Single rodent mesohabenular axons release glutamate and GABA

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The lateral habenula (LHb) is involved in reward, aversion, addiction and depression through descending interactions with several brain structures, including the ventral tegmental area (VTA). The VTA provides reciprocal inputs to LHb, but their actions are unclear. Here we show that the majority of rat and mouse VTA neurons innervating LHb coexpress markers for both glutamate signaling (vesicular glutamate transporter 2; VGluT2) and GABA signaling (glutamic acid decarboxylase; GAD, and vesicular GABA transporter; VGaT). A single axon from these mesohabenular neurons coexpresses VGluT2 protein and, surprisingly, establishes symmetric and asymmetric synapses on LHb neurons. In LHb slices, light activation of mesohabenular fibers expressing channelrhodopsin2 driven by VGluT2 (Slc17a6) or VGaT (Slc32a1) promoters elicits release of both glutamate and GABA onto single LHb neurons. In vivo light activation of mesohabenular terminals inhibits or excites LHb neurons. Our findings reveal an unanticipated type of VTA neuron that cotransmits glutamate and GABA and provides the majority of mesohabenular inputs.

Converging evidence demonstrates that descending LHb efferent pathways regulate VTA activity and play a prominent role in learning, motivation, reward and aversion1–11. In contrast, the role of ascending VTA projections to the LHb are not well understood, although anatomical studies delineated this pathway over 30 years ago12. The detection of tyrosine hydroxylase–positive (TH+) fibers in the rat LHb originating from the VTA13,14 has led to the assumption that dopamine (DA) from the VTA is released in the LHb and the suggestion that DA plays a prominent role within this structure. However, anatomical studies in the rat show that most VTA neurons targeting the LHb (mesohabenular neurons) lack TH12,14,15, indicating that the mesohabenular inputs are mostly nondopaminergic. In addition, recent mouse studies show that optogenetic stimulation of LHb fibers arising from VTA TH neurons results in the release of GABA without detectable release of DA11. Findings from other mouse studies suggest that some non-TH mesohabenular neurons may be glutamatergic, as VTA neurons expressing VGluT2 innervate the LHb16,17. These observations underscore the complexity of the VTA projections to the LHb, the molecular and synaptic compositions of which are unclear.

We used a combination of tract tracing, molecular, microscopy, electrophysiological, pharmacological and optogenetic techniques to identify (i) the types of VTA neurons projecting to LHb, (ii) the axon terminals and synaptic architectures that these neurons establish with LHb neurons, (iii) the neurotransmitter-release capacities of mesohabenular axon terminals, and (iv) the firing patterns of LHb neurons following activation of mesohabenular axon terminals. We found that (i) the majority of VTA neurons projecting to LHb belong to a previously unrecognized neuronal phenotype that coexpresses transcripts encoding VGluT2 and GAD; (ii) mesohabenular axon terminals exhibit a previously unrecognized type of synaptic architecture in which a single axon terminal coexpresses VGluT2 and VGaT, establishes both asymmetric and symmetric synapses, expresses AMPA receptors postsynaptic to asymmetric synapses, and expresses GABA_A receptors postsynaptic to symmetric synapses; (iii) mesohabenular terminals monosynaptically cotransmit glutamate and GABA onto LHb neurons, a type of neurotransmission that had not been previously demonstrated in the adult mammalian brain; and (iv) mesohabenular terminals evoke inhibitory and excitatory changes in the firing rates of LHb neurons in vivo.

RESULTS
Most mesohabenular neurons are VGluT2+ GAD+
To determine the molecular composition of rat VTA projections to the LHb, we used a combination of neuroanatomical approaches. It has been established that TH+ and TH− mesohabenular neurons are localized to the anteromedial VTA12,14,15,17. As this region contains many neurons that express VGluT2 mRNA18 (VGluT2+), we initially determined the degree of expression of TH protein or VGluT2 mRNA in mesohabenular neurons. Rat mesohabenular neurons were labeled in vivo by injection of the retrograde tracer FluoroGold (FG) into the LHb (Supplementary Fig. 1a,b). These FG neurons were phenotyped on the basis of cellular expression of TH or VGluT2 in the FG-labeled neurons, we divided mesohabenular neurons into four classes: VGluT2+ TH− (67.6 ± 0.4%; 418 of 619); VGluT2+ TH+ (24.9 ± 0.3%; 154 of 619);
Most mesohabenular axon terminals are VGluT2+ VGaT+

To determine whether LHB neurons receive dual glutamatergic and GABAergic projections, we processed rat LHB samples for detection of VGluT2 and VGAT by immunoelectron microscopy. We identified three types of axon terminals, with different frequencies (Supplementary Fig. 5): VGluT2+ VGAT− axon terminals (33.5 ± 1.3%; 468 of 1,410), VGluT2− VGAT+ axon terminals (13.4 ± 1.5%; 194 of 1,410) and VGluT2+ VGAT+ axon terminals (53.1 ± 0.9%; 748 of 1,410). At the synaptic level (Supplementary Fig. 5), single axon terminals formed asymmetric (putative excitatory\textsuperscript{19}) synapses, symmetric (putative inhibitory\textsuperscript{19}) synapses and puncta adhaerentia (adhesion sites between axon terminals and dendrites\textsuperscript{19,20}). Thus the LHB is the first recognized structure in which the majority of innervations are from dual glutamatergic-GABAergic neurons that form both symmetric and asymmetric synapses.

After establishing that the LHB has a large population of VGluT2+ VGAT+ axon terminals, we next used a transgenic mouse line to determine whether the VGluT2+ GABA+ mesohabenular neurons are a source of some of the VGluT2+ VGAT+ terminals present in the LHB. To selectively tag mesohabenular axon terminals derived from VTA VGluT2 neurons, we delivered a Cre-inducible adeno-associated vector (AAV) encoding channelrhodopsin2 (ChR2)-mCherry (AAV-DIO-ChR2-mCherry) into the VTA of VGluT2− mice (VGluT2− mice; Fig. 2a). By three-dimensional reconstruction of confocal images from triple fluorescent immunolabeled LHB, we found that the majority of mCherry-labeled mesohabenular axon terminals coexpressed VGluT2 and VGaT (69.07% ± 1.13%; 1,415 of 2,043; Fig. 2b–l). A smaller population of mesohabenular axon terminals contained VGluT2 without VGaT (28.18% ± 1.09%; 572 of 2,043) or VGAT without VGluT2 (2.8 ± 0.1%; 56 of 2,043) (Fig. 2b–l). These findings indicate that the majority of axon terminals from mesohabenular VGluT2+ GABA+ neurons are endowed with the capability for vesicular accumulation of both glutamate and GABA in the same terminals. By immunoelectron microscopy, we confirmed expression of VGluT2 in mCherry-labeled mesohabenular axon terminals (Fig. 2m). These mCherry-labeled VGluT2+ terminals established asymmetric synapses with dendritic spines or dendrites, and they synapsed on more than one postsynaptic structure (Fig. 2m and Supplementary Fig. 6). We also detected the expression of VGaT in mCherry-labeled mesohabenular axon terminals. As in the rat, some mouse mesohabenular terminals (Fig. 2n) exhibited puncta adhaerentia. Ultrathin serial section analysis of these terminals revealed...
that single mCherry-labeled VGAT+ axon terminals established both asymmetric and symmetric synapses on LHb neurons (Fig. 2n–p and Supplementary Fig. 7). These ultrastructural findings provide further evidence that VGluT2+ GABA+ mesohabenular neurons have the capacity for the vesicular accumulation of each of glutamate and GABA in the same axon terminals and that these terminals can simultaneously establish asymmetric and symmetric synapses. VGluT2 and VGAT are likely sorted to individual vesicles21, as emerging evidence indicates that these types of transporters have specific sorting signals determined by selective motifs in their molecular structures22.

We next applied confocal fluorescence and electron microscopy to determine whether glutamate and GABA receptors in the LHb are postsynaptic to single mesohabenular axon terminals. By three-dimensional reconstruction of confocal images of triple fluorescent immunolabeled LHb, we found that mCherry-labeled mesohabenular axon terminals were present in the vicinity of both GABAA receptors and glutamate type 1 AMPA receptors (GluR1) (Fig. 3a,b). By immunoelectron

| Table 1 Mesohabenular neurons expressing VGluT2 mRNA, GAD mRNA or TH immunoreactivity |
|-----------------------------------------------|
|    | VGluT2+ | VGluT2+ | VGluT2+ | VGluT2+ | VGluT2- | VGluT2- | VGluT2- | VGluT2- |
|    | GAD+ TH+ | GAD+ TH+ | GAD+ TH+ | GAD+ TH+ | GAD+ TH+ | GAD+ TH+ | GAD+ TH+ | GAD+ TH+ |
| Rat 2 | 55.3% (n = 105) | 25.8% (n = 49) | 4.7% (n = 9) | 7.4% (n = 14) | 2.6% (n = 5) | 1.6% (n = 3) | 0.5% (n = 1) | 2.1% (n = 4) |
| Rat 3 | 59.8% (n = 98) | 22.6% (n = 37) | 8.5% (n = 14) | 4.9% (n = 8) | 2.4% (n = 4) | 0.6% (n = 1) | 0% (n = 0) | 1.2% (n = 2) |
| Rat 5 | 55.3% (n = 68) | 20.3% (n = 25) | 7.3% (n = 9) | 6.5% (n = 8) | 5.7% (n = 7) | 2.4% (n = 3) | 0.8% (n = 1) | 1.6% (n = 2) |
| Mean ± s.e.m. | 56.8 ± 1.5% | 22.9 ± 1.6% | 6.9 ± 1.1% | 6.3 ± 0.7% | 3.6 ± 1.1% | 1.5 ± 0.5% | 0.4 ± 0.2% | 1.7 ± 0.3% |

Percentages refer to the numbers of FG retrogradely labeled VTA neurons coexpressing VGluT2 mRNA, GAD mRNAs or TH immunofluorescence divided by the total number of FG-labeled VTA neurons. Numbers in parentheses indicate number of counted neurons of that phenotype. FG-VTA cell counting was performed on 9 or 10 sections each from rats 2, 3 and 5 (Supplementary Fig. 1), between bregma –4.9 mm and –6.1 mm.
Figure 3 Presence of both glutamatergic and GABAergic receptors postsynaptic to single mesohabenular axon terminals. (a) Cre-inducible AAV-DIO-ChR2-mCherry vector injected into VTA of VGluT2-Cre mice. Mesohabenular axon terminals (ATs) were identified by mCherry expression and examined under confocal (b) or electron microscopy (c-f). (b) Mesohabenular fiber expressing mCherry (red). Single focal planes are shown for immunolabeling of GluR1-containing AMPA receptors (green), GABA<sub>A</sub> receptor (blue) and a merge. Far right panel is a three-dimensional reconstruction of merged z-stacks showing GluR1 (arrow) and GABA<sub>A</sub> receptors (arrowheads) proximal to an mCherry-labeled mesohabenular fiber. (c,d) LHb sections from a VGluT2-ChR2-mCherry mouse showing mCherry detection by immunoperoxidase labeling (scattered dark material) and detection of either GluR1 or GABA<sub>A</sub> receptor by immunogold. Experiments were repeated successfully three times. (c) Detection of GluR1 (black arrowhead) at the asymmetric synapse (black arrow) postsynaptic to an mCherry-labeled AT that simultaneously forms a symmetric synapse (blue arrow). (d) Detection of GABA<sub>A</sub> receptor (blue arrowhead) at the symmetric synapse (blue arrow) postsynaptic to an mCherry-labeled AT that simultaneously forms an asymmetric synapse (black arrow). Experiments were repeated successfully three times. (e,f) Rat LHb serial sections. Detection of GluR1 receptor by immunogold and detection of GABA<sub>A</sub> receptor by immunoperoxidase (scattered dark material). A single AT is seen establishing two asymmetric synapses (black arrows) with postsynaptic GluR1 receptors (black arrowheads) and simultaneously establishing a symmetric synapse (blue arrows) with postsynaptic GABA<sub>A</sub> receptors (blue arrowheads). Axon terminals are outlined by red dots and LHb dendrites (De) by white dots.

Microscopy, we found that single mCherry-labeled mesohabenular axon terminals had GluR1 postsynaptic to their asymmetric synapses and GABA<sub>A</sub> receptors postsynaptic to their symmetric synapses (Fig. 3c,d). We also observed this arrangement of a single mesohabenular axon terminal establishing asymmetric synapses containing postsynaptic GluR1 and establishing symmetric synapses containing postsynaptic GABA<sub>A</sub> receptors in the rat LHb (Fig. 3e,f). From our anatomical findings, we concluded that, in the mouse and in the rat, VTA projections to LHb are capable of accumulating each of glutamate and GABA into synaptic vesicles within a single axon terminal that establishes asymmetric synapses with postsynaptic glutamate receptors and symmetric synapses with postsynaptic GABA receptors.

Mesohabenular cotransmission of glutamate and GABA

The synaptic organization of mesohabenular axon terminals detailed above led us to hypothesize that mesohabenular terminals cotransmit glutamate and GABA onto LHb neurons. To test this hypothesis, we performed intracellular recordings from individual LHb neurons in brain slices obtained from mice expressing ChR2-eYFP under the control of the VGluT2 promoter (VGluT2-ChR2 mice; Fig. 4a,b). Light stimulation (5 ms) of ChR2-eYFP mesohabenular fibers evoked synaptic currents that displayed both a fast inward current and a slower outward current in eight of nine recorded LHb neurons (six VGluT2-ChR2 mice; Fig. 4c,d). LHb neurons were voltage-clamped at −70 mV (near the null potential for chloride) to isolate the inward synaptic current and at −50 mV to isolate the outward current. The light-evoked inward current was selectively blocked by the AMPA-receptor antagonist NBQX (5 µM) without affecting the light-evoked outward current (Fig. 4e,f). These findings indicate that the inward synaptic currents are mediated by glutamatergic transmission. In contrast, the GABA<sub>A</sub>-receptor antagonist picrotoxin (50 µM) blocked the light-evoked outward currents (Fig. 4c,f) and enhanced the light-evoked glutamatergic inward currents (Fig. 4c,f). These findings indicate that the outward currents were mediated by GABAergic transmission. The short latency and low jitter of light-evoked currents (Supplementary Fig. 8a,b), together with the persistence of the light-evoked GABAergic outward current during NBQX application (Fig. 4e), suggests that the GABAergic outward current is mediated by monosynaptic GABA release rather than polysynaptic glutamate-driven GABA release. To test whether light stimulation of ChR2-eYFP mesohabenular terminals results in both monosynaptic inward currents and outward currents, LHb neurons from VGluT2-ChR2 mice or rats injected in the VTA with a nonspecific calcium/calmodulin-dependent kinase-α (CaMKII<sub>α</sub>-ChR2-eYFP vector (CaMKII<sub>α</sub>-ChR2 rats) were recorded with and without tetrodotoxin (TTX)23–25. We replicated the inward glutamatergic currents and outward GABAergic currents in both VGluT2-ChR2 mice and CaMKII<sub>α</sub>-ChR2 rats in response to light stimulation of ChR2-eYFP fibers in the absence of TTX (Supplementary Fig. 8c). TTX eliminated both inward and outward light-evoked currents, and both inward and outward light-evoked currents were recovered by blocking the delayed rectifier potassium channel with 4-aminopyridine (Supplementary Fig. 8d). Thus, both glutamate and GABA are released monosynaptically from mesohabenular axon terminals.
To confirm glutamatergic and GABAergic cotransmission by mesohabenular terminals from dual VGluT2-ChR2, GABA+ VTA neurons, we next performed intracellular recordings from individual LHB neurons in brain slices obtained from mice expressing ChR2-eYFP under the control of the VGaT promoter (VGaT-ChR2 mice; Fig. 4g). As in the results obtained with VGluT2-ChR2 mice, light stimulation of VGaT-ChR2 mesohabenular fibers evoked inward currents that were blocked by NBQX (1 μM) without affecting the light-evoked outward currents (Fig. 4h). Furthermore, in the same LHB neurons, light-evoked outward currents were also blocked by picrotoxin (50 μM) (Fig. 4h). Thus, using two transgenic mouse lines and wild-type rats, we have demonstrated that in the adult rodent brain axon terminals from the VTA cotransmit glutamate and GABA onto LHB neurons. Although this type of cotransmission has been postulated to occur in the CNS in adulthood\textsuperscript{26}, these results provide the first direct evidence for such a synaptic response.

Firing patterns evoked by mesohabenular activation in vivo

To determine the net effect of mesohabenular currents upon LHB firing patterns, we recorded from spontaneously active LHB neurons in urethane-anesthetized mice (VGluT2-ChR2 mice and VGaT-ChR2 mice; Fig. 5a) and in CaMKIIα-ChR2 rats (Fig. 5b). To mimic the burst firing output of nondopaminergic VTA neurons in response to rewarding or aversive events\textsuperscript{27,28}, mesohabenular fibers were optically stimulated using parameters that evoked burst firing in VGluT2 neurons (10-ms pulses of 473-nm light presented every 2 s; Supplementary Fig. 9). This stimulation evoked inhibitory or excitatory firing patterns in LHB neurons, characterized as follows: (i) a
Figure 5 In vivo optical stimulation of mesohabenular inputs evokes inhibition in most LHb neurons and elicits excitation in some. (a) Cre-inducible AAV-DIO-ChR2-eYFP vector injected into VTA of VGluT2::Cre (VGluT2-ChR2) mice or VGaT::Cre (VGaT-ChR2) mice. (b) AAV-CaMKIIa-ChR2-eYFP vector injected into VTA of CaMKIIa-ChR2 rats. (c–f) LHb single-unit recordings and local optical stimulation (10-ms pulse; blue bars). (c) Four responses were found: a fast inhibition followed by slow return to pre-stimulation activity (inhibition), a fast inhibition followed by excitation (inhibition-excitation), a fast, brief excitation (excitation) or a fast, brief excitation followed by inhibition (excitation-inhibition). Top panel shows rasters of timing of action potentials (black dots) for all optical stimulation trials and peristimulus time histograms (bottom) aligned to optical stimulation onset. Inset (right) shows outlined portion of excited neurons’ latency and jitter spike times in response to light stimulation. (d–f). Summary of light-evoked LHb responses (duration panels show mean ± s.e.m.) from VGluT2-ChR2 mice (n = 59 neurons from 16 mice; d), VGaT-ChR2 mice (13 neurons from 5 mice; e) and CaMKIIa-ChR2 rats (21 neurons from 9 rats; f).

short latency decrease in firing rate followed by slow return to pre-stimulation firing rates (inhibition); (ii) a short latency decrease in firing rate followed by increased firing rates (inhibition-excitation); (iii) a short latency increase in firing rate (excitation) or (iv) a short latency increase in firing rate followed by decreased firing rates (excitation-inhibition) (Fig. 5c). Approximately 70% of LHb neurons were initially inhibited and 30% were initially excited by single brief light stimulations of mesohabenular fibers, and this was independent of both the rodent species and the ChR2 promoter (Fig. 5d–f). 38 of 54 LHb neurons from 13 VGluT2-ChR2 mice, 11 of 13 LHb neurons from 4 VGaT-ChR2 mice and 16 of 21 LHb neurons from 7 CaMKIIa-ChR2 rats. The inhibited and excited LHb neurons were broadly distributed throughout the LHb (Supplementary Fig. 10). The light-evoked inhibition of LHb neurons in VGluT2-ChR2 mice was significantly reduced by local application of the GABAA receptor antagonists bicuculline (20.7 ± 6.3% of baseline firing rate before bicuculline application versus 93.7 ± 20.3% of baseline firing rate after bicuculline application) or picrotoxin (20.9 ± 4.8% of baseline firing rate before picrotoxin application versus 70.8 ± 16.4% of baseline firing rate after picrotoxin application; Fig. 6). Though GABAA receptor antagonists can act on small-conductance potassium channels29, the picrotoxin and bicuculline sensitivity of mesohabenular inhibition further supports the GABA release capabilities of VTA VGluT2+ GABA+ projections to LHb.

We next examined the light-evoked excitation of LHb neurons in VGluT2-ChR2 mice. Two different components of the excitation were uncovered by the intra-LHb administration of a cocktail of AMPA and NMDA receptor antagonists (CNQX and AP5): an early excitation sensitive to CNQX–AP5 (mean ± s.e.m. response magnitude 73.1 ± 14.3 spikes during baseline and 28.2 ± 3.7 spikes following CNQX–AP5 administration; Fig. 7) and a delayed excitation insensitive to CNQX–AP5 (Fig. 7a–d). These findings demonstrate that stimulation of mesohabenular VGluT2+ GABA+ fibers is capable of increasing the spiking activity of LHb neurons by glutamate release and that some of the excitation is independent of glutamate receptor activation. In conclusion, the molecular and synaptic architecture of the VGluT2+ GABA+ mesohabenular projection is organized to integrate signals that result in complex functional changes in LHb neurons.

DISCUSSION

We demonstrate using both anatomical and electrophysiological approaches that the major ascending projection from VTA to LHb arises from a newly identified type of neuron that coexpresses VGluT2 and GAD mRNA. VGluT2-expressing VTA neurons are known to be highly heterogeneous30, and the discovery of the VTA VGluT2+ GABA+ neurons herein provides further evidence that glutamatergic VTA neurons participate in diverse functions. Within the LHb, single axon terminals from these VGluT2+ GABA+ neurons express vesicular transporters responsible for accumulating both glutamate (via VGluT2) and GABA (via VGaT). Postsynaptic to a single mesohabenular axon terminal are both GABA receptors and GluR1 receptors. Consistent with these anatomical findings, selective activation of mesohabenular terminals evokes both monosynaptic glutamate
**Figure 6** Mesohabenular light-evoked inhibition is sensitive to GABA<sub>A</sub> antagonists in vivo. (a) Delivery of a Cre-inducible AAV-DIO-ChR2-eYFP vector into the VTA of VGluT2::Cre mice. An optical fiber and a drug barrel (filled with a GABA<sub>A</sub> antagonist, either 0.5 mM picrotoxin or 0.5 mM bicuculline) were glued to a glass electrode. Light pulses (473 nm; 10 ms) were delivered every 2 s. (b–e) Inhibition of a LHb neuron in response to 10-ms mesohabenular fiber light stimulation (trials 1–200; raster in b, peristimulus time histogram (PSTH) in c). Local infusion of bicuculline (50 nl) blocked mesohabenular light-evoked inhibition (trials 201–400, raster in b, PSTH in d). The inhibition was recovered 20 min after application of bicuculline (trials 401–800, raster in b, PSTH in e). (f–h) Population analysis of mesohabenular light-evoked inhibition. (f) Percentage change in firing rate evoked by mesohabenular fibers light stimulation before (baseline) and after application of artificial cerebral spinal fluid (ACSF). ACSF had no effect on light-evoked inhibition (paired t-test, t(4) = 1.62; P = 0.18, n = 5 neurons from 3 mice). (g) Percentage change in firing rate evoked by mesohabenular fiber light stimulation before (baseline) and within 20 min of local infusion of bicuculline. Bicuculline significantly attenuated mesohabenular light-evoked inhibition (paired t-test, t(5) = 3.2; P = 0.024, n = 6 neurons from 3 mice). (h) Percentage change in firing rate evoked by mesohabenular fiber light stimulation before (baseline) and within 20 min of local infusion of picrotoxin. Picrotoxin significantly attenuated mesohabenular light-evoked inhibition (paired t-test, t(6) = 3.0; P = 0.03, n = 7 neurons from 6 mice). *P < 0.05.

Cotransmission of glutamate and GABA from VTA to LHb

Cotransmission of glutamate and GABA extends previous findings demonstrating cotransmission of glutamate and dopamine, or GABA and dopamine, from VTA neurons. Because LHb appears to lack local inhibitory circuitry, we hypothesize that cotransmission of glutamate and GABA from mesohabenular axons may in some cases facilitate the shunting of excitatory currents, thereby balancing excitation and inhibition. Cotransmission of glutamate and GABA from VTA to LHb is likely a consequence of their unique synaptic architecture, which we have shown here to be a surprisingly common type of synaptic structure in the LHb. We found that the majority of LHb axon terminals coexpressed VGluT2 and VGlut, and that more than three-quarters of the GABA axon terminals coexpressed VGluT2. Thus, the LHb is the first recognized brain structure in which the majority of GABAergic inputs are also glutamatergic. The present data indicate that the VTA is a major source of these glutamate-GABA inputs to LHb. In addition, the mesohabenular axon terminals expressing both VGluT2 and VGlut that simultaneously establish asymmetric (associated with synaptic excitation) and symmetric (associated with synaptic inhibition) synapses represents a newly identified synaptic arrangement.

Consistent with dual asymmetric and symmetric synapses from a single mesohabenular glutamatergic/GABAergic axon terminal, in vivo activation of these axon terminals evokes either fast inhibition that may be followed by excitation or fast excitation that may be followed by inhibition. LHb neurons postsynaptic to mesohabenular axon terminals showed coexpression of GABA<sub>A</sub> receptors and GluR1-containing AMPA receptors, which are likely to contribute to the detected multimodal responses of LHb neurons after activation of mesohabenular terminals. The multimodal responses of LHb neurons may reflect differential activation of postsynaptic LHb glutamate or GABA receptors after release of glutamate and GABA from the same mesohabenular axon terminal. In addition, the degree of shunting of glutamatergic responses by the concurrent release of GABA may depend on the activity pattern or resting membrane potential of individual postsynaptic LHb neurons. Regardless of the mechanism, our findings demonstrate that whereas glutamate release consistently resulted in increased LHb firing rates, GABA release resulted in either inhibition of firing alone or initial inhibition followed by a rebound excitation of firing, suggesting that a major function of the mesohabenular pathway is to provide a temporally controlled change in LHb neuron firing rates.

**Potential implications**

The timescale of changes in firing rate in LHb neurons evoked by activation of mesohabenular terminals is similar to the timescale of changes in firing rate by LHb neurons and nondopaminergic VTA neurons in response to rewards, punishers, their predictors and other salient stimuli, suggesting a role for mesohabenular neurons in these processes. Moreover, rodent models of depression and addiction have shown alterations in the molecular composition of LHb glutamate and GABA receptors, which are both postsynaptic to single mesohabenular axon terminals, raising the possibility that
Figure 7 Mesohabenular light-evoked excitation is sensitive to glutamate receptor antagonists in vivo. (a,b) Two examples of mesohabenular light-evoked excitation. Left top and bottom panels show results from LHb neurons before and immediately after local infusion of a cocktail of the AMPA and NMDA receptor antagonists CNQX (50 µM) and AP5 (100 µM). Right, examples of raw signals. In the neuron shown in a, CNQX-AP5 blocked the mesohabenular-evoked excitation. The excitation that occurred during the laser stimulation (blue shading) in the neuron shown in b was blocked by CNQX-AP5; a secondary delayed excitation remained. (c–e) Population analysis of mesohabenular light-evoked excitation. (c) Changes in absolute spiking activity of LHb neurons. CNQX-AP5 decreased both spontaneous and mesohabenular light-evoked excitation. (d) After normalizing the firing activity of LHb neurons by their pre-stimulus spontaneous activity, two components of mesohabenular light-evoked excitation were evident: a fast component sensitive to CNQX-AP5 and a delayed component insensitive to CNQX-AP5. (e) Quantitative analysis of the mesohabenular light-evoked excitation. All LHb neurons showed a decrease in the number of spikes evoked by stimulation of mesohabenular fibers (paired t-test, t(6) = 5.8; P = 0.0007, left panel; n = 7 neurons from 4 VGluT2-ChR2 mice). In five neurons, CNQX-AP5 produced a larger decrease in mesohabenular light-evoked excitation relative to their spontaneous activity. In three neurons, CNQX-AP5 produced a decrease in the spontaneous activity that was larger than that observed upon mesohabenular light-evoked excitation, resulting in an increase in the percentage change (right panel). ***P < 0.001.

mesohabenular system plays a role in these pathologies. Considering the complexity of depression and addiction, disorders involving VTA and LHb dysfunction, the discovery of a single, anatomically defined pathway capable of exciting or inhibiting LHb neurons provides insight into the interactions of brain regions responsible for these disorders.

METHODS

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

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

M.M., D.H.R. and C.A.M.-A. conceived this project. H.-L.W. and D.H.R. performed retrograde tract tracing and in situ hybridization experiments. Cellular characterization and analysis were done by M.M., H.-L.W., C.A.M.-A. and D.H.R. S.Z. performed confocal and electron microscopy experiments, the result of which were analyzed by M.M. and S.Z. C.R.L. and A.E.H. designed the whole-cell electrophysiological experiments. A.E.H. performed these experiments and A.E.H. and C.R.L. analyzed them. C.A.M.-A. and D.H.R. performed and analyzed in vivo electrophysiological recording experiments. M.M., C.A.M.-A. and D.H.R. wrote the manuscript with the contribution of all authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals and surgical procedures. All animal procedures were performed in accordance with NIH Guidelines and approved by the NIDA Animal Care and Use Committee.

Retrograde tracer injections. Male Sprague-Dawley rats (10–15 weeks; 400–450 g) were anesthetized with Equithesin (3.3 ml/kg, i.p.) in a physiological saline solution. FluoroGold (FG: 1% in cacadoylate buffer, pH 7.5) was delivered unilaterally into the lateral habenula (LHb) (−3.4 mm anteroposterior (AP), 0.9 mm mediolateral (ML) and 5.4 mm dorsoventral (DV) from skull) iontophoretically through a stereotactically positioned glass micropipette (inner tip diameter 18–36 μm) by applying 1 μA current in 5 s at 10 s intervals for 15 min. After each injection, the micropipette was left in place for an additional 10 min to prevent backflow. Following surgery, rats were single-housed with a reversed 12 h:12 light/dark cycle and perfused a week later.

Virus injections. Male VGlut2-T2-ires:Cre (Slc17a8tm2(cre)Lowl/J) or Vgat-T-IRES::Cre (Slc32atm2(cre)Lowl/J) mice (6–12 weeks; 20–30 g) (both in C57BL/6j background from The Jackson Laboratories, Bar Harbor, ME) and male Sprague-Dawley rats (5–10 weeks; 260–280 g; Harlan) were anesthetized with 1–5% isoflurane. Cre-dependent AAV1-EF1α-Chr2-rEYFP (for electrophysiology) or AAV5-Elf1α-Chr2-mCherry (for microscopy) was injected in the ventral tegmental area (VTA) of mice (350 nl, 100 nl/min, −3.2 mm AP, 0.0 mm ML, −4.3 mm DV from skull for electrophysiology; 200 nl, 100 nl/min, −3.4 mm AP, ±0.2 mm ML, −4.3 mm DV from skull for microscopy). Rats were injected in VTA with AAV1-CamKIIα-Chr2-eYFP (500 nl, 100 nl/min, −5.4 mm AP, −2.0 mm ML at 10°, −8.0 mm DV from skull). The CamKIIα virus was chosen to infect all types of VTA neurons. Injections were made using the UltraMicro Pump with Micro 4 controller, 10-μl Nanofil syringes and 35 gauge needles (WPI Inc, Sarasota, FL). Syringes were left in place for 10 min following injections to minimize diffusion. Following surgery, mice were housed in groups of 1–5, and rats were single housed, with a reversed 12 h:12 light/dark cycle. Six weeks after viral injections, animals were perfused or used for recordings.

Retrograde trac tract and in situ hybridization. Tissue preparation. Rats were anesthetized with chloral hydrate (0.5 ml/kg) and perfused transcardially with α-dimethylaminopropyl (DA) tetrahydrochloride (DAB) and 0.005% H2O2. Sections were mounted on coated slides. Slides were dipped in Ilford K.5 nuclear tract emulsion (Polysciences; 1:1 dilution in double-distilled water) and exposed in the dark at 4 °C for 3–4 weeks before development.

Data analysis of in situ hybridization studies. Sections were viewed, analyzed and photographed with bright-field and epiluminescence microscopy using an Olympus BX51 microscope. Neurons were observed in each traced region at high power (20 × or 40 × objective lenses) and marked electronically. Subdivisions of the VTA were traced according to previously described methods [18]. FG-VGlut2, FG-GAD and FG-VGluT2-GAD labeled material was analyzed using epiluminescence to increase the contrast of silver grains identically to the manner reported previously by our laboratory [8,33,40]. For VGlut2 in situ hybridization procedures, FG fluorescent cells containing or lacking TH fluorescent signal were photographed before processing for in situ hybridization. For all radioactive in situ hybridization methods (VGluT2 mRNA or GAD mRNA), a cell was considered to express transcripts when its soma contained concentric aggregates of silver grains above background level. FG-labeled cells expressing VGlut2 mRNA, GAD mRNA and TH were counted separately. FG (brown DAB labeled) and radioactive in situ hybridization (silver grains) double-labeled material was analyzed by the following procedure: (i) silver grains corresponding to VGlut2 mRNA or GAD mRNA expression were focused; (ii) the path of epiluminescence light was blocked without changing the focus, and (iii) bright-field light was used to determine whether a brown neuron, expressing FG in focus, contained the aggregates of silver grains seen under microscopy. FG (brown DAB product), nonradioactive GAD in situ hybridization (purple alkaline phosphatase product), and radioactive VGlut2 in situ hybridization (silver grain) triple-labeled material was analyzed by the same procedure but, in addition, a cell was considered to express DIG-labeled transcripts (GAD mRNA) when its soma was the same shape as a DAB-labeled FG cell. Neurons were counted when the stained cell was at least 5 μm in diameter. Pictures were adjusted to match contrast and brightness by using Adobe Photoshop (Adobe Systems). Cell counting was completed blind of injection site by three scorers, and the inter-rater reliability was 95.6%. Any FG-labeled neuron located outside the VTA was excluded from analysis. Number of rats analyzed was based on previous studies in our lab using radioactive detection of VGlut2 mRNA from rat VTA neurons [18,33,40].

Confocal and electron microscopy. VGlut2-T2-ires:Cre mice were anesthetized with chloral hydrate (35 mg/100 g) and perfused transcardially with a fixative solution containing 4% PF with 0.15% glutaraldehyde and 1% picric acid in 0.1 M PB. VGlut2 mRNA expression was focused under epiluminescence microscopy, and the path of epiluminescence light was blocked without changing the focus, and bright-field light was used to determine whether a brown neuron, expressing FG in focus, contained the aggregates of silver grains seen under microscopy. FG (brown DAB product), nonradioactive GAD in situ hybridization (purple alkaline phosphatase product), and radioactive VGlut2 in situ hybridization (silver grain) triple-labeled material was analyzed by the same procedure but, in addition, a cell was considered to express DIG-labeled transcripts (GAD mRNA) when its soma was the same shape as a DAB-labeled FG cell. Neurons were counted when the stained cell was at least 5 μm in diameter. Pictures were adjusted to match contrast and brightness by using Adobe Photoshop (Adobe Systems). Cell counting was completed blind of injection site by three scorers, and the inter-rater reliability was 95.6%. Any FG-labeled neuron located outside the VTA was excluded from analysis. Number of rats analyzed was based on previous studies in our lab using radioactive detection of VGlut2 mRNA from rat VTA neurons [18,33,40].

Immuno labeling for light microscopy. To determine whether the injected transduced virus was confined to VTA neurons and reporter gene mCherry was expressed in the LHb, every fifth section of the VTA or LHb was used to detect mCherry by immunohistochemistry. Sections were incubated with a blocking solution (4% bovine serum albumin (BSA) in PB supplemented with 0.3% Triton-X-100) for 1 h. Sections were then incubated with mouse anti-mCherry antibody (632543; Clontech Laboratories Inc., Mountain View, CA, 1:50 dilution) and rabbit anti-GluR1 antibody (GluR1C-Rb-Af692-1; Millipore, Billerica, MA, 1:200 dilution) or a cocktail of mouse anti-mCherry antibody, guinea pig anti-GluR1 antibody (GluR1C-Rb-Af692-1; Millipore, Billerica, MA, 1:200 dilution) and rabbit anti-Thy1 antibody (clone 2461, Millipore, Billerica, MA, 1:200 dilution) for 1 h at RT with biotinylated goat anti-rabbit secondary antibody (1:200; BA1000, Vector Laboratories, Burlingame, CA) and fluorescein-conjugated donkey anti-mouse antibody (1:50; 715-095-151, Jackson ImmunoResearch, West Grove, PA) for 1 h at 30 °C. Sections were then incubated in PB supplemented with 4% PF while sections were imaged. After image collection, sections were processed using the following staining protocol: (i) silver grains corresponding to radioactive detection of VGlut2 mRNA or GAD mRNA (transcripts encoding the two isoforms of glutamic acid decarboxylase (GAD65 or GAD67)) or double in situ hybridization (for radioactive detection of VGlut2 mRNA and nonradioactive detection of GAD mRNA) were visualized under epiluminescence microscopy, and the path of epiluminescence light was blocked without changing the focus, and bright-field light was used to determine whether a stained neuron, expressing FG in focus, contained the aggregates of silver grains seen under electron microscopy. FG (brown DAB product), nonradioactive GAD in situ hybridization (purple alkaline phosphatase product), and radioactive VGlut2 in situ hybridization (silver grain) triple-labeled material was analyzed by the same procedure but, in addition, a cell was considered to express DIG-labeled transcripts (GAD mRNA) when its soma was the same shape as a DAB-labeled FG cell. Neurons were counted when the stained cell was at least 5 μm in diameter. Pictures were adjusted to match contrast and brightness by using Adobe Photoshop (Adobe Systems). Cell counting was completed blind of injection site by three scorers, and the inter-rater reliability was 95.6%. Any FG-labeled neuron located outside the VTA was excluded from analysis. Number of rats analyzed was based on previous studies in our lab using radioactive detection of VGlut2 mRNA from rat VTA neurons [18,33,40].

Fluorescence microscopy and three-dimensional analysis. Coronal LHb sections were incubated for 1 h at RT with biotinylated goat anti-mouse secondary antibody (BA-9200, 1:200, Vector Laboratories), rinsed with PB and incubated with avidin-biotinylated horseradish peroxidase for 1 h. Sections were then incubated with 0.05% DAB and 0.003% H2O2. The specificity of primary anti-mCherry antibodies was demonstrated by the lack mCherry immuno labelling in brain sections from mice injected with saline solution without mCherry vector.

Fluorescence microscopy and three-dimensional analysis. Coronal LHb sections were incubated for 1 h in PB supplemented with 4% BSA and 0.3% Triton X-100. Sections were then incubated with a cocktail of mouse anti-mCherry antibody, guinea pig anti-VGlut2 antibody (VGlut2-T2-GP-Af240-1; Frontier Institute Co., Ltd, Japan, 1:500) and rabbit anti-VGAT antibody (VGAT-Rb-Af440; Frontier Institute Co., Ltd, Japan, 1:500) or a cocktail of mouse anti-mCherry antibody, guinea pig anti-GABA_A receptor antibody (GABAARa1-GP-Af440; Frontier Institute Co., Ltd, Japan, 1:500 dilution), rabbit anti-GluR1 antibody (GluR1C-Rb-Af692-1; Frontier Institute Co., Ltd, Japan, 1:200 dilution) overnight at 4 °C. Specificity
of primary antibodies has been previous shown. After PB rinsing, sections were incubated in either of two cocktails of fluorescent secondary antibodies (all raised in donkey; Jackson ImmunoResearch Laboratories Inc., 1:100): (i) Alexa Fluor 488 anti-guinea pig (706-545-14), Alexa Fluor 594 anti-mouse (715-585-151) and Alexa Fluor 647 anti-rabbit (711-605-152), or (ii) Alexa Fluor 488 anti-rabbit (711-545-152), Alexa Fluor 594 anti-mouse (715-585-151) and Alexa Fluor 647 anti-guinea pig (706-585-148). After rinsing, sections were mounted with Vectashield mounting medium (H1000; Vector Laboratories) on slides and air-dried. Fluorescence images were collected with Olympus FV1000 Confocal System (Olympus, Center Valley, PA). Images were taken sequentially with different lasers with 100 × oil immersion objectives and z-axis stacks were collected at intervals of 0.1 µm. Imaris microscopy software (Bitplane Inc., South Windsor, CT) was used to analyze z-stacks of confocal images from 3 mice (62 × 62 × 5 µm for each image, 4 images from each mouse between bregma −1.22 mm and −2.18 mm) to obtain three-dimensional quantification of axon terminals expressing mCherry, VGluT2 or VGaT. The same confocal images were analyzed with Amira microscopy software (Visualization Sciences Group, Burlington, MA) to obtain three-dimensional reconstruction of axon terminals. These software packages implemented quantification blindly. Differences in numbers of VGluT2+ VGaT+, VGluT2+ VGaT−, and VGluT2− VGaT+ mesohabenular terminals were analyzed in GraphPad prism 5. We analyzed over 2,000 mesohabenular axon terminal samples from 3 mice, which provided statistical power to detect small effects.

Because the data distribution was skewed, we used the nonparametric Friedman test to analyze differences between the three mesohabenular axon terminal phenotypes. Post hoc Dunn's multiple comparison tests were used to analyze differences between specific axon terminal phenotypes.

Electron microscopy. Coronal LHb sections were rinsed with 0.1 M PB (pH 7.3), incubated with 1% sodium borohydride in PB for 30 min, rinsed in PB and then incubated with blocking solution (1% normal goat serum (NGS), 4% BSA in PB supplemented with 0.02% saponin) for 30 min. Sections were incubated with cocktail of primary antibodies: mouse anti-mCherry + guinea pig anti-VGluT2 (1:400), mouse anti-mCherry (1:1,000) + rabbit anti-VGAT (1:400), mouse anti-mCherry + rabbit anti-GluR1 (1:200), mouse anti-mCherry + guinea pig anti-VGluT2 receptor (1:200), rabbit anti-GluR1 + anti-VGluT2 receptor, guinea pig anti-VGluT2 + rabbit-anti-VGAT, guinea pig anti-VGluT2 or rabbit anti-VGAT. All primary antibodies were diluted in PB with 1% NGS, and incubations were for 24 h at 4°C. All gold-coupled secondary antibodies (Nanoprobes Inc., Stony Brook, NY) were coupled to 1.4-nm gold particles and used at 1:100 dilution. Sections were rinsed and incubated overnight at 4°C in the corresponding secondary antibodies: biotinylated goat anti-mouse antibody (for mCherry detection) + anti-guinea pig IgG Fab′ fragment gold-coupled (catalog number 205; for VGluT2 detection) or biotinylated goat anti-mouse antibody (for VGaT detection) + anti-rabbit IgG gold-coupled (catalog number 203; for VGAT detection), biotinylated goat anti-mouse antibody (for mCherry detection) + anti-rabbit IgG gold-coupled (for GluR1 detection), biotinylated goat anti-mouse antibody (for GluR1 detection) + anti-guinea pig IgG Fab′ fragment gold-coupled (for GABA_A receptor detection), biotinylated goat anti-rabbit antibody (for GluR1 detection) + anti-guinea pig IgG Fab′ fragment gold-coupled (for GABA_A receptor detection), biotinylated goat anti-guinea pig antibody (for GABA_A receptor detection) + anti-rabbit IgG gold-coupled (for GluR1 detection), biotinylated goat anti-rabbit antibody (for VGluT2 detection) + anti-rabbit IgG gold-coupled (for VGluT2 detection), biotinylated goat anti-guinea pig antibody (for VGluT2 detection) + anti-rabbit IgG gold-coupled (for VGluT2 detection), or anti-rabbit IgG gold-coupled (for VGluT2 detection). Silver enhancement of the gold particles was done using the Nanoprobe Silver Kit (2012; Nanoprobes Inc.). Sections were then incubated in avidin–biotinylated horseradish peroxidase complex (1:100 dilution) in PB for 2 h at RT and incubated with 0.025% DAB and 0.003% H_2O_2 in PB. Sections were fixed with 0.5% osmium tetroxide in PB for 25 min and contrasted in 1% uranyl acetate for 35 min. Sections were dehydrated and resin embedded, and ultrathin section were prepared as we previously detailed. Sections were examined and photographed using a Tecnai G2 12 transmission electron microscope (FEI Company, Hillsboro, OR) equipped with a digital micrograph 3.4 camera (Gatan, Inc., Pleasanton, CA).

Ultrastructural analysis. Serial thin sections of LHb (bregma −1.22 mm to −2.18 mm) were analyzed. Synaptic contacts were classified according to their morphology and immunolabeling, and were photographed at magnifications of 6,800–13,000×. The morphological criteria used for identification and classification of cellular components observed in these thin sections were as previously described. Type 1 synapses, referred to here as asymmetric synapses, were defined by the presence of contiguous synaptic vesicles in the presynaptic axon terminal and a postsynaptic density (PSD) more than 40 nm thick. Type II synapses, referred to here as symmetric synapses, were defined by the presence of contiguous synaptic vesicles in the presynaptic axon terminal and a thin PSD. Puncta adhaerentia, mechanical adhesion sites between axon terminals and dendrites, were defined by the absence of synaptic vesicles in the presynaptic axon terminal and a PSD. Serial sections were obtained to confirm the type of synapse. In the serial section analyses of VGluT2 and VGAT expression, a terminal containing more than five immunogold particles was considered an immunogold-positive terminal. Pictures were adjusted to match contrast and brightness using Adobe Photoshop (Adobe Systems Incorporated, Seattle, WA). Data collection and analysis were performed blind to the conditions of the experiments.

Whole-cell recordings of LHb neurons. Slice preparation. Male VGluT2-ChR2 mice (n = 8), VGAT-ChR2 mice (n = 3) and CaMKII-eGluR2 rats (n = 3) were euthanized by isoflurane followed by rapid decapitation. The brains were then removed and placed into a chilled (4 °C) and aerated (95% O_2/5% CO_2) modified artificial cerebrospinal fluid (ACSF, consisting of (in mM) NaCl, 87; KCl, 2.5; MgCl_2, 2.5; CaCl_2, 0.5; NaH_2PO_4, 1.25; t-glucose, 25; sucrose, 75; NaHCO_3, 25. Coronal slices containing the LHb were cut (280 µm) using a vibratome. Slices were transferred to a holding chamber containing normal ACSF consisting of (in mM) NaCl 126; KCl 3.0; MgCl_2 1.5; CaCl_2 2.4; NaH_2PO_4 1.2; t-glucose, 11.0; NaHCO_3 26, saturated with 95% O_2/5% CO_2, at 35 °C for 20–25 min and subsequently maintained at RT for ≥1 h before recordings.

Intracellular recordings. Slices were submerged in a low-volume (~170 µl) recording chamber (RC-22; Warner Instruments, Hamden, CT) and continuously superfused at 2 ml/min with warm (30–32 °C) ACSF containing 40 µM d-(-)-2-amino-5-phosphonopentanoic acid (d-APV) to block NMDA receptors (control medium). Visualization of LHb neurons was performed using an upright microscope equipped with epifluorescence and differential interference contrast infrared microscopy (Axioskop-2, Carl Zeiss). Recording electrodes (3–5 MΩ) were filled with an intracellular solution consisting of (in mM) potassium glutamate, 140; KCl, 5; HEPES, 10; EGTA, 0.2; MgCl_2, 2; Mg-ATP, 4; Na_2-GTP, 0.3; Na_2-phosphocreatine, 10; pH 7.2. The intracellular solution also contained 0.2% bicytin. Neurons were sampled randomly from the LHb. Whole-cell voltage clamp recordings were performed using a Multiclamp 700B (Molecular Devices, Sunnyvale, CA). Data were recorded using WinLTP (WinLTP Ltd, Bristol, UK) and an A/D board (National Instruments PCI-6251). Optical stimulation was delivered to the slice via an optical fiber (200 µm core, Thorlabs, Newton, NJ) coupled to a 150-mW, 473-nm diode-pumped solid-state continuous-wave laser system (OEM Laser Systems Salt Lake City, UT). Stimulation consisted either of a single 5-msec pulse or of paired 5-msec pulses delivered 50 msec apart. Output of the laser was <2 mW. Light-evoked responses and the effects of AMPA receptor antagonist (NBQX) or GABA_A receptor antagonist (picrotoxin) on these responses were recorded over several holding potentials (between −45 mV and −80 mV). Order of presentation of NBQX and picrotoxin was counterbalanced and randomly assigned. All drugs were dissolved in ACSF. Paired t-tests compared the average light-evoked response in control medium to the minimum light-evoked response (peak drug effect) in drug medium. Light-evoked changes were predicted to either have no change or be abolished by drug application. The expected s.d. of pre versus post drug effects used in power calculations was 40, based on analysis of previous results. Using this criterion, a minimum of four pairs of neurons receiving both pre and post drug examination provided adequate power to detect large effects.

To evaluate monosynaptic transmission from VTA to LHb, whole-cell recordings of LHb neurons were performed under conditions described above without AMPA receptor or GABA_A receptor antagonists. Light-evoked responses were recorded at −50 mV and −70 mV holding potentials in control medium and subsequently in the presence of TTX (1 µM), followed by TTX with 4-AP (200 µM). Latency was calculated from the onset of the light pulse to 10% of the peak response. Jitter was calculated as the s.d. of the latency measured from 8–10 responses. A two-way repeated measures ANOVA was used to test for significant differences in percentage of light-evoked current from control conditions.
within rodent (CaMKIIα-ChR2 rats or VGluT2-ChR2 mice) and TTX condition (TTX or TTX/4AP). Sidak adjusted pairwise comparisons were used to examine differences between TTX conditions within each rodent species. Data collection and analysis were not performed blind to the conditions of the experiments. Following intracellular recordings, slices were stored in a 4% PF solution overnight at 4 °C, rinsed with PB and incubated in Alexa Fluor 546 streptavidin (1:500, Invitrogen) for 2 h at RT.

**In vivo single-unit recordings of LHb and VTA neurons.** In vivo recordings. Male VGluT2-ChR2 mice (n = 32), VGaT-ChR2 mice (n = 5) and CaMKIIα-ChR2 rats (n = 9) were anesthetized with urethane (1.4 g/kg, i.p.) and mounted in a stereotaxic frame. A hole was drilled in the skull to access the LHb or VTA. Glass micropipettes (6–10 MΩ) filled with 2.0% pontamine sky blue (BDH Chemicals, Poole, England) in 3 M NaCl were used for recordings. Glass electrodes were used to shield against any potential light-induced artifacts that can be observed with metal electrode recordings alone. A 100-µm-core multimode fiber was glued 350 µm dorsal to the glass electrode tip and connected to a 473-nm DPSS laser (OEM laser systems, Bluffdale, UT). Signals were amplified using a Neuro Data IR283A (Cygnus Technology, Delaware Water Gap, PA) and BrownLee 440 amplifier. Neuronal activity was bandpass filtered between 300 and 3,000 Hz. The biopotentials were digitized using a Power 1401 Analog-Digital Converter (Cambridge Electronic Design, Cambridge, UK). Spontaneously active or optogenetically driven neurons were recorded and analyzed. LHb recordings were obtained from rats between −3.0 to −3.8 mm AP, 0.5 to 1.0 mm ML and −4.3 to −5.4 DV and from mice between −1.2 to 2.0 mm AP, 0.2 to 0.6 mm ML and −2.9 to −3.6 mm DV. Entrance into the LHb was demarcated by passage through the hippocampus. Immediately following the hippocampus a rise in background noise was observed through the ventricle, after which background noise subsided and neurons were recorded in the LHb with electrophysiological properties as previously described. VTAs were recorded from VGluT2-ChR2 mice between −3.1 to −3.5 mm AP, 0 to 0.5 mm ML and −4.2 to −4.6 mm DV. Entrance into the VTA was demarcated by passage through a layer of fast-firing neurons dorsal to the VTA. Neurons were randomly sampled by slowly lowering the micropipette through the LHb or VTA while 10-16 pulses of 473-nm light (20 mW) were applied every 2 s. After encountering a neuron, baseline activity was recorded for 2 to 5 min, and peristimulus time histograms were constructed at 5-ms resolution bins, with a window width of 0.5 s before and 1 s after the stimulus onset. Fidelity was defined as the percentage of occasions in which laser stimulation evoked spiking in the recorded neuron. Signal to noise ratio was typically >5:1. After recordings, animals were perfused with 4% PF, and LHb and VTA cryosections (30 µm) were prepared to verify the deposit of the micropipette. Staining with a mouse anti-GFP antibody (1:400, JL-8, Clontech, Mountain View, CA), as described for mCherry detection. Neurons recorded external to the LHb or VTA were excluded from the analysis. The specificity of primary anti-GFP antibodies was demonstrated by the lack GFP immunolabeling in brain sections from mice injected with saline solution without GFP.

**Local drug infusion.** A double-barrel pipette was assembled for local injection of drug while simultaneously recording LHb neurons. The recording pipette was similar to that described above in the *in vivo* recording section and protruded approximately 130 µm beyond the injection tip (25–30 µm internal diameter). A 100-µm diameter optic fiber was glued 350 µm dorsal to the electrode tip. The injection pipette was filled with 50 nM bicuculline, 500 nM picrotoxin, a cocktail of 50 µM CNQX and 100 µM AP-5, or vehicle. All drugs were dissolved in ACSF. After isolating a single LHb neuron, spontaneous activity was recorded to establish baseline activity, followed 100–200 10-ms, 473-nm light pulses at 20 mW to determine responses to optical activation of VTA fibers on LHb activity. After 5 min of baseline activity, drug was delivered (50–200 nl) using brief pulses of pneumatic pressure (Picospritzer, General Valve, Fairfield NJ), and the drug effects were assessed every 10 min.

**Data analysis.** For single 10-ms, 473-nm light pulses, peristimulus time histograms (PSTHs) of 5 ms bin width of LHb or VTA activity were generated. PSTHs were analyzed to determine excitatory and inhibitory epochs. Mean and s.d. of counts per bin were determined for a baseline period, defined as the 500-ms epoch preceding stimulation. The onset of excitation was defined as the first bin for which the mean value exceeded mean baseline activity by 2 s.d., and response offset was determined as the time at which activity had returned to be consistently within 2 s.d. of baseline. Inhibition was defined as an epoch of at least 20 ms in which the mean count per bin was at least 30% less than that during baseline. Excitatory and inhibitory response magnitudes (R mags) were normalized for different levels of baseline impulse activity, allowing comparison of drug effects on evoked responses independent of effects on baseline activity. R mags for excitation were calculated with the following equation: Excitation R mag = (counts in excitation epoch) − (mean counts per baseline bin × number of bins in excitation epoch). Some neurons exhibited a secondary response: either excitation after inhibition or inhibition after excitation. Neurons were categorized by the first occurring response. We only analyzed the initial response that occurred after optical activation. Sample size was determined as described in the “Intracellular recordings” sections. Data collection and analysis were not performed blind to the conditions of the experiments.

**Statistics.** Parametric statistics were used after verifying compliance for normal distribution and equal variance assumptions. One-way and two-way ANOVA were used to compare between-groups effects. Repeated-measures ANOVAs were used to compare within-group effects across time. Normality was assessed using the D’Agostino and Pearson omnibus normality test. Equality of variance was assessed with the Bartlett’s test for equal variances. The sphericity of the matrix assumption for repeated-measures ANOVA was assessed with the Mauchly sphericity test; when the outcome of the test was significant, the F values were corrected using the Greenhouse–Geisser approach. Newman-Keuls *post hoc* test was used to establish differences in means after the ANOVA. When requirements for parametric statistics were not met, a Kruskal–Wallis test was used to compare between-group differences.

A **Supplementary Methods** checklist is available.

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