Supporting Information

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SI Experimental Procedures

Subjects. Male Sprague-Dawley rats, aged 6 to 8 wk for virus injection and cannula placement and aged 10 to 12 wk for behavioral and electrophysiological studies, were housed two per cage and kept on a 12/12 h light/dark cycle (lights on/off at 0700/1900 hours). All procedures involving animals were approved by the Institutional Animal Care and Use Committees of the University of California, San Diego.

Virus Preparation. The cDNA for the oChIEF variant of ChR2 was a gift from the laboratory of Roger Tsien (deceased), University of California, San Diego, La Jolla, CA. To make pAAV-hSyn-GFP, we PCR amplified eGFP from pEGFP-N1 vector and cloned it into pAAV-hSyn vector. pAAV-hSyn-eArch3.0-eYFP was kindly provided by Karl Deisseroth, Stanford University, Stanford, CA. Recombinant AAVs were prepared as previously described (1). Viral titers were determined by real-time quantitative PCR methods and ranged from 1×10^{12} to 1×10^{13} genome copies per milliliter.

Surgery. Rodents were anesthetized with isoflurane for stereotaxic injection of AAVs into the LHb [anterior-posterior (AP) −3.4 mm, medial-lateral (ML) ±0.7 mm, dorsal-ventral (DV) −4.85 to −5.0 mm]. A total of 0.4 to 0.5 μL of virus was injected over an 8- to 10-min period. At the end of the injection, the pipet remained at the site for 5 min to allow for diffusion of the virus into the surrounding tissue. An optic fiber cannula was implanted just above the RMTg (AP −7.0, ML 1.6 mm, DV −7.6 mm with an 8° angle) and secured to the skull with dental cement reinforced with surgical screws. For fiber photometry (FP) and optogenetic manipulations, rats were implanted with 400-μm [.48 numerical aperture (NA)] and 200-μm (.22 NA) diameter fibers, respectively. Rats were injected with 5 mg carprofen (a nonsteroidal antiinflammatory drug) per kilogram body weight after surgery.

Behavioral Assays.

FST. Rats were placed for 20 min in a cylinder of water (water temperature, 25 to 26 °C; cylinder, 30 cm in diameter and 40 cm high; water depth, set to prevent rats from touching the bottom with their hind limbs). Rat behavior during the FST was videotaped using a PC6EX3 infrared camera (SuperCircuits) at 3.74 frames per second. Mobility values were determined using significant motion pixel analysis, an automated and unbiased method to analyze animal motion (2). Immobility in the FST, calculated from a five-point smoothing of significant motion pixel data, correlated well with immobility estimated by a human observer (Fig. S1). An immobility bout was defined as a period where values <0.8 SD of the mean lasted at least 2 s. In a few cases where an animal spent a significant fraction of the test immobile, this threshold was adjusted to <0.6 SD from the mean to better reflect the animal's behavior. Bouts were excluded if another identified bout fell within the baseline period or if the identified bout abutted the beginning or end of the trial. Movement data were cut into 10-s segments, with a 5-s baseline.

During the FST, rats were connected to an optic fiber patch cord. For photostimulation of oChIEF, we alternated between 2-min periods without and with 25-Hz optical stimulation (5-ms optical pulse duration). The vast majority of LHb projection neurons target one midbrain aminergic nucleus (3). Thus, stimulation experiments are not expected to affect LHb outputs other than to the RMTg. For experiments with eArch3.0-eYFP, constant green light was administered during photomanipulation periods (2 min). While the use of eArch3.0 can increase spontaneous release, evoked release is reduced (4). Our observation that the opposite behavioral effects are seen in the experiments using eArch3.0, compared with use of oChIEF, suggests that evoked release is more important than spontaneous transmitter release in transmitting behaviorally relevant information at this synapse.

OF. Rats were placed for 10 min in an arena (70 × 45 × 40 cm), and movement was videotaped from above using a PC6EX3 infrared camera (SuperCircuits) at 3.74 frames per second. Mobility was analyzed as described for the FST. Rats were connected to the laser, and we alternated between 2-min periods without and with 25-Hz optical stimulation (5-ms optical pulse duration). Periods with light stimulation were compared with the mean of flanking no-light periods to account for the general downward trend in mobility as a function of time.

Operant conditioning training. Rats were trained and tested in a modular operant test chamber (Med Associates). During the training and testing period, rats were kept on a restricted water schedule (2 h ad libitum daily). Rats were initially trained to associate a reward (20 to 30 μL of 10% sucrose) with a light above the dispenser receptacle before being trained to press a lever to obtain a reward (20 to 30 μL of 10% sucrose per lever press) in a fixed ratio schedule with one reward per lever press (FR1). Each FR1 session lasted 30 min, and rats that successfully learned to press the lever to obtain rewards were selected for subsequent PR testing.

Progressive ratio lever press. Rats were tested in a PR schedule as described elsewhere (5). Briefly, sucrose rewards (20 to 30 μL of 7% sucrose) were earned with increasing number of lever presses. The final number of lever presses that produced a reward represented the BP value. All PR sessions were performed with rats connected to optic fiber cable; optical stimulation consisted of trains of 5-ms pulses delivered at 25 Hz (1 s on/1 s off).

Sucrose preference test. Rats were habituated to the two-bottle (tap water or 1% sucrose) paradigm for 24 h. During testing (30 min), water-deprived (24 h) rats were connected to an optic fiber cable. Testing alternated (daily) with or without optical stimulation trains (25 Hz, 1 s on/1 s off). Consumption (water and sucrose solution) was calculated by weight [total grams of liquid intake per gram of body weight]. Total liquid consumed was calculated as amount of liquid per gram body weight; sucrose preference was calculated as sucrose solution consumed/total liquid consumed.

Real-time place preference test. The real-time place preference test was performed using previously described methods (6). In brief, after a 6-min baseline period, optical stimulation (continuous 25 Hz, 5 msec pulse duration) was delivered while the animal was in one context (defined as "context A"). For the next 14 min, optical stimulation of the LHb→RMTg occurred whenever the rat was located in context A. Optical stimulation was stopped when the animal was in the other side of the cage (context B). Preference scores were measured by taking (time spent in context A minus time spent in context B)/total time. Student's *t* test compared avoidance score from period 14–20 min (*ii*) to baseline period (0–6 min; *i*).

Rotarod. Rats were initially trained to remain on the rod (Ugo Basile Rota-Rod) at low speed (5 rpm) for 5 min (day 1). Subsequently, rats connected to optic fiber cable alternated daily trials (without or with optical stimulation) on rotarod (20 rpm on day 2; 30 rpm on day 3). Optical stimulation was started 10 s before rats were placed on the rod. A minimum of a 30-min rest period was given between trials to minimize motor fatigue. On day 4, rats alternated trials with rod speed ramped from 2 to

15 rpm over 20 s or from 2 to 30 rpm over 30 s; test was stopped at 25 or 30 s, respectively, when rats remained on the rod during and after the speed ramp.

FP. To record fluorescence signals from GCaMP6s, light from a 470-nm LED (Doric Lenses) was bandpass filtered (FB470-10; Thorlabs), collimated, reflected by a dichroic mirror (DMLP550R), and focused by a 20× objective (N.A. 0.4; RMS20X; Olympus). Excitation power was adjusted so as to get 35 to 50 μW of 470-nm light at the tip of the patch cord. Emitted GCaMP6s fluorescence was bandpass filtered (FF01-535/22-25; Semrock) and focused on the sensor of a CCD camera (Hamamatsu Orca). FP and movement data were aligned by simultaneously triggering the 470-nm LED and the start of the video capture. The end of the fiber was imaged at 27 to 42 Hz (binned 8×). Mean value of a region of interest covering the cross-section of the fiber was calculated using ImageJ. These data were exported to MATLAB for further analysis. This experiment was replicated with two independent groups of rats. Data were pooled across experiments.

Correlation Analysis. FP data were corrected for heat-induced LED decay and photobleaching of GCaMP6s by fitting the data to a double-exponential decay curve. FP data were segmented about the onset of the previously determined bouts of immobility, binned so that the number of frames in an FP segment equaled that of a movement segment, and the Pearson's correlation coefficient for each immobility bout was then calculated. For each rat, on average, we detected 42 ± 8 immobility bouts. Data are presented as mean of the average r values across rats.

In Vivo Recordings. Juxtacellular in vivo recordings were done as previously described (7). In brief, 4 wk after injection of AAV-oChIEF-tdTomato into the LHb, rats were anesthetized and mounted on a custom-made stereotaxic frame with an adjustable angle, to hold the head in a fixed position during the recording. The body temperature was regulated by a heating pad. Using aseptic surgical tools, the skull was exposed and a hole (~3 mm) was made, centered at AP −7.0 mm and ML 2.6 mm. The recording electrode was a glass pipet (15 to 20 mΩ) filled with 0.5 M NaCl. The recording electrode was connected to an Axopatch-1D amplifier. The signal was amplified (1,000×), filtered (2 kHz) and digitized at 10 kHz using an InstruTECH A/D interface. Data were acquired using custom software written in Igor Pro (WaveMetrics), and spikes were detected using a custom-written MATLAB script. For optical stimulation, an optic fiber was glued to the glass pipet so that the tip of the fiber was 500 μm above the tip of the glass pipet to form an optrode. The optic fiber was connected to a 473-nm solid-state laser diode (Shanghai Laser & Optics Century Co.). The optrode was slowly lowered in at a 14° angle after the start of stimulation. When spike activity was detected (DV −7 to −7.8 from the top of the brain), photostimulation was evoked using 25-Hz light pulses for 10 s per 30-s sweep. All rats were perfused after the recordings and the position of the recording site verified.

Statistical Analyses. All statistical analyses were completed using MATLAB (MathWorks, 2016a) or GraphPad Prism 6 (GraphPad Software). Behavioral data were analyzed using two-sided paired (when intraanimal comparisons were made) or unpaired (when group means were compared) Student's t test. For FP data, Pearson's correlations were performed using MATLAB, which provided a P value.

Exclusion of animals or data points. Several rats were excluded post hoc in the rare case that an optical fiber was misplaced or if expression of the construct of interest was off-target or low. Exclusion was done blind to both the animal's identification and the behavioral results.

FP recordings. During recordings of neural activity from axon terminals (while a rat was vigorously moving in the FST), a patch cord occasionally became loose from its connection with the rat. In such cases, portions of the recording sessions (or an entire recording session) were excluded. These determinations were made through a combined analysis of the motion-capture video and the fluorescence recordings.

Data and Code Availability. Codes used for fiber photometry analysis are available at https://github.com/NotAnHerb/LHbRMTg.

1. Shabel SJ, Proulx CD, Piriz J, Malinow R (2014) Mood regulation. GABA/glutamate co-release controls habenula output and is modified by antidepressant treatment. *Science* 345:1494–1498.
2. Kopec CD, et al. (2007) A robust automated method to analyze rodent motion during fear conditioning. *Neuropharmacology* 52:228–233.
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7. Nabavi S, et al. (2014) Engineering a memory with LTD and LTP. *Nature* 511:348–352.

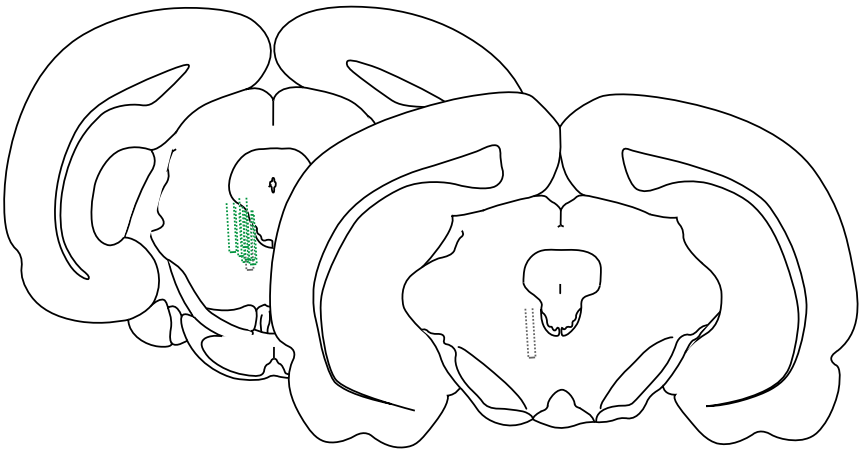

Fig. S1. Location of optical fiber placement in the RMTg of rats injected with AAV-hSyn-GCaMP6s (depicted in green) or AAV-hSyn-eGFP (depicted in gray). Position relative to Bregma, from left to right (in mm): −6.7, −6.0.

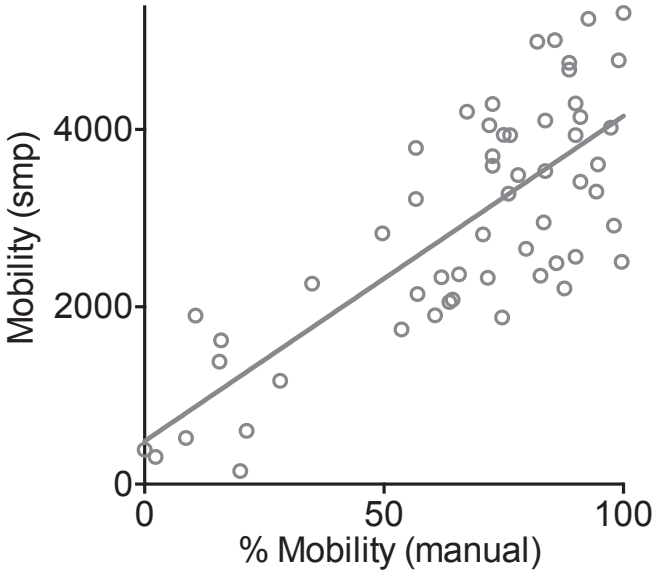

Fig. S2. Significant motion pixel analysis is an accurate predictor of mobility in the FST. Plot of mobility as determined by significant motion pixel analysis vs. a human observer. Each open circle is a different rat tested in the FST. $r = 0.77$; $P < 0.0001$.

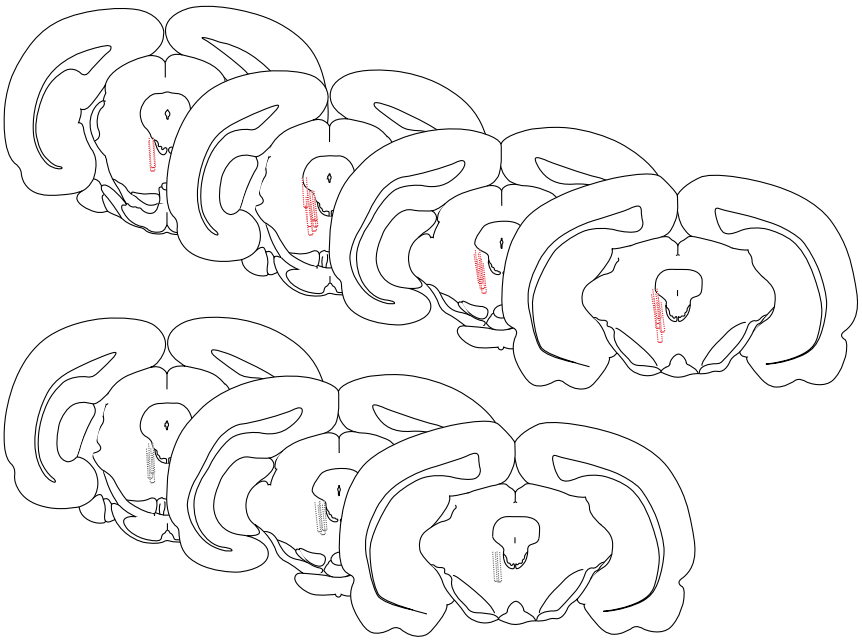

Fig. S3. Location of optical fiber placement in the RMTg of rats injected with AAV-hSyn-oChIEF-tdTomato (depicted in red) and AAV-hSyn-tdTomato (depicted in gray). Position relative to Bregma for AAV-hSyn-oChIEF-tdTomato [from left to right (in mm): −7.0, −6.7, −6.3, −6.0] and AAV-hSyn-tdTomato [from left to right (in mm): −6.7, −6.3, −6.0].

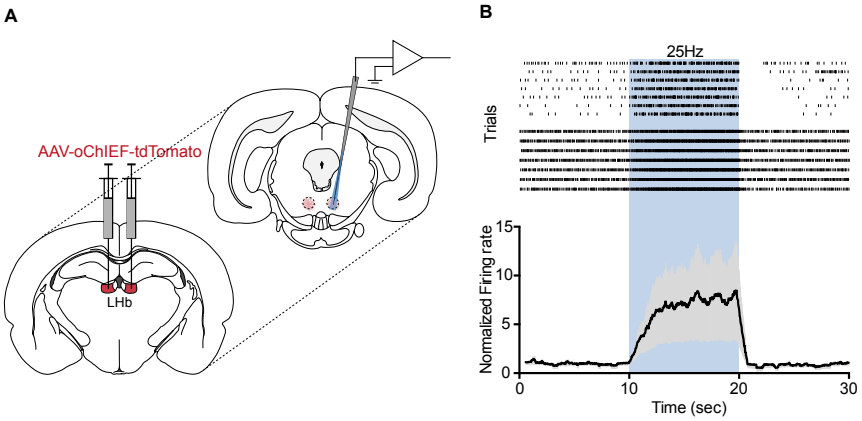

Fig. S4. Optogenetic stimulation of the LHb→RMTg pathway increases firing of RMTg neurons. (*A*) AAV encoding the light-sensitive cation channel oChIEF-tdTomato was injected into the LHb, and juxtacellular recordings of RMTg neurons were made. (*B*, *Top*) Two representative rasterplots of juxtacellular recordings of single RMTg neurons in anesthetized rats. (*B*, *Bottom*) Plot of average normalized firing (black line indicates mean; gray shading indicates SEM; $n = 6$). Fold increase in firing during stimulation of LHb terminals, 8.5 ± 3.5; $P < 0.05$, paired Student's t test.

Fig. S5. Location of optical fiber placement in the RMTg of rats injected with AAV-hSyn-eArch3.0-eYFP (depicted in yellow) and AAV-hSyn-eGFP (depicted in gray). Position relative to Bregma, from left to right (in mm): −6.7, −6.3, −6.0.