, 62.1 ± 3.93 s, N = 7; control, 78.1 ± 4.47 s, N = 5; t = 1.96, df = 10, P = 0.078, unpaired two-tailed Student’s t test; Fig. 3h). State-dependent optogenetic activation of the LH GABA-TRN circuit precisely reproduced behavioral changes observed following activation of LH GABA cells bodies (compare Fig. 1i and Fig. 3f), suggesting that the TRN nuclei is the primary target of LH GABA cells in arousal control during NREM sleep.

In addition, and consistent with the widespread projections of LH GABA cells in the locus coeruleus (LC), medial septum (MS) and dorsal thalamus (PV; Fig. 4a and Supplementary Fig. 1a,b,d), we found that optogenetic stimulation of LH GABA terminals in the MS had no effect on arousal from NREM sleep, whereas their activation in the LC induced arousal from both NREM and REM sleep (Fig. 4b,c). In addition, LH GABA terminals in the PV nuclei induced arousal from NREM, but not REM, sleep with substantially lower efficacy than the TRN (Fig. 4b,c). These latter data suggest the existence of some functional heterogeneity amongst LH GABA arousal cells. They further confirm that TRN, rather than dorsal thalamus or LC, is primarily involved in fast arousal control from NREM sleep.

Silencing of TRN cells causes arousal
Our results indicate an causal link between the transient activation of LH GABA-TRN circuit and NREM sleep-to-wake transitions, suggesting that a transient inhibition of TRN cells is required for arousal transition as suggested in vivo (Fig. 3b). To test this hypothesis, we transduced TRN cells with ArchT-YFP or YFP (control) AAVs and implanted bilateral optical fibers above the anterior TRN for in vivo optical silencing of LH GABA neurons while recording cortical EEG and EMG signals (Fig. 5a). Expression of the transgene was selective to the TRN, but was absent from adjacent thalamic nuclei, which do not contain VGAT-expressing neurons (Fig. 5b). Consistent with our previous results, we found that optical silencing of TRN cells during NREM sleep induced rapid sleep-to-wake transitions in ArchT-YFP compared with control animals (ArchT, 18.74 ± 1.5 s, N = 6; control, 64.06 ± 6.2 s, N = 5; t = 8.54 df = 8, P = 0.073, unpaired two-tailed Student’s t test;
Silencing of LHGABA–TRN stabilizes NREM sleep

Does silencing of LHGABA cells, and their projections to TRN in particular, promote NREM sleep? To test this, we transduced LH of Tg(VGAT)–IRESCre mice with AAVs and implanted bilateral optical fibers above the anterior TRN for in vivo optical silencing while recording cortical EEG and EMG signals (Fig. 6a,b). Optical continuous pulses were started 10 s after the onset of NREM and continued until the next transition (that is, wake or REM sleep; Fig. 6b–d). We found that bilateral optogenetic silencing of LHGABA terminals in the anterior part of the TRN (using continuous optical illumination; Fig. 6c,d) significantly prolonged the duration of NREM sleep episode in ChETA compared with control mice (ArchT, 117.0 ± 12.13 s, N = 4; control, 59.21 ± 3.14 s, N = 4; or spontaneous NREM bout duration ±5.3 s, N = 4, t = 3.12, df = 3.94, P = 0.0001, repeated measures followed by Bonferroni post hoc test; Fig. 6e). Notably, time-frequency analysis showed that optical silencing induced a significant increase of the amplitude of delta oscillations (0.5–4 Hz; ArchT, 0.118 ± 0.006, N = 4; control, 0.00872 ± 0.005 s, N = 4; t = 4.42, P = 0.0052, unpaired two-tailed Student’s t test; Fig. 6f,g), a hallmark of sleep homeostasis.37 No changes were observed in the spindle frequency domain (ArchT, 0.013 ± 0.0017, N = 4; control, 0.0169 ± 0.0017 s, N = 4; t = 1.16, P = 0.28, unpaired two-tailed Student’s t test; Fig. 6h). Thus, silencing of LHGABA cells facilitates maintenance of NREM sleep and enhances the process of recovery of sleep.

Activation of LHGABA–TRN induces emergence from anesthesia

Notably, the changes in thalamocortical oscillations and cellular activity associated with a transition from sleep to wakefulness share some characteristics with those occurring during emergence from anesthesia.8 In particular, experimental evidence has linked the thalamus, and the TRN in particular, to various states of consciousness, including anesthesia.8 General anesthetics (barbiturates, propofol, etc.) at clinically relevant concentrations disinhibit TRN neuron activity and cause a concomitant reduction in firing rate in the thalamus.8,58 We therefore hypothesized that the LHGABA–TRN arousal circuit could be a key structure in modulating thalamocortical oscillations during anesthesia, as well as controlling emergence from anesthesia. To test this, we optogenetically activated the LHGABA–TRN terminals during progressive isoflurane exposure (0–1.2%; Fig. 7a) while recording EEG activity and loss of righting reflex (LORR), a surrogate for loss of consciousness in humans (see ref. 39). Animals were optically stimulated once stable burst-suppression mode was recorded from the cortex (Fig. 7b,c). We found that a single 5-s bilateral optical stimulation caused a significant increase of the total duration of burst activity in

Figure 7 Activation of the LHGABA–TRN circuits induced cortical arousal and emergence from anesthesia. (a) Schematic representation of the in vivo polysomnographic recording set up in anesthetized chamber. (b) Higher time resolution of the EEG traces shown in c depicted by the boxes for control condition (1) and ChETA-EYFP–positive animals (2) after 5-s light stimulation of the TRN. These illustrate changes at the level of cortical EEG of burst-suppression ratio after stimulation during anesthesia (1–1.2% isoflurane). (c) Representative EEG and EMG recordings and heat map EEG power spectrum before, during and after single 5-s optical stimulation (horizontal blue bars) in EYFP (left) and ChETA-EYFP (right) animals during deep anesthesia. Note the muscle tone that occurred in ChETA-EYFP (two of five animals). (d,e) Mean duration ± s.e.m. of burst total time (d) and burst suppression ratio (e) before and after bilateral optical stimulation delivered during the EEG burst in control (white) and ChETA-EYFP (black) (N = 4 mice, 3 repetitions per animal). (f) Mean duration ± s.e.m. of burst total time before and after bilateral optical stimulation delivered during isoelectric period in control (white) and ChETA-EYFP (black) (N = 4 mice, 3 repetitions per animal). *** P < 0.0001 using a two-tailed Student’s t test.
the cortex (ChETA, 35.0 ± 3.7 s, N = 4; control, 12.1 ± 4.3 s, N = 4; in a total time of 60 s 2 min after stimulation, t = 4.08, df = 6, P = 0.0065, unpaired two-tailed Student's t test; Fig. 7d). This change persisted for ~2 min before returning to burst-suppression mode (Fig. 7e). In some cases (two of four animals), optical stimulation during bursting led to emergence from anesthesia accompanied by limb movements and a lack of LORR. In contrast, optogenetic stimulation delivered during iso-electric activity had no effects (ChETA, 13.7 ± 2.03 s N = 4; control, 8.53 ± 1.74 s, N = 4; in a total time of 60 s 2 min after stimulation, t = 1.94, df = 6, P = 0.1003, unpaired two-tailed Student's t test; Fig. 7f), consistent with their inhibition during that phase of the burst-suppression mode40. This result further implicates the TRN in regulating LHGABA activation of the thalamocortical system.

**DISCUSSION**

The implication of TRN neurons in generating spindle oscillations (11–15 Hz) that characterizes early sleep stages28,36,41 and consolidated NREM sleep25 suggests a possible role of this structure in modulating the transitions between sleep-wake states and their maintenance.

We found that a subset of LHGABA cells send monosynaptic connections to TRN cells when both antero- and retrograde tracing experiments. Based on a qualitative analysis of this mapping, we found that LHGABA cells exerted a strong GABAmediated inhibitory action on TRN cells during spontaneous NREM sleep-to-wake transitions. To the best of our knowledge, this is the first identification of a functional GABAergic input to the TRN, as previously suggested in vitro20–22, although other inhibitory inputs of intra- or extra-hypothalamic origin may also be involved. The chemical nature of this subset of TRN-projecting LHGABA neurons remains unclear. Our results suggest that these cells do not include Lepr-expressing GABA neurons, as their activation resulted in an opposite effect and promote NREM sleep. Furthermore, in agreement with the literature32 and our previous work27, it is unlikely that these cells coexpress melanin-concentrating hormone peptide, the main marker of LH inhibitory cells, or hypocretins or orexins, as those two cell populations send scarce projections and/or fibers of passage to the TRN region27,42,43. Consistent with our in vivo recordings indicating that the activity of LH cells is strongly modulated across NREM sleep-to-wake transitions31, we demonstrated that optogenetic activation of the LHGABA-TRN circuit promotes rapid wakefulness and cortical arousal selectively during NREM sleep and anesthetized states, respectively, as measured by a prominent change in thalamocortical oscillations (that is, sudden decrease of the amplitude of <4-Hz oscillations and burst-suppression mode, respectively). This latter result emphasizes the potency of the LHGABA-TRN circuit to control thalamocortical oscillations during robust and global burst-suppression cortical oscillations associated with anesthesia8.

Our in vivo experiments revealed important conceptual findings. First, wakefulness transitions induced by activation of the LHGABA-TRN circuit were faster than those induced by optogenetic activation of hypothalamic hypocretin or orexin neurons (LHhcrt)26, and similar to electrical stimulations of the LH area49 or optogenetic activation of norepinephrine neurons from the locus coeruleus (LCNE)45. Both the number of synapses in these circuits (LHGABA-TRN versus LHhcrt-LCNE-thalamus/neocortex) and their functional nature (feedforward inhibition versus sequential excitation) might be responsible for their temporal difference in controlling arousal. Second, the LHGABA-TRN arousal circuit is specific to NREM sleep, as its activation during REM sleep did not result in any noticeable behavioral transitions. This is in contrast with optogenetic activation of LHhcrt26 and LCNE45 neurons, which induce arousal both from NREM and REM sleep. Consistent with the fact that NREM and REM sleep are controlled by different neurocircuits2,3, these results strongly suggest a high degree of specialization, rather than redundancy, among arousal circuits of the mammalian brain. Third, if optical activation of LHGABA-TRN coincides with isoelectric point of the EEG burst-suppression mode—that is, when TRN cells are hyperpolarized in anesthetized animals—cortical burst-suppression mode remains unchanged, whereas cortical arousal systematically followed when optical stimulation was time-locked with a burst phase. This latter result emphasizes the critical temporal organization of LHGABA-TRN synaptic activity for controlling the activity of the thalamocortical projections.

It is well established that the TRN has a key role in controlling thalamocortical network oscillations, attention and consciousness36, but it has not traditionally been considered to be an important node in sleep-wake circuitries. Our results indicate that the rapid arousal that occurs following activation of the LHGABA-TRN circuit is associated with a transient silencing of TRN cell activity. Our results further suggest that a virtual hyperpolarization of TRN cells is required for a temporally coordinated arousal during NREM sleep. During NREM sleep-to-wake transitions, increased activity of LHGABA cells exerts a strong inhibition onto TRN cells, which progressively result in a dis-inhibition of thalamocortical (TC) cells and a switch of TC cell membrane potential from a hyperpolarized (DOWN) to a more depolarized (UP) state1,46–48. This slow depolarization of TC is associated with the presence of cholinergic, serotonin, norepinephrine and histamine inputs from the brainstem and hypothalamus, respectively, which in parallel also inhibit TRN cells47. Thus, it is likely that subcortical inputs are integrated to LHGABA cell signals in the TRN, which further controls the activity of thalamic centers, including the intralaminar thalamic (IL) nuclei49. Notably, we found that direct activation of LHGABA-IL projections resulted in a slower arousal, suggesting a modulatory, rather than a switch, function. Specialization among thalamic circuitries controlling sensory integration, attention, arousal or wakefulness awaits further investigation.

Our results indicate that the discharge of LHGABA cells is relatively transient at the transition between NREM sleep and wakefulness, suggesting the existence of inhibitory inputs to LHGABA cells. One candidate circuit is a subset of TRN cells that project directly back to the LH area, where they exert a rapid (<2 ms) and strong top-down inhibitory action on a large population (~66%) of LH cell50. Alternatively, TC glutamatergic inputs to TRN cells will indirectly provide a negative feedback to TC cells1,11. These negative feedback loops would repress the firing of LHGABA or TC cells, respectively, and avoid uncontrolled hyperactivation of the thalamo-cortical network and possible pathological states (for example, epilepsy)7. The implication of local and extra-hypothalamic sleep-wake transmitters inputs to LHGABA cells projecting to TRN awaits further investigation. In turn, reactivation of LHGABA cells can occur through local glutamatergic neurons (for example, hypocretins and orexins cells), as well as extra-hypothalamic arousal circuits from the brainstem. Further functional investigation of the LH-TRN pathway during wakefulness is required to fully elucidate its importance in regulating attention and consciousness.

**METHODS**

Methods and any associated references are available in the online version of the paper.
AUTHOR CONTRIBUTIONS

All of the authors designed the experiments. C.G.H. collected and analyzed anatomical, electrophysiological, behavioral, polysomnographic and optostimulation data. S.J. collected behavioral, polysomnographic and optostimulation preliminary data. M.C.C. and A.P. collected and M.C.C., A.P. and T.K. analyzed in vivo LH and TRN electrophysiological and optostimulation data. All of the authors discussed the results and C.G.H., M.C.C., A.P. and T.K. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. Mice used for all experiments (heterozygous Tg(VGAT-Cre) mice were maintained on a 129/Sv x C57BL/6J genetic background) were housed in individual custom-designed polycarbonate cages at constant temperature (22±1°C), humidity (30–50%), and circadian cycle (12-h light-dark cycle, starting at 8 a.m.). Food and water were available ad libitum. Animals were treated according to protocols and guidelines approved by McGill University and the Canadian Council of Animal Care, the Veterinary office of the Canton of Bern, Switzerland, and the Landesamt für Gesundheit und Soziales (LaGeSo, Berlin).

Only male mice were used in the behavioral experiments. Mice were housed in groups of 2–4 animals per cage before experiments and after virus injections. After implantations all mice were housed individually. Animals were habituated to the EEG/optical stimulations in their home cages (30 x 20 cm) and kept plugged for the duration of the experiments. Animals were kept in a rectangular box, 50 x 30 cm and were allowed the mice to freely explore an enclosure during in vivo electrophysiology experiments.

Plasmid and viral targeting. 6-week-old Tg(VGAT):CRE-IREs-Cre male or female mice were anesthetized with isoflurane (5% induction, 1.2–1.5% maintenance) and then placed on a small animal digital stereotaxic frame (David Kopf Instruments). Only male mice were used for behavioral experiments whereas both male and female mice were used for anatomical and in vitro electrophysiological experiments. Mice were randomly assigned to viral injection. 0.6 µl of recombinant AAVs carrying Ef1α-DIO-ChETA-EYFP or 0.5 µl control Ef1α-DIO-EYFP vector (plasmids were kindly provided by K. Deisseroth; virus vectors were packaged at Vollum Vector Core, University of Washington) were bilaterally injected in the lateral hypothalamus through an internal 28G cannula (Plastics One) connected to a microinfusion pump (Harvard Apparatus, model 1200) at a rate of 50 nl min−1, as previously described.26,51,52 Inhibition of TRN cells was done by virally targeting the TRN (anteroposterior (AP), −0.85 mm; mediolateral (ML), ±1.7 mm; dorsoventral (DV) 3.5 mm) using injections of 0.5 µl of each Ef1α-DIO-ArchT-EYFP Ef1α-DIO-EYFP (controls). Inhibition of LH_GABA terminals in the TRN was done by injecting Ef1α-DIO-ArchT-EYFP or Ef1α-DIO-EYFP (controls) AA V in the LH (AP, −1.4 mm; mediolateral (ML), ±1 mm; dorsoventral (DV) 5.4 mm). These virus vectors were packaged and titered by the University of North Carolina Vector Core Facility.

Slice preparation. P14 Tg(VGAT):CRE-IREs-Cre mice were injected bilaterally using 0.5 µl of Ef1α-DIO-ChETA-EYFP AAV in LH stereotactic coordinates adapted to young animals (AP, −1.40, ML: ±0.90, DV: 5.35). After completion of the virus injection, mice were returned to their home cage with the mother for 15–20 d. All mice were anesthetized using isoflurane inhalation. Brains of the virus injection, mice were returned to their home cage with the mother and visualized with an upright microscope (BX51WI, Olympus) equipped with a 40× water-immersion objective, differential interference contrast optics, and a near-infrared fluorescence camera (Eksio Blue, Q imaging), and the cells were then identified under IR-DIC. For studies investigating the postsynaptic targets of LH_GABA neurons, slices containing the TRN were observed under both green light excitation and IR-DIC at both 4× and 40× to visualize YFP-positive fibers, only TRN cells in close proximity to fluorescent fibers were selected for recordings. TRN cells were identified based on the electrophysiological fingerprints and anatomical location.

Electrophysiological recordings and data analysis. Both current-clamp and voltage-clamp configurations were used to describe the electrophysiological characteristics in response to activation of photocurrents in both, GABAergic LH cells, and postsynaptic currents of TRN cells in response to activation of LH_GABA fibers. Whole-cell configuration was done using micropipettes prepared from borosilicate glass capillaries (1.0 mm OD, 0.58 mm ID) using a horizontal puller (P-97, Sutter Instruments), with resistances between 3–5 MΩ. Somatic whole-cell current and voltage clamp recordings from Vgat−/− CRE neurons were obtained using patch recording pipettes containing (in mM): 120 potassium gluconate, 20 KCl, 10 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 7 phosphocreatine di-Tris, 2 MgCl2, 0.2 ethylene glyco-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 4 Na+, 3 ATP, 0.3 GTP-Tris (pH adjusted to 7.20–7.26 using KOH, 275–285 mOsm). For postsynaptic experiments TRN cells were patched using pipette solution containing (in mM): 108 potas-sium gluconate, 2 mM MgCl2, 8 mM sodium gluconate, 8 mM KCl, 2.5 mM K2-EGTA, 4 mM K2-ATP and 0.3 mM Na2-GTP buffered with 10 mM HEPES. Access resistance was monitored every 2 min and cells in which changes were greater than 15% of the initial value (8–10 MΩ) were discarded. TRN cells were voltage-clamped at a holding potential of −70 mV and in the presence of the AMPA antagonist, CNQX (20 μM). Quantification of the efficacy of somatic responses was done by calculating the total number of action potentials over 10× consecutive stimulation at different frequencies in cells which resting membrane potential was above −58mV. We kept all cells at a holding potential of −60 mV thought out the experiment.

For the postsynaptic currents, we use a pair-pulse stimulation paradigm with 50 ms at a rate of 0.2 Hz. We recorded evoked potentials at 50% of the maximum response. For the pharmacological manipulations to assess the action of GABA release from presynaptic LH_GABA terminals on TRN cells, we recorded baseline of 5–10 min before perfusing with bicuculline methiodide (BIC, 10 μM) to block GABA A receptors 10 μM of GABA A blocker bicuculline. Cells were recorded for 10 min in the presence of the clocker and then washed out. All salts and powders were purchased from Sigma-Aldrich, except for 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), DL-(-)-2-amino-5-phosphono pentanoic acid (AP-5), and BIC (10 μM) (Ascent Scientific). In vitro electrophysiological data were discarded if intrinsic cell properties (resting membrane potential, input resistance) were more than 3SDs outside group mean, and/or if recording stability changed by >15%, as monitored by series resistance measurements throughout the course of the experiment (mean series resistance was 17±1 MΩ).

Surgical procedures for sleep experiments. As described previously,6,52,53 8–10-week-old male Tg(VGAT):CRE-IREs-Cre mice were chronically implanted with bilateral 200-µm fiber implants above the lateral hypothalamic (MFC_200/245.0.17.5_) and fibers above the PVf (MFC_200/245.0.17.5_) cortexes. EMG signals were recorded from four electrodes on the frontal (AP, −2 mm; ML, ±2.5 mm) and temporal (AP, 3.5 mm; ML, ±3 mm) cortices. EMG signals were recorded from two electrodes inserted in the neck musculature to record postural tone. After surgical procedures, mice were allowed to recover in an individual housing cage for at least 2 weeks. After 1 additional week of acclimation to the EEG-EMG recording set up, an optical patch cord (MFP_200/230/900-0.37_2m_FC-ZF1,25) and a zirconia sleeve (ID = 1.25; Doric Lenses) were connected permanently on the fiber implant. Black nail polish was applied on the plug to black out the light during the optoge netic stimulation. The same methods of implantation and habituation were used for animals transduced with ArchT-EYFP in the TRN and LH, and implanted with optical fibers in the TRN.

For the stimulation of the LH_GABA targets, a bilateral fiber implant was implanted above the TRN at two different locations (reticular thalamic nucleus; Anterior: AP, −0.85 mm, ML, 1.70 mm and DV, −3.4 mm; N = 6 ChETA and N = 6 control; Posterior AP, −1.34 mm, ML, ±2.0 mm and DV, −3.2 mm; N = 7 ChETA and N = 6 control). A single optical fiber with a 6.5° angle was implanted near the medial septum (AP, 0.86 mm, ML, 0.60 mm and DV, −3.62 mm; N = 4 ChETA and N = 4 controls). A single optical fiber was implanted above the PVf (paraventricular thalamic nucleus, AP, −0.86 mm, ML, 0.60 mm and DV, −3.55 mm; N = 4 ChETA and N = 4 controls). A bilateral optical fiber was implanted above the LC (Locus coeruleus AP, −5.45 mm, ML, ±1.0 mm and DV, −3.55 mm;
For all EYFP and ArchT-EYFP transduced animals, sorting was performed automatically followed by manual clusters adjustment. Spike trains were extracted and represented by the first three principle components. Action potentials were detected in a band-pass filtered signal (0.8–5 kHz). Events of the wide-band signal to 1250 Hz. Further data processing was performed by custom-written MATLAB (Mathworks) algorithms as described elsewhere.

Signal processing and data analysis. Electrophysiological signals were processed using Neurophysiological Data Manager (NDManager, http://neurosuite.sourceforge.net/). LFP was obtained by low-pass filtering and down-sampling of the wide-band signal to 1250 Hz. Further data processing was performed by custom-written MATLAB (Mathworks) algorithms as described elsewhere.

Laser pulse time stamps and borders of stimulation epochs were detected. Action potentials were detected in a band-pass filtered signal (0.8–5 kHz). Events of the wide-band signal to 1250 Hz. Further data processing was performed by custom-written MATLAB (Mathworks) algorithms as described elsewhere.

Polysonomographic recordings and state transitions. All sleep experimental procedure took place between 12:00 and 19:00 (light onset at 8:00). EEG and EMG signals derived from the surgically implanted electrodes were amplified (Grass Instruments) and digitized at 512 Hz using sleep recording software (Vital Recorder, Kissei Comtec). The signals were digitally filtered and spectrally analyzed by fast Fourier transformation. Polysonomographic recordings were scored using sleep analysis software (SleepSign for Animal, Kissei Comtec). All scoring was performed manually based on the visual signature of the EEG and EMG waveforms, as well as the power spectra of 5-s epochs, as previously described. We defined wakefulness as desynchronized low-amplitude EEG and high tonic EMG activity with phasic bursts. We defined NREM sleep as synchronized, high-amplitude, low-frequency (0.5–4 Hz) EEG and highly reduced EMG activity compared with wakefulness with no phasic bursts. We defined REM sleep as having a pronounced theta rhythm (6–9 Hz) and a flat EMG. State transitions were identified when EEG/EMG criterion change were predominant for more than 50% of the epoch duration (that is, 2 s). As previously described, polysonomographic scorings were tested by two independent scorers and was found to lie in a 95% confidence interval.

In vivo optogenetic stimulations of LH and TRN areas were conducted during NREM or REM sleep using real-time EEG/EMG detection after 10 s of the onset of NREM sleep and 5 s of REM sleep and described in ref. 27. Note that animals with aberrant circadian distribution of sleep-wake cycle (<10%) were discarded from this study and that genetic and viral manipulations did not disrupt spontaneous sleep-wake architecture compared to naive and wild type animals.

For sleep recordings performed in combination with the in vivo electrophysiology, sleep scoring was performed in Spike 5, using the SleepScoring v1.01 script. The statistical significance of comparisons was determined by t test (or adjusted for multiple comparisons) or Kruskal Wallis test; P < 0.05 was considered to indicate significance.

Optogenetic stimulation. For in vitro electrophysiology stimulation, somatic and postsynaptic currents were evoked by delivering 5-ms square pulses of blue light (473 nm) delivered to the acute brain slice. We use a diode-pumped solid-state lasers (DPSSL, Laserglow) connected to a 200 μm optical fiber (ThorLabs) aimed at the target region. Pulse durations and frequencies were triggered via a built-in TTL circuit controlled by Clampex 10.3 software package. Light intensity was tested before each experiment, and was calibrated to emit 5 mW from the fiber tip. For in vivo optogenetic stimulation electrophysiological experiments, a 3-m-long fiber optic patch cord with protective tubing (Thorlabs) was connected to a chronically implanted optical fiber with a zirconia sleeve (Precision Fiber Products) during optical stimulation. The patch cord was connected to a 473 nm DPSSL laser (RA7100SF1X, Laserglow Technologies) with an FC/PC adapter. The laser output was controlled using a stimulus generator and MC Stimulus software (Multichannel Systems). Optical stimulation of LH GABA-TRN projection consisted of blue light (473 nm) light pulses at a light power output of 25–30 mW from the tip of the patch cord measured with a power meter (PM100D, Thorlabs). For the characterization of TRN cells response to stimulation of LH GABA-TRN projections, blue light was delivered at 1, 5 or 20 Hz for 40 s each, or in 2-s pulses during 2 min. For the characterization of LH cell responses to stimulation of LH GABA cell bodies, blue light of 5-ms pulses for 10 s at various frequencies was delivered with light power output of 25–30 mW from tips of the optic fibers, as mice freely explored an enclosure.

Optical stimulation during polysomnographic recordings. Animals were kept tethered with the EEG recording cable and the fiber optic patch cord with protective tubing as described above. During the stimulation session, animals were left to sleep and during sleep periods, light was delivered at 5-ms square pulses at 1, 20 Hz or 1-s continuous light (473 blue laser) using a Master 9 pulse generator (A.M.P. Instruments, LTD) (ChETA experiments) or continuously for 5 s in the case of the inhibition of the TRN (593-nm yellow laser) and anesthesia experiments (light activation of LH GABA terminals with 473 blue laser). Similarly to the inhibition of the LH GABA terminals in the TRN experiment, was done by delivering light continuously throughout the duration of NREM sleep. During the experiment, frequencies of optogenetic stimulations (1 and 20 Hz) were randomly delivered at either NREM or REM sleep onset in Tg(VGAT);::ires-Cre and control mice. Animals with no viral expression, abnormal sleep-wake cycle (3 s.d. outside group mean) or optical fiber/probe implants outside the target area were discarded from the study. Based on these criteria, 4 ChETA, 3 EYFP, 1 ArchT transduced Tg(VGAT);::ires-Cre animals were excluded from the study. Data collection and analysis were not performed blind to the conditions of the experiments.

Spectral EEG/EMG analysis. For all EYFP and ArchT-EYFP transduced mice used for state-specific stimulations, EEG power spectra were computed for 6–10 stimulated events per animal (more than 20-s duration) and EEG activity recorded during these events was digitized and subjected to the fast Fourier transform (512 points) using SleepSign software. Mean spectral density of all the stimulated events per animal was sorted to successive 0.062-Hz frequency bands between 0 and 100 Hz, then each frequency band was normalized to the sum of the power of the entire range (0–100 Hz). Frequency EEG bands are defined as delta (0.5–4 Hz), theta (6–9 Hz), alpha (9–12 Hz), spindle (11–15 Hz), sigma (12–20 Hz), low gamma (20–60 Hz) and fast gamma (60–100 Hz). Time-frequency power spectrums (inhibition of LH GABA projections in the anterior TRN and anesthesia experiment) were computed using Matlab and the Chronux signal processing toolbox.
Anesthesia experiments. Male mice transduced with YFP and ChETA viral vectors (N = 4 in each group) and used in sleep recording experiments were anesthetized using an induction chamber equipped with gas sampling analyzer (DATEX) to monitored the isoflurane delivered and keeping stable levels of anesthetic throughout the experiments. We recorded 5 min of baseline when the mice were awake. Following that, we induced the mice to an anesthetized state (LORR and in the EEG recording characterized by a burst suppression mode) at a level of isoflurane 1–1.2%; and recorded for 10 min of stable anesthesia (concentration of isoflurane and burst suppression ratio were constant and stable, respectively). Animals were optically stimulated with a single pulse of 5-s continuous illumination in triplicate session. We quantify the total length of each burst (Fig. 7d). Burst suppression ration was calculated by taking the total burst duration time divided by the total time of isoelectric EEG signal. Values were computed taking epochs of 2 min before and 4 min after the stimulation. We compared the response between animals transduced with ChETA and YFP viruses. Burst length was calculated using Spike 2 (Cambridge electronic design limited data acquisition and analysis software). A burst was detected if it has value superior to 2x the standard deviation of the mean amplitude of isoelectric EEG signal. Power spectrum was done using a custom-made Matlab script.

Immunohistochemistry. After deep anesthetization with ketamine/xylazine/acepromazine (100, 16 and 3 mg per kg, respectively, intraperitoneal injection) and perfused transcardially with 1× phosphate-buffered saline (PBS)-heparine 0.1%, pH 7.4, followed by 4% paraformaldehyde in PBS. The brains were extracted, postfixed overnight in the same fixative at 4 °C, and cryoprotected in 30% sucrose dissolved in PBS for an additional 24 h at 4 °C. Each brain was sectioned at 40 μm using a cryostat (Leica Microsystems) and collected in PBS with 0.1% Triton X-100 (PBST). For double immunolabeling, brain sections from virally-transduced mice were washed in PBST for 10 min at 22–23 °C. Sections were then incubated in a blocking solution composed of PBST with 4% bovine serum albumin for 1 h at room temperature. Sections were first incubated for 48 h at 4 °C in a rabbit anti-YFP (1:5,000, Ab290, Abcam) in blocking solution. For Figures 2a and 5a,b, colabeling with mouse anti-GAD67 (1:2,000, MAB546, Millipore, Lot # 2350525) was used followed by 5 × 10 min in 1× in PBST, sections were incubated in a solution of Alexa 488 Donkey anti-rabbit IgG secondary antibody (1:1,000, A21206, Invitrogen) and Alexa 555 goat anti-mouse IgG secondary antibody and in PBST for 1 h at 22–23 °C. Sections were washed 3 × 10 min in 1× PBST, mounted on glass slides, and coverslipped with Fluoromount-G (100–500, Southern Biotech). Quantification of colocalization was performed on adjacent sections containing LH and throughout the brain in a total of N = 13 brains included in Supplementary Figure 1b–d.

Brains from silicon probe recordings were fixed overnight in 4% paraformaldehyde, equilibrated in 1% PBS for an additional night and finally cut in 50-μm slices using an oscillating tissue slicer (EM5400, Electron Microscopy Science). Brain slices were mounted (Fluoromount Aqueous Mounting Medium, Sigma-Aldrich).

Fluorescence in situ hybridization. Virally transduced brains from 8-week-old male or female mice (N = 4) were flash frozen. Brains were cut in coronal sections (16 μm) and mounted onto glass slides for further hybridization with GAD-67 antisense probe followed by immunodetection of YFP fluorescent protein (antibody described above), as described27. Colocalization was quantified by counting the total number of cells in the field of view using a 40x objective that were GAD-67+/YFP+. Experiment was repeated five times. Brain sections that had no fluorescent expression in the LH area (one mice) were discarded for the quantification shown in Supplementary Figure 1c.

Retrograde labeling. Male or female Tg (VGAT):::ires cre mice were injected with 0.5 μl of AAVdJ Ef1α-DIO-EYFP at 6 weeks of age. 2 weeks later red fluorescence retrograde beads (Lumafour) were injected in the anterior part of the TRN (AP, −0.85 mm; ML, 1.70 mm; DV, −3.4 mm). After an additional 2 weeks of incubation, brains were collected as describe above for immunohistochemical labeling. A separate set of mice were injected with AAV2 Ef1α-DIO-mCherry at 6 weeks and with LT-HSV-EGFP lentivirus at 8 weeks. Brains were collected, perfused and processed for immunohistochemistry (see above) 2 weeks after the lentiviral injection.

Microscopy. Non-confocal images were collected on a Axios Observer Carl Zeiss fluorescence microscope using fluorescent reflected light except for images in Supplementary Figures 4a and 7a were taken on an Olympus BX 61 microscope (2×/0.06 NA 10×/0.3 NA, 20×/0.5 NA, dry). Confocal images were collected on a LSM 710 Carl Zeiss confocal microscope. Digital images were minimally processed using Image J or Zen to enhance brightness and contrast for optimal representation of the data. All digital images were processed in the same way between experimental conditions to avoid artificial manipulation between different data sets.

Statistical analysis. Statistical analyses of electrophysiological properties and synaptic responses were assessed using repeated measures ANOVAs, paired t tests, and significant effects were investigated using pairwise multiple comparisons using Student-Newman-Keuls method for parametric data, unless otherwise indicated. Data are presented as mean ± s.e.m.

The statistical significance of comparisons for sleep/silicon multiunit recordings was determined by t test (t adjusted for multiple comparisons) or Kruskal Wallis test; P < 0.05 were considered to indicate significance. All data were analyzed using Prism 5.0 (GraphPad Software), Clampfit 10.3 (Axon Instruments) unless otherwise mentioned. Data were exported into Adobe Illustrator CS3 (Adobe Systems) for preparation of figures. Data distribution was assumed to be normal but this was not formally tested. Experimental sample size were defined based on previous studies26,27,35. No statistical methods were used to pre-determine sample sizes.

A Supplementary Methods Checklist is available.

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