Inhibitory Input from the Lateral Hypothalamus to the Ventral Tegmental Area Disinhibits Dopamine Neurons and Promotes Behavioral Activation

Highlights
- Activating GABAergic LH-VTA supports positive reinforcement
- GABAergic LH-VTA activation inhibits VTA GABA neurons
- GABAergic LH-VTA activation increases dopamine release in the NAc
- Activating glutamatergic LH-VTA causes avoidance and suppresses DA release in the NAc

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In Brief
Nieh et al. demonstrate that inhibitory inputs from the lateral hypothalamus disinhibit dopamine neurons in the ventral tegmental area to increase motivated behaviors, including approach and social interaction. In contrast, excitatory projections suppress dopamine release and promote avoidance.
Inhibitory Input from the Lateral Hypothalamus to the Ventral Tegmental Area Disinhibits Dopamine Neurons and Promotes Behavioral Activation

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SUMMARY

Projections from the lateral hypothalamus (LH) to the ventral tegmental area (VTA), containing both GABAergic and glutamatergic components, encode conditioned responses and control compulsive reward-seeking behavior. GABAergic neurons in the LH have been shown to mediate appetitive and feeding-related behaviors. Here we show that the GABAergic component of the LH-VTA pathway supports positive reinforcement and place preference, while the glutamatergic component mediates place avoidance. In addition, our results indicate that photoactivation of these projections modulates other behaviors, such as social interaction and perseverant investigation of a novel object. We provide evidence that photostimulation of the GABAergic LH-VTA component, but not the glutamatergic component, increases dopamine (DA) release in the nucleus accumbens (NAc) via inhibition of local VTA GABAergic neurons. Our study clarifies how GABAergic LH inputs to the VTA can contribute to generalized behavioral activation across multiple contexts, consistent with a role in increasing motivational salience.

INTRODUCTION

Dopamine (DA) release from ventral tegmental area (VTA) DA neurons promotes goal-directed behavior (Gallistel et al., 1985; Grace et al., 2007; Phillips et al., 2003), enhances the salience of environmental stimuli (Berridge and Robinson, 1998; Everitt et al., 1999; Wyvell and Berridge, 2000), increases behavioral vigor (Niv et al., 2007; Salamone et al., 1994, 2005), and mediates the reinforcing properties of reward (Di Chiara and Imperato, 1988; Roberts and Koob, 1982; Wise, 2006). Importantly, excitotoxic lesions of the lateral hypothalamus (LH) evoke similar pathologies to those observed after DA depletion, including aphagia (Grossman et al., 1978; Stricker et al., 1978), which suggests that LH input to the VTA is a critical circuit element in modulating motivation, perhaps via its action on VTA DA neurons. Indeed, the LH provides one of the most robust inputs to the VTA (Phillipson, 1979; Watabe-Uchida et al., 2012).

The LH has been historically implicated in both reward processing (Hoebel and Teitelbaum, 1962; Olds and Milner, 1954) and feeding behaviors (Anand and Brobeck, 1951; Burton et al., 1976; Powley and Keesey, 1970). The cells that comprise the LH-VTA projection are diverse: glutamatergic, GABAergic, and/or peptidergic in nature. Several studies have shown modulatory effects of LH peptidergic populations on the VTA, including orexin/hypocretin (Borgland et al., 2006; Harris et al., 2005) and neurotensin (Kempadoo et al., 2013; Opland et al., 2013). While these studies clearly demonstrate that the peptidergic LH-VTA circuit modulates reward and motivation, recent studies have also highlighted the importance of GABAergic and glutamatergic neuronal populations in the LH. Jennings and colleagues identified a GABAergic population in the LH, independent of the melanin-concentrating hormone (MCH) and orexin/hypocretin populations, that encodes reward seeking or feeding (Jennings et al., 2015).

Additionally, we recently demonstrated that activation of the GABAergic LH projection to the VTA increases feeding, while the glutamatergic projection may play more of a regulatory role (Nieh et al., 2015). However, as previous studies have shown, feeding behavior can be driven by either the motivation to escape the negative affective state of hunger (Betley et al., 2015) or the motivation to obtain food as a primary reinforcer (Jennings et al., 2015). Our first goal was to determine whether the motivation to engage in feeding behavior evoked by GABAergic LH-VTA stimulation was due to the aversive drive state associated with hunger (negative valence) or the rewarding properties associated with food (positive valence).

Furthermore, previous studies have shown that nonspecific hypothalamic activation via electrical stimulation can elicit feeding, drinking, gnawing, motor effects, as well as sexual behaviors (Singh et al., 1996; Valenstein et al., 1968). As a result, our second goal was to investigate whether LH-VTA stimulation was specific to controlling feeding or generalizable across multiple motivated behaviors.
Finally, LH projections to the VTA likely influence motivation by modulating the activity of DA neurons. It has been suggested that activation of the glutamatergic component of the LH-VTA projection provides excitatory drive onto VTA DA neurons (Kempadoo et al., 2013; You et al., 2001). Kempadoo and colleagues showed that NMDA blockade in the VTA attenuates the ability of neuropeptide-expressing LH-VTA projections to drive reward seeking (Kempadoo et al., 2013). However, it is unknown how LH input to the VTA modulates DA release in downstream targets, because the VTA is also a heterogeneous structure and contains dopaminergic, GABAergic, and glutamatergic cell types (Dobi et al., 2010; Nair-Roberts et al., 2008). Therefore, our third goal was to elucidate the downstream effects of GABAergic and glutamatergic LH-VTA inputs on DA neurotransmission.

RESULTS

Activation of the GABAergic or Glutamatergic LH-VTA Projection Promotes Approach or Avoidance, Respectively

To study the effect of GABAergic LH-VTA activation on behavior, we injected AAV$_V$-DIO-ChR2-eYFP or AAV$_V$-DIO-eYFP into the LH of vesicular GABA transporter (VGAT):Cre mice, and we placed an optic fiber over the VTA to illuminate LH GABAergic axon terminals (Figure 1A and see Figure S1 available online). To test whether stimulating the GABAergic component of the LH-VTA projection (LHGABA-VTA) would support place preference or avoidance, we placed mice into a three-chamber apparatus where one side of the chamber was paired with blue light stimulation (473 nm, 10 Hz, 20 mW, 5-ms pulses), is shown.

LHGABA-VTA:eYFP mice had a significantly greater difference score (percentage time spent in stimulation side minus percentage time spent in non-stimulation side) than LHGABA-VTA:eYFP mice (n = 8 ChR2, n = 10 eYFP; two-tailed, unpaired Student’s t test, **** p < 0.0001). LHGABA-VTA:ChR2 mice made significantly more responses at the active nose poke paired with blue light stimulation (473 nm, 10 Hz, 20 mW, 5-ms pulses, 1-s duration) than the inactive nose poke as compared with eYFP controls (n = 6 ChR2, n = 8 eYFP; two-way ANOVA revealed a group x nose poke interaction, F$_{1,12}$ = 19.40, p = 0.0009; Bonferroni post hoc analysis, *** p < 0.001).

LHglut-VTA mice had a significantly lower difference score than LHglut-VTA:eYFP mice in the RTPP/A assay (n = 7 ChR2, n = 9 eYFP; two-tailed, unpaired Student’s t test, * p = 0.0175). Optical stimulation did not have any significant effect on ICSS in LHglut-VTA:ChR2 mice compared with eYFP controls (n = 7 ChR2, n = 6 eYFP; two-way ANOVA: group x nose poke interaction, F$_{1,11}$ = 0.05, p = 0.8307).

Error bars indicate ± SEM. See also Figures S1 and S2.

Figure 1. Photostimulation of the GABAergic LH-VTA Projection Promotes Approach, while Activation of the Glutamatergic LH-VTA Projection Promotes Avoidance

(A) VGAT::Cre mice were injected with AAV$_V$-DIO-ChR2-eYFP or AAV$_V$-DIO-eYFP into the LH, and an optic fiber was implanted over the VTA.

(B) Representative track from the real-time place preference/avoidance (RTPP/A) assay of an LHGABA-VTA:ChR2 mouse moving through an open chamber, where one side was paired with blue light stimulation (473 nm, 10 Hz, 20 mW, 5-ms pulses), is shown.

(C) LHGABA-VTA:ChR2 mice had a significantly greater difference score (percentage time spent in stimulation side minus percentage time spent in non-stimulation side) than LHGABA-VTA:eYFP mice (n = 8 ChR2, n = 10 eYFP; two-tailed, unpaired Student’s t test, **** p < 0.0001).

(D) LHGABA-VTA:ChR2 mice made significantly more responses at the active nose poke paired with blue light stimulation (473 nm, 10 Hz, 20 mW, 5-ms pulses, 1-s duration) than the inactive nose poke as compared with eYFP controls (n = 6 ChR2, n = 8 eYFP; two-way ANOVA revealed a group x nose poke interaction, F$_{1,12}$ = 19.40, p = 0.0009; Bonferroni post hoc analysis, *** p < 0.001).

(E) VGLUT2::Cre mice were injected with AAV$_V$-DIO-ChR2-eYFP or AAV$_V$-DIO-eYFP into the LH, and an optic fiber was implanted over the VTA.

(F) Representative track from the RTPP/A assay of an LHglut-VTA:ChR2 mouse is shown.

(G) LHglut-VTA:ChR2 mice had a significantly lower difference score than LHglut-VTA:eYFP mice in the RTPP/A assay (n = 7 ChR2, n = 9 eYFP; two-tailed, unpaired Student’s t test, * p = 0.0175).

(H) Optical stimulation did not have any significant effect on ICSS in LHglut-VTA:ChR2 mice compared with eYFP controls (n = 7 ChR2, n = 6 eYFP; two-way ANOVA: group x nose poke interaction, F$_{1,11}$ = 0.05, p = 0.8307).

Error bars indicate ± SEM. See also Figures S1 and S2.
stimulation (473 nm, 10 Hz, 20 mW, 5-ms pulses; Figure 1B). Surprisingly, we found that LHGABA-VTA:ChR2 mice spent significantly more time in the chamber paired with stimulation than the chamber without stimulation when compared with their eYFP counterparts (Figures 1B and 1C). In addition, to test whether LHGABA-VTA activation could support intracranial self-stimulation (ICSS), we placed mice into an operant chamber with an active and inactive nose-poke operandum. An active nose-poke response was paired with a compound light/sound cue and optogenetic stimulation (473 nm, 10 Hz, 20 mW, 5-ms pulses, 1-s duration), and an inactive nose-poke response was paired only with a cue. LHGABA-VTA:ChR2 mice made significantly more responses in the active nose poke compared with the inactive nose poke—an effect not observed in the eYFP controls (Figure 1D). These data show that mice prefer LHGABA-VTA stimulation and are willing to perform an instrumental response in order to receive that stimulation.

To determine how activation of the glutamatergic component of the LH-VTA projection (LHglut-VTA) influences motivation, we used the same optogenetic approach and behavioral assays described above in vesicular glutamate transporter 2 (VGLUT2):Cre mice (Figures 1E and S1). In contrast to the robust preference supported by LHGABA-VTA stimulation, activation of the glutamatergic projection was avoided by mice in the real-time preference/avoidance (RTPP/A) assay (Figures 1F and 1G). Consistent with these results, LHglut-VTA:ChR2 mice did not show a preference for the active nose poke in the ICSS task (Figure 1H). Taken together, these data suggest that activation of the glutamatergic component of the LH-VTA projection supports avoidance.

**GABAergic and Glutamatergic Components of the LH-VTA Pathway Distinctly Modulate Motivated Behaviors**

Next we sought to determine whether stimulation of the LHGABA-VTA projection could drive other behaviors in addition to feeding and approach. To assess the effect of LHGABA-VTA stimulation on social interaction, VGAT::Cre mice with the same surgical injections and implants as described above were placed in a cage with a novel juvenile male or adult female intruder (Figure 2A; Movies S1 and S2). Time spent engaging in social interaction with (B) juvenile male intruders compared with LHGABA-VTA:ChR2 mice showed increased time spent interacting with (B) juvenile male intruders compared with LHGABA-VTA:ChR2 mice (Figures 1E and S1). In contrast to the robust preference supported by LHGABA-VTA stimulation, activation of the glutamatergic component of the LH-VTA projection (LHglut-VTA) influences motivation, we used the same optogenetic approach and behavioral assays described above in vesicular glutamate transporter 2 (VGLUT2):Cre mice (Figures 1E and S1). In contrast to the robust preference supported by LHGABA-VTA stimulation, activation of the glutamatergic projection was avoided by mice in the real-time preference/avoidance (RTPP/A) assay (Figures 1F and 1G). Consistent with these results, LHglut-VTA:ChR2 mice did not show a preference for the active nose poke in the ICSS task (Figure 1H). Taken together, these data suggest that activation of the glutamatergic component of the LH-VTA projection supports avoidance.

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(F) To examine the effects of GABAergic and glutamatergic LH-VTA stimulation on motivational salience, mice were placed into an open-field chamber with four zones, each containing a novel object. Mice were allowed to freely explore the chamber for 1 hr while receiving blue light stimulation (473 nm, 20 Hz, 20 mW, 5-ms pulses) for 3-min epochs at 3-min intervals. (G) LHGABA-VTA:ChR2 mice had a significantly greater difference score in time spent investigating the novel objects (ON-OFF) than their eYFP counterparts (n = 7 ChR2, n = 8 eYFP; two-tailed, unpaired Student’s t test, *p = 0.0070). (H) LHglut-VTA:ChR2 mice had a significantly lower difference score in time spent investigating the novel objects (ON-OFF) than their respective eYFP counterparts (n = 8 ChR2, n = 7 eYFP; two-tailed, unpaired Student’s t test, *p = 0.0250). (I) LHGABA-VTA:ChR2 mice had a significantly lower difference score for the number of zone crossings (ON-OFF) than their eYFP counterparts (n = 7 ChR2, n = 8 eYFP; two-tailed, unpaired Student’s t test, *p = 0.0080). (J) LHglut-VTA:ChR2 mice had a significantly higher difference score for the number of zone crossings (ON-OFF) than their respective eYFP counterparts (n = 8 ChR2, n = 7 eYFP; two-tailed, unpaired Student’s t test, *p = 0.0372). Error bars indicate ± SEM. See also Figures S1 and S2.
(e.g., grooming, investigating the face or hind regions, or mounting of the intruder) was measured for three consecutive 3-min epochs, during which blue light (473 nm, 20 Hz, 20 mW, 5-ms pulses) was used to activate LH(GABA) VTA projections throughout the second epoch. LH(GABA) VTA:ChR2 mice spent significantly more time interacting with both juvenile (Figure 2B) and female intruders (Figure 2C) during the stimulation epoch as compared with eYFP controls. In contrast, while we did not detect any significant difference in interaction with juvenile intruders between LH(Glu) VTA:ChR2 mice and their controls (Figure 2D), we did find that LH(Glu) VTA:ChR2 mice spent significantly less time interacting with female intruders during the stimulation epoch as compared with their controls (Figure 2E).

These data, together with our previous work (Nieh et al., 2015), suggest that the LH-VTA projection plays a role in multiple motivated behaviors, including feeding, approach/avoidance, and social interaction, with the GABAergic component promoting behavioral responding and the glutamatergic component suppressing it. Thus, we hypothesized that, instead of playing a specific role in modulating each of these behaviors individually, the LH-VTA pathways might serve to change the overall motivational level in the animal, which could be manifested as the investigation of any salient target, regardless of what that target object may be (e.g., food or social stimulus).

To test this, we placed experimental mice into an open field with four chambers, each containing a novel object (Figure 2F). Mice were allowed to explore the open field for 1 hr and were stimulated using blue light (473 nm, 20 Hz, 20 mW, 5-ms pulses) for 3-min epochs at 3-min intervals. Our goal was to determine if mice would spend more or less time with the most salient object, in this case the most proximal object, upon LH(GABA) VTA or LH(Glu) VTA stimulation. We quantified the time spent investigating the objects and found that LH(GABA) VTA:ChR2 mice spent significantly more time investigating the objects during optical stimulation compared with eYFP controls (Figure 2G), while LH(Glu) VTA:ChR2 mice spent significantly less time investigating male intruders during the stimulation epoch as compared with their controls (Figure 2H). This revealed that LH GABA-VTA stimulation induced more c-Fos (an immediate early gene used to indicate recent neural activity) and tyrosine hydroxylase (TH; the rate-limiting enzyme in DA synthesis) in the VTA of mice that had received either GABAergic or glutamatergic LH-VTA stimulation (Figure 4A). This revealed that LH(GABA) VTA-stimulation induced more c-Fos+ DA (TH+) neurons than LH(Glu) VTA-stimulation (Figure 4B), suggesting that stimulation of the LH(GABA) VTA pathway enhances the activity of VTA DA neurons.

To explore the necessity of this projection in feeding, we placed food-restricted mice into an empty chamber with two cups, one of which contained a moist food pellet (Figure 3A). In addition to a significant group × epoch effect (Figure 3B), LH(GABA) VTA:NpHR mice showed a significantly larger decrease in percentage of time spent feeding during optical inhibition from the baseline epoch compared with eYFP controls (Figure 3C). However, LH(Glu) VTA:NpHR mice did not show any change in time spent feeding upon optical inhibition compared with their eYFP controls (Figures 3D and 3E). In the four-chamber novel object test (Figure 3F), unrestricted LH(GABA) VTA:NpHR mice spent significantly less time investigating the objects (Figure 3G) and made significantly more zone crossings (Figure 3H) during optical inhibition compared with eYFP controls. No significant differences were found upon LH(Glu) VTA-inhibition (Figures 3I and 3J).

**Modulation of DA Release in the Nucleus Accumbens by LH-VTA Projections**

We next examined the consequence of LH(GABA) VTA and LH(Glu) VTA activation on the activity of dopaminergic and non-dopaminergic neurons in the VTA. We quantified the co-expression of c-Fos (an immediate early gene used to indicate recent neural activity) and tyrosine hydroxylase (TH; the rate-limiting enzyme in DA synthesis) in the VTA of mice that had received either GABAergic or glutamatergic LH-VTA stimulation (Figure 4A). In contrast, LH glut-VTA activation (Figure 4J) caused a decrease in current at the oxidation potential for DA, indicative of a decrease in DA neurotransmission in the NAc (Figures 4G–4I and S4C). In many subjects, evoked DA release was composed primarily of individual phasic DA release events, or transients (Figures 4D and S4B), which are indicative of phasic firing of VTA DA neurons (Dreyer et al., 2016; Owesson-White et al., 2012). To further confirm recorded signals as DA, mice were administered the D2 receptor antagonist raclopride, which is known to increase [DA] and DA transients in the NAc (Andersson et al., 1995; Aragona et al., 2008). In the presence of D2 receptor antagonism, LH(GABA) VTA stimulation significantly increased DA neurotransmission in the NAc (Figures 4G–4I and S4C).

In contrast, LH(Glu) VTA activation (Figure 4J) caused a decrease in current at the oxidation potential for DA, indicative of a decrease in DA neurotransmission in the NAc, leading to a significant reduction in [DA] at baseline (Figures 4K–4M and S4D) and after D2 receptor blockade (Figures 4N–4P and S4E). Consistent with the idea that LH(Glu) VTA activation results in the suppression of activity in NAc-projecting VTA DA neurons, stimulation offset often evoked a phasic DA transient response.
In addition, LHGABA-VTA:NpHR mice had a significantly lower difference score in time spent feeding (ON-first OFF) compared with eYFP controls (n = 8 NpHR, n = 9 eYFP; two-tailed, unpaired Student's t test, \( p = 0.0202 \)).

The number of zone crossings (ON-OFF) was quantified for three consecutive 3-min epochs, with the second epoch paired with yellow light inhibition (589/593 nm, constant, 5 mW).

Figure 3. Inhibition of the GABAergic LH-VTA Projection of Mice in a Motivated State Suppresses Behavioral Response

(A) Food-restricted mice were placed into an empty chamber with two cups, one of which held a moist food pellet while the other was empty. Time spent feeding was quantified for three consecutive 3-min epochs, with the second epoch paired with yellow light inhibition (589/593 nm, constant, 5 mW).

(B) There was a significant interaction of optical inhibition on time spent feeding in LHGABA-VTA:NpHR mice relative to eYFP controls (n = 8 NpHR, n = 9 eYFP; two-way ANOVA revealed a group \( \times \) epoch interaction, \( F_{2,30} = 4.46, p = 0.0202 \)).

(C) In addition, LHGABA-VTA:NpHR mice had a significantly lower difference score in time spent feeding (ON-first OFF) compared with eYFP controls (n = 8 NpHR, n = 9 eYFP; two-tailed, unpaired Student's t test, \( p = 0.0210 \)).

(D and E) No effect of optical inhibition was found in LHglut-VTA:NpHR mice and their controls on (D) % time spent feeding (n = 10 NpHR, n = 7 eYFP; two-way ANOVA: group \( \times \) epoch interaction, \( F_{2,30} = 0.17, p = 0.8484 \)), or in (E) difference score (n = 10 NpHR, n = 7 eYFP; two-tailed, unpaired Student’s t test, \( p = 0.5963 \)).

(F–H) In the (F) four-chamber novel object test, (G) LHGABA-VTA:NpHR mice had a significantly lower difference score in investigation time (ON-OFF) than eYFP controls (n = 7 NpHR, n = 8 eYFP; two-tailed, unpaired Student’s t test, \( p = 0.0305 \)), while (H) LHglut-VTA:NpHR mice were not detectably different from their eYFP controls (n = 10 NpHR, n = 7 eYFP; two-tailed, unpaired Student’s t test, \( p = 0.5358 \)).

(I) LHGABA-VTA:NpHR mice also had a significantly greater difference score in the number of zone crossings (ON-OFF) than eYFP controls (n = 8 NpHR, n = 8 eYFP; two-tailed, unpaired Student’s t test, \( *** p < 0.0001 \)).

(J) LHglut-VTA:NpHR mice showed no difference from their eYFP controls (n = 10 NpHR, n = 7 eYFP; two-tailed, unpaired Student’s t test, \( p = 0.3247 \)). Error bars indicate \pm \ SEM. See also Figures S2 and S3.

Effects of GABAergic LH-VTA Stimulation on DA Neurotransmission Occur via Disinhibition in the VTA

Our previous work demonstrated that GABAergic neurons in the VTA receive both monosynaptic GABAergic and glutamatergic input from the LH (Nieh et al., 2015), and previous studies have shown that VTA GABA neurons inhibit VTA DA neurons (Tan et al., 2012; van Zessen et al., 2012). Together with our results from FSCV, we hypothesized that activation of the GABAergic projection from the LH elicits DA release in the NAc by suppressing the inhibition of VTA DA neurons by local VTA GABA neurons.

To test this hypothesis, we simultaneously photostimulated the GABAergic LH-VTA projection while recording the neural activity of VTA GABA neurons. To achieve this, we used a combination of the red-shifted depolarizing opsin ChrimsonR (Klapoetke et al., 2014) and the genetically encodable calcium indicator GCaMP6m (Chen et al., 2013). We injected VGAT::Cre mice with AAVp2-hSyn-FLEX-ChrimsonR-tdTomato into the LH and AAVp2-CAG-FLEX-GCaMP6m into the VTA and implanted two optic fibers over the VTA (Figures 5A–5C). This enabled us to shine yellow (593-nm) light into the VTA through one optic fiber to activate GABAergic axon terminals arising from the LH expressing ChrimsonR, while shining low levels of blue light (473 nm, 30–80 \( \mu \)W, constant) through the second optic fiber to excite GCaMP6m expressed in VTA GABA neurons, and measure emitted green (525-nm) fluorescence using fiber photometry (Gunaydin et al., 2014). In control mice, we injected AAVp2-DIO-eYFP into the VTA instead of AAVp2-CAG-FLEX-GCaMP6m to observe changes in fluorescence that could be due to movement-related or other artifacts. In awake mice, freely moving in their home cage, we activated the LHGABA-VTA projection with either 20 Hz (593 nm, 5–10 mW, 5-ms pulses, 1-s duration) or constant yellow light (593 nm, 5–10 mW, 1-s duration) and observed a significant decrease in emitted fluorescence compared with pre-stimulation fluorescence and fluorescence from control mice (Figures 5D and 5E). This significant decrease in fluorescence reflects a decrease in VTA GABA neural activity and suggests that LHGABA-VTA stimulation significantly reduces activity in VTA GABA neurons.

Finally, we performed whole-cell patch-clamp recordings from VTA TH+ (DA) and TH− (putative GABA) neurons in VGAT::Cre and VGLUT2::Cre mice (Figure 6A). This revealed that the amplitudes of inhibitory postsynaptic currents (IPSCs) elicited by LHGABA-VTA stimulation were significantly greater in putative input from the LH (Nieh et al., 2015), and previous studies have shown that VTA GABA neurons inhibit VTA DA neurons (Tan et al., 2012; van Zessen et al., 2012). Together with our results from FSCV, we hypothesized that activation of the GABAergic projection from the LH elicits DA release in the NAc by suppressing the inhibition of VTA DA neurons by local VTA GABA neurons.

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Figure 4. Optogenetic Activation of the GABAergic LH-VTA Projection Increases, while Activation of the Glutamatergic LH-VTA Projection Suppresses, DA Release in the NAc

(A) Representative confocal images from the VTA of LHGABA-VTA:ChR2 (top) and LHglut-VTA:ChR2 (bottom) mice show c-Fos+ (red) and TH+ (yellow) neurons in the VTA after photostimulation (473 nm, 20 Hz, 20 mW, 5-ms pulses, 10-min duration).

(B) Proportion of DA (TH+) neurons (left) and TH/C0 neurons (right) that either co-express or do not co-express c-Fos after LHGABA-VTA or LHglut-VTA photostimulation. Mice that received LHGABA-VTA stimulation showed a significantly greater proportion of cells co-expressing TH and c-Fos compared with mice that received LHglut-VTA stimulation (chi-square = 21.77, ****p < 0.0001).

(C) VGAT::Cre mice were injected with AAV5-DIO-ChR2-eYFP into the LH, and an optic fiber was implanted over the VTA. Anesthetized fast-scan cyclic voltammetry (FSCV) recordings were obtained from the nucleus accumbens (NAc).

(D–F) Optical activation of the LHGABA-VTA projection evoked DA release in the NAc. (D) Representative false color plot shows an increase in current at the oxidation potential for DA (\(0.65 \text{ V}\)) upon LHGABA-VTA photostimulation (473 nm, 20 Hz, 20 mW, 5-ms pulses, 10-s duration), which is also evident in the (E) averaged population data after conversion into DA concentration. (F) Quantification of extracellular DA concentration ([DA]) as area under the curve shows that LHGABA-VTA stimulation caused a significant increase in DA release in the NAc (compared with pre-stimulation; \(n = 6\) mice; two-tailed, paired Student’s t test, **p = 0.0013).

(G–I) Under D2 receptor blockade (intraperitoneal [i.p.] raclopride), LHGABA-VTA stimulation also increased NAc DA neurotransmission, as seen in the (G) representative color plot and (H) averaged population data. (I) Quantification of [DA] as area under the curve revealed a significant increase in DA release under D2 receptor blockade (\(n = 6\) mice; two-tailed, paired Student’s t test, **p = 0.0037).

(J) VGLUT2::Cre mice were prepared for FSCV as described above for VGAT::Cre mice.

(legend continued on next page)
Similarly, the amplitudes of excitatory postsynaptic currents (EPSCs) elicited by LH\textsuperscript{GABA}-VTA stimulation were also significantly greater in putative GABA neurons compared with DA neurons in the VTA (Figure 6C). These data suggest that, although the LH sends excitatory and inhibitory projections to both DA and GABA neurons in the VTA (Nieh et al., 2015), the relative strengths of these inputs are greater onto putative GABA neurons. Taken together, our data support a model wherein activating an inhibitory projection from the LH to the VTA supports appetitive behaviors though inhibition of VTA GABA neurons, which causes disinhibition of DA neurons to increase DA release in the NAc (Figure 6D).

DISCUSSION

The Role of LH Inhibitory Input onto GABAergic Neurons in the VTA

The LH projection to the VTA has been well studied for its involvement in reward processing and feeding behaviors (Bielawa and Shizgal, 1986; Kempadoo et al., 2013; Nieh et al., 2015; Stuber and Wise, 2016). The glutamatergic component of the LH-VTA projection has been proposed to be responsible for supporting positive reinforcement. Specifically, it has been suggested that glutamatergic fibers from the LH traveling to the VTA might contribute to LH- and VTA-evoked self-stimulation (You et al., 2001). Additionally, NMDA receptor antagonism in the VTA has been shown to block optogenetically induced ICSS of LH-VTA projections, implicating the involvement of glutamate release from the LH to the VTA (Kempadoo et al., 2013).

However, our findings contradict this notion and instead demonstrate that the GABAergic component of the LH-VTA pathway mediates the reward-related properties observed in this circuit. This is evidenced by our finding that mice will self-stimulate for GABAergic LH-VTA stimulation, but not glutamatergic LH-VTA stimulation (Figures 1D and 1H). Furthermore, photostimulation of LH\textsuperscript{GABA}-VTA is preferred, while photostimulation of LH\textsuperscript{glut}-VTA is avoided (Figures 1B, 1C, 1F, and 1G).

As a result, our findings counter the interpretation proposed by Kempadoo and colleagues (2013) and may be reconciled by evidence that infusion of NMDA receptor antagonists into the VTA is known to prevent spontaneous burst-firing in DA neurons (Chergui et al., 1993; Grace et al., 2007; Johnson et al., 1992). Therefore, an alternative interpretation is that their manipulation not only blocked glutamate action from the LH but also prevented burst-firing of DA neurons. The model for glutamatergic activation of VTA playing the major role in generating reward-related behaviors was attractive because of the known influence of VTA DA stimulation on positive reinforcement. However, our experiments present evidence for the inhibitory projection to the VTA as the principal mediator of appetitive behaviors. This apparent paradox—in which an inhibitory input to the VTA causes DA release in the NAc to cause behavioral activation—was resolved by our finding that GABAergic LH inputs are stronger onto putative GABA neurons in the VTA than DA neurons (Figure 6) and that stimulating this projection inhibits these VTA GABA neurons (Figure 5), thereby allowing for disinhibition of DA neurons projecting to the NAc.

Our study follows experiments from other groups showing that animals are willing to self-administer GABAergic agonists into the VTA (David et al., 1997; Ikemoto et al., 1997, 1998). At the time, the reason why animals would do this was not well understood, but it was known that GABA\textsubscript{A} receptors were expressed on both VTA DA neurons (Sugita et al., 1992) and VTA GABA neurons (Rick and Lacey, 1994). Johnson and North (1992) first hypothesized that mu-opioid receptor agonists, such as morphine, act in the VTA via disinhibition through GABA neurons, while Bocklisch and colleagues showed that cocaine also can disinhibit VTA DA neurons through potentiation of inhibitory NAc projections to VTA GABA neurons (Bocklisch et al., 2013). Our results are generally consistent with other recent studies indicating the role for LH GABA neurons (Jennings et al., 2015) and their projection to the VTA (Barbano et al., 2016) in supporting positive reinforcement and appetitive behaviors, though nuances in behavior may be attributed to our targeting a more anterior portion of the LH.

Our work is the first to show direct relationships among activating LH GABA projections to the VTA, the suppression of GABA neuron activity in the VTA, and downstream DA release in the NAc.

Noteworthy Nuances

Because the medial/lateral location of DA neurons within the VTA has been shown to indicate a difference in projection target, with DA neurons in medial VTA projecting to the NAc medial shell and medial prefrontal cortex (mPFC) and DA neurons in lateral VTA projecting to the NAc lateral shell (Lammel et al., 2008, 2011, 2012), we generated maps with the location of each TH+ or TH− cell we recorded from in Figure 6, with the area of the symbol proportional to the recorded EPSC or IPSC (Figure S5). However, there did not appear to be any differences in the medial/lateral locations of the recorded TH+ cells with respect to amplitude.

As a result of the gnawing behavior that occurs in an empty chamber, we conducted the RTPPA and ICSS experiments at
10 Hz instead of 20 Hz to minimize the amount of gnawing that might confound the results (read more on gnawing in Nieh et al., 2015). There appeared to be less gnawing in the resident-intruder and novel object assays, likely due to the presence of very salient stimuli, so 20 Hz stimulation was used to maximize the effect. Voltammetry experiments showed that LH\(^{\text{GABA}}\)-VTA or LH\(^{\text{Glu}}\)-VTA stimulation at either 10 or 20 Hz evoked the same pattern of DA release and suppression, respectively (Figures 4 and S4).

The LH-VTA Circuit as an Environment-Dependent Modulator of Motivational Salience

While both the LH and VTA have long been identified as areas involved in feeding and reward, we show evidence that activation of individual components of the LH-VTA projection also can modulate social behaviors. Valenstein and colleagues proposed the notion of substitutability based on their observations that animals will eat, drink, or gnaw upon LH stimulation dependent on the availability of food, water, or a wooden block, respectively (Valenstein et al., 1968). Other studies using electrical stimulation also have reported that LH activation can evoke locomotor effects, gnawing, ejaculation, and aggression (Albert et al., 1979; Singh et al., 1996), and, more recently, Navarro and colleagues showed that stimulating specifically the GABAergic neurons in the LH can induce consummatory behaviors toward saccharin, water, or wood (Navarro et al., 2016). Our results showing that stimulation of GABAergic LH inputs to the VTA causes DA release in the NAc also brings into conversation a large field involved in the study of DA as a substrate for behavioral activation, initiation vigor, arousal, and motivational salience (Berridge and Robinson, 1998; Horvitz, 2000; Ko and Wanat, 2016; Salamone and Correa, 2012). Several studies have shown that subsecond fluctuations in ventral striatal DA are enhanced prior to the performance of an instrumental action (Collins et al., 2016; Hamid et al., 2016; Howe et al., 2013), which is consistent with the idea that DA signaling supports motivated approach behavior (Di Ciano et al., 2001; Saunders and Robinson, 2012).

Our present results support these ideas as a whole, in that neither LH stimulation nor DA release in the NAc is specific to individual behaviors, such as feeding, but may instead cause an increase in many different behaviors by supporting a change in the motivational state of the animal. In our study, we showed that GABAergic LH-VTA stimulation causes DA release in the NAc, commensurate with a motivational state change in the animal, and caused the animal to obtain, approach, and/or investigate salient stimuli. The context of the environment and the nature of the stimulus determined which action the animal would take. In the social interaction task, wherein the salient stimulus was the intruder mouse, GABAergic LH-VTA stimulation promoted interaction with the intruder (Movies S1 and S2), and in the four-chamber novel object task, wherein the salient stimulus

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was the most proximal object, GABAergic LH-VTA stimulation induced increased investigation of the object (Figure 2).

Importantly, glutamatergic LH-VTA stimulation suppressed interaction with intruders, reduced investigation of objects, caused avoidance in the RTPP/A assay, and decreased DA release in the NAc. As a result, the glutamatergic LH-VTA component also could be modulating motivation levels in order to promote avoidance. However, because our experiments in this study only focused on rewarding or neutral target stimuli, future experiments should explore how glutamatergic LH-VTA stimulation/inhibition affects behavior in the presence of aversive target stimuli. While glutamatergic LH-VTA inhibition did not appear to have any significant effects in the experiments of this study, we speculate that, in an assay where animals must avoid an aversive stimulus, glutamatergic LH-VTA stimulation may suppress the animal’s motivation to avoid that stimulus.

LH-VTA as Part of a Distributed Neural Circuit

Importantly, optogenetic activation may not recapitulate the physiological role of a given projection. While photostimulation of the GABAergic input from LH to VTA produced robust changes, the photoinhibition induced relatively modest changes in behavior. This may be due to a floor effect, or, more likely, it reflects that the LH input to the VTA is only one of multiple contributing factors that influence VTA activity and subsequent behavioral changes.

Another important note is that terminal stimulation does not rule out the possibility of antidromic activation. Thus, it is possible that activation of LH-VTA terminals can cause antidromic activation of the cells bodies in the LH, which could recruit other downstream structures, including the bed nucleus of the stria terminals, dorsal raphe, amygdala, and lateral habenula (Berk and Finkelstein, 1982; Saper et al., 1979). In addition, while we have recorded DA levels in the NAc as a result of activating the GABAergic or glutamatergic components of the LH-VTA projection, it is unknown whether these projections also have an effect on DA levels in dorsal striatum and/or prefrontal cortex. Considering DA innervation in the dorsal striatum also plays a role in feeding (Szczypka et al., 1999, 2001) and

![Figure 6. GABAergic and Glutamatergic LH Projections Are Stronger onto Putative GABA Neurons than DA Neurons in the VTA](image)
compulsive behaviors (Ito et al., 2002; Vanderschuren et al., 2005), future experiments studying the differences in DA release in dorsal/ventral striatum from LH-VTA stimulation would provide another level of insight into this circuit.

Additionally, the GABAergic LH-VTA projection synapses onto both GABA and DA neurons in the VTA, even if the primary input is onto VTA GABA neurons (Figures 5 and 6). It is also possible that within the GABAergic LH-VTA projection, there may be further subdivisions that uniquely contribute to distinct motivated behaviors (e.g., feeding, drinking, and sex), but, by stimulating the entire projection, we are activating these motivated behaviors together. In addition, disinhibiting DA neurons by activating GABAergic LH-VTA inputs is physiologically different from directly activating DA neurons. A single GABA interneuron in the VTA could have widespread effects onto many DA neurons simultaneously. By activating the GABAergic LH-VTA input, we also may be causing peptideergic co-release within the VTA or via axon collaterals, since a subset of GABA-expressing LH neurons also express peptides such as neuropeptide Y (Leinninger et al., 2009; Opland et al., 2013).

**Conclusions**

Homeostasis can be maintained with three elements (Cannon, 1929). The first detects the current state of the system (detector), the second compares the current state to the set point (evaluator), and the third adjusts the state of the system toward the set point (adjuster), where the set point is defined as the optimal state of any given system.

We previously showed that stimulating the LH-VTA projection can cause mice to seek a sugar reward even in the face of a negative consequence (Nieh et al., 2015). In this study, we showed that the GABAergic component of this projection is positively reinforcing and increases behavioral activation generalizable across multiple motivated behaviors. One explanation is that activating this projection may be simulating the rewarding value that is then attributed to the most salient proximal stimulus. Another possible explanation is that the LH may play the role of the evaluator within a homeostatic circuit, integrating inputs from the periphery and upstream cortical areas (Berthoud and Münnich, 2011; Diorio et al., 1993) to compute differences between the current state and the target set points, and the VTA may play the role of the adjuster, enhancing or suppressing DA release to generate downstream motor action. Taken together, our manipulations of the LH-VTA projection may either circumvent the detection and evaluation elements in a homeostatic model or increase motivation by an anatomically distinct reward-related system. Therefore, in contrast to other neural populations that cause feeding due to hunger when stimulated, such as the agouti-related peptide (AGRP) cells of the arcuate nucleus (Belt et al., 2015), LH-GABA_VTA stimulation appears to evoke feeding by increasing the motivation for food reward.

Thus, we conjecture that the GABAergic LH-VTA component is more likely to be involved in disorders such as compulsive eating, where the primary cause of overeating is not hunger. Importantly, because inhibiting this projection suppresses feeding when animals are in a highly motivated state, the GABAergic LH-VTA pathway could serve as an important target for drug action in the treatment of these disorders. Furthermore, our data show that this projection not only modulates feeding but also other appetitive behaviors. As a result, a hyperactive population of LH-VTA GABA neurons could not only induce overeating or compulsive eating and thus elevate food intake to maladaptive levels but could also potentially lead to compulsive behaviors toward other stimuli. This idea that a malfunction in one neural population may result in compulsive behaviors toward multiple stimuli may be a root cause in a subset of addictive disorders in human patients, given the observed comorbidity of binge eating disorder with compulsive buying (Faber et al., 1995) or pathological gambling with substance abuse (Black and Moyer, 1998; Cunningham-Williams et al., 1998).

In conclusion, our study elucidates how the GABAergic and glutamatergic LH-VTA components can work together to produce approach and avoidance behaviors by modulating motivational state through midbrain DA release, and it identifies a possible target for therapeutic intervention in compulsive eating and other addictive disorders.

**EXPERIMENTAL PROCEDURES**

All procedures were in accordance with guidelines from the NIH and approved by the MIT Institutional Animal Care and Use Committee.

**Targeting GABAergic and Glutamatergic LH-VTA Projections for Optogenetic Stimulation**

Male VGAT::Cre and VGLUT2::Cre mice were injected with AAV5-DIO-ChR2-eYFP, AAV5-DIO-NpHR-eYFP, or AAV5-DIO-eYFP into the LH, and an optic fiber was implanted directly above the VTA.

**FSCV to Detect DA Release upon LH-VTA Activation**

A carbon-fiber electrode was lowered into the NAc to locations where optical activation of the LH-VTA circuit evoked changes in DA release. Recordings were obtained under resting (baseline) conditions and after the administration of raclopride (D2 receptor antagonist).

**Photometry to Determine the Effect of GABAergic LH-VTA Photoactivation on VTA GABA Neurons**

Male VGAT::Cre mice were injected with AAV4-hSyn-FLEX-ChrimsonRedTomato into the LH and AAV4-CAG-FLEX-GCaMP6m into the VTA with two optic fibers implanted above the VTA. Yellow light was used to activate GABAergic LH-VTA terminals, while blue light was used to activate GABA cells in the VTA expressing GCaMP6m.

For more information, please refer to the Supplemental Experimental Procedures.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, five figures, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2016.04.035.

**AUTHOR CONTRIBUTIONS**

E.H.N. performed stereotaxic virus/implant surgeries, behavioral and photometry experiments, histology, confocal imaging, and data analysis. C.M.V.W. performed FSCV recordings, histology, confocal imaging, and data analysis. G.A.M. performed patch-clamp recordings and confocal imaging. K.N.P. performed behavioral experiments, histology, and data analysis. R.W. performed behavioral experiments. E.M.I. and G.A.M. assisted with histology. R.W., K.N.P., E.M.I., C.M.V.W., and C.A.L. performed cell counting. E.H.N., C.M.V.W., and K.M.T. designed the experiments and wrote the manuscript. All authors contributed to the editing and revision of the manuscript.
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based on stimulation parameters and behavioural response characteristics. Brain Res. Bull. 41, 399–408.


Supplemental Information

Inhibitory Input from the Lateral Hypothalamus
to the Ventral Tegmental Area Disinhibits Dopamine
Neurons and Promotes Behavioral Activation

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Nieh, Vander Weele et al., Figure S2

**Figure S2**

A. LHGABA-VTA and LHglut-VTA: Velocity (cm/s) (ON-OFF) for RTPP/A.

B. LHGABA-VTA and LHglut-VTA: Four-Chamber Novel Object Velocity (cm/s) (ON-OFF).

C. LHGABA-VTA: RTPP/A Time Spent (%) (ON-OFF).

D. NpHR eYFP: Nose Pokes (h⁻¹) for Active and Inactive.

E. LHglut-VTA: RTPP/A Time Spent (%) (ON-OFF).

F. NpHR eYFP: Nose Pokes (h⁻¹) for Active and Inactive.

G. Juvenile Intruder Interaction Time (%): LHGABA-VTA and LHglut-VTA.

H. Female Intruder Interaction Time (%): LHGABA-VTA and LHglut-VTA.

I. LHGABA-VTA and LHglut-VTA: Velocity (cm/s) (ON-OFF) for RTPP/A.

J. LHGABA-VTA and LHglut-VTA: Four-Chamber Novel Object Velocity (cm/s) (ON-OFF).
Nieh, Vander Weele et al., Figure S3
SUPPLEMENTAL INFORMATION

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Related to Figures 1 and 2. (A) Estimated injection sites in the LH for AAV5-DIO-ChR2-eYFP in VGAT::Cre (blue) and VGLUT2::Cre (red) mice, as well as for AAV5-DIO-eYFP in eYFP controls (grey). (B) Location of optic fiber tips implanted in VGAT::Cre (blue) and VGLUT2::Cre (red) ChR2+ mice, as well as in eYFP controls (grey). (C) Low- (top) and high- (bottom) magnification confocal images from representative LH\textsuperscript{GABA}:ChR2 and (D) LH\textsuperscript{glut}:ChR2 mice showing the expression of ChR2 in the LH at three different AP coordinates. Dotted boxes indicate approximately where high-magnification 40x images were taken. (E) Quantification of the percentage of LH neurons that were ChR2+ at three different AP coordinates in LH\textsuperscript{GABA}:ChR2 (n=10) and (F) LH\textsuperscript{glut}:ChR2 (n=10) mice. (G) No significant differences were found between expression at different AP coordinates in LH\textsuperscript{GABA}:ChR2 and LH\textsuperscript{glut}:ChR2 mice (two-way ANOVA: AP-coordinate effect, $F_{2,36}=1.38$, $p=0.2651$; group effect, $F_{1,18}=0.01$, $p=0.9256$). Error bars indicate ±SEM.

Figure S2. Related to Figures 1, 2, and 3. (A) LH\textsuperscript{GABA}-VTA:ChR2 mice had a significantly lower difference score in velocity (ON-OFF) than their eYFP counterparts, while LH\textsuperscript{glut}-VTA:ChR2 mice had a significantly higher difference score than their eYFP counterparts in the real-time place preference/avoidance (RTPP/A) assay (n=8 LH\textsuperscript{GABA}-VTA:ChR2, n=10 LH\textsuperscript{GABA}-VTA:eYFP; two-tailed, unpaired Student’s t test, ***$p=0.0006$; n=7 LH\textsuperscript{glut}-VTA:ChR2, n=9 LH\textsuperscript{glut}-VTA:eYFP; two-tailed, unpaired Student’s t test, **$p=0.0064$) and (B) the four-chamber novel object task (n=7 LH\textsuperscript{GABA}-VTA:ChR2, n=8 LH\textsuperscript{GABA}-VTA:eYFP; two-tailed, unpaired Student’s t test, **$p=0.0049$; n=9 LH\textsuperscript{glut}-VTA:ChR2, n=7 LH\textsuperscript{glut}-VTA:eYFP; two-tailed, unpaired Student’s t test, **$p=0.0064$).
test, *p=0.0242). (C) No significant differences were found in LH\textsuperscript{GABA}-VTA:NpHR mice when compared with eYFP controls in the RTPP/A (n=9 NpHR, n=9 eYFP; two-tailed, unpaired Student’s t test, p=0.9956) or (D) intracranial self-stimulation (ICSS; n=9 NpHR, n=9 eYFP; two-way ANOVA: group x epoch interaction, F\textsubscript{1,16}=1.89, p=0.1887) assays. (E) No significant differences were found in LH\textsuperscript{glut}-VTA:NpHR mice when compared with eYFP controls in the RTPP/A (n=10 NpHR, n=6 eYFP; two-tailed, unpaired Student’s t test, p=0.6206) or (F) ICSS (n=10 NpHR, n=7 eYFP; two-way ANOVA: group x epoch interaction, F\textsubscript{1,15}=0.09, p=0.7744) assays. (G) No significant differences were found in social interaction with either juvenile (n=7 LH\textsuperscript{GABA}-VTA:NpHR, n=8 LH\textsuperscript{GABA}-VTA:eYFP; two-way ANOVA: group x epoch interaction, F\textsubscript{2,26}=0.25, p=0.7840; n=10 LH\textsuperscript{glut}-VTA:NpHR, n=7 LH\textsuperscript{glut}-VTA:eYFP; two-way ANOVA: group x epoch interaction, F\textsubscript{2,30}=0.80, p=0.4570) or (H) female intruders (n=9 LH\textsuperscript{GABA}-VTA:NpHR, n=8 LH\textsuperscript{GABA}-VTA:eYFP; two-way ANOVA: group x epoch interaction, F\textsubscript{2,30}=0.93, p=0.4060; n=10 LH\textsuperscript{glut}-VTA:NpHR, n=7 LH\textsuperscript{glut}-VTA:eYFP; two-way ANOVA: group x epoch interaction, F\textsubscript{2,30}=0.09, p=0.9154) in the social interaction assay for either LH\textsuperscript{GABA}-VTA:NpHR or LH\textsuperscript{glut}-VTA:NpHR mice compared with their respective eYFP controls. (I) There were no significant differences in velocity for either LH\textsuperscript{GABA}-VTA:NpHR or LH\textsuperscript{glut}-VTA:NpHR mice when compared with their eYFP controls in either the RTPP/A assay (n=9 LH\textsuperscript{GABA}-VTA:NpHR, n=9 LH\textsuperscript{GABA}-VTA:eYFP; two-tailed, unpaired Student’s t test, p=0.7362; n=10 LH\textsuperscript{glut}-VTA:NpHR, n=6 LH\textsuperscript{glut}-VTA:eYFP; two-tailed, unpaired Student’s t test, p=0.5514) or (J) the four-chamber novel object task (n=8 LH\textsuperscript{GABA}-VTA:NpHR, n=8 LH\textsuperscript{GABA}-VTA:eYFP; two-tailed, unpaired Student’s t test, p=0.1187; n=10 LH\textsuperscript{glut}-VTA:NpHR, n=7 LH\textsuperscript{glut}-VTA:eYFP; two-tailed, unpaired Student’s t test, p=0.3124). Error bars indicate ±SEM.

**Figure S3. Related to Figure 3.** (A) Estimated injection sites in the LH for AAV\textsubscript{S}-DIO-NpHR-eYFP in VGAT::Cre (purple) and VGLUT2::Cre (orange) mice, as well as for AAV\textsubscript{S}-DIO-eYFP in eYFP controls (grey). (B) Location of optic fiber tips implanted in VGAT::Cre (purple) and
VGLUT2::Cre (orange) NpHR+ mice, as well as in the eYFP controls (grey). (C) Low- (top) and high- (bottom) magnification confocal images from representative LH\textsuperscript{GABA}:NpHR and (D) LH\textsuperscript{glut}:NpHR mice showing the expression of NpHR in the LH at three different AP coordinates. Dotted boxes indicate approximately where high-magnification 40x images were taken. (E) Quantification of the percentage of LH neurons that were NpHR+ at three different AP coordinates in LH\textsuperscript{GABA}:NpHR (n=9) and (F) LH\textsuperscript{glut}:NpHR mice (n=10). (G) No significant differences were found between expression at different AP coordinates in LH\textsuperscript{GABA}:NpHR and LH\textsuperscript{glut}:NpHR mice (two-way ANOVA: AP-coordinate effect, F\textsubscript{2,34}=0.18, p=0.8375; group effect, F\textsubscript{1,17}=2.85, p=0.1094). Error bars indicate ±SEM.

**Figure S4. Related to Figure 4.** (A) Locations of FSCV electrode tips in the NAc of VGAT::Cre (blue; n=6 mice; n=6 baseline recording locations, n=6 raclopride recording locations) and VGLUT2::Cre (red; n=6 mice, n=5 baseline recording locations, n=6 raclopride recording locations) mice. Recording locations were reconstructed using electrode track and recording depth (distance from brain surface). (B) Representative false color plots from each subject under baseline recording conditions and (C) with raclopride in LH\textsuperscript{GABA-VTA:ChR2} mice. (D) Representative false color plots for each recording site under baseline recording conditions and (E) with raclopride in LH\textsuperscript{glut-VTA:ChR2} mice. (F, G, H) 10-Hz LH\textsuperscript{GABA-VTA} photostimulation (473 nm, 20 mW, 5-ms pulses, 10-s duration) evoked DA release in the NAc under baseline, resting conditions. (F) Representative false color plot showing dopamine release in response to stimulation, which is also evident in the (G) averaged population data after conversion into DA concentration. (H) Quantification of extracellular dopamine concentration ([DA]) as area under the curve shows that 10-Hz LH\textsuperscript{GABA-VTA} activation caused a significant increase in DA release in the NAc (compared with pre-stimulation) (n=4 mice; two-tailed, paired Student’s t test, *p=0.0145). (I, J, K) Under D2 receptor blockade (raclopride, I.P.), 10-Hz LH\textsuperscript{GABA-VTA} activation increased NAc DA neurotransmission as seen in the (I) representative color plot and (J)
averaged population data. (K) Quantification of [DA] as area under the curve did not show a significant increase in evoked release, possibly due to high variability (n=4 mice; two-tailed, paired Student’s t test, p=0.1973). (L, M, N). Similar to 20-Hz stimulation, 10-Hz LH\textsuperscript{glut}-VTA stimulation (473 nm, 20 mW, 5-ms pulses, 10-s duration) caused a pause in NAc DA release under resting, baseline conditions. (L) Representative false color plot shows a decrease in DA release in response to stimulation. 10-Hz stimulation offset was often accompanied by a small “rebound” DA increase, which is also observed in the (M) averaged population data after conversion to [DA]. (N) Quantification of [DA] as area under the curve shows that 10-Hz LH\textsuperscript{glut}-VTA activation caused a significant decrease in [DA] in the NAc under resting conditions (n=5 mice; two-tailed, paired Student’s t test, *p=0.0178). (O, P, Q) Under the influence of raclopride, 10-Hz LH\textsuperscript{glut}-VTA activation inhibited NAc DA release observed in the (O) representative color plot and (P) population average. (Q) Quantification of [DA] shows that LH\textsuperscript{glut}-VTA activation caused a trending decrease in [DA] under D2 receptor blockade (n=6 mice; two-tailed, paired Student’s t test, #p=0.0624). Error bars indicate ±SEM.

**Figure S5. Related to Figure 6.** Horizontal brain maps of (A) dorsal and (B) ventral VTA slices showing the locations of all recorded TH+ (filled) and TH- (open) neurons with the areas of the circles indicating the relative amplitudes of the recorded inhibitory postsynaptic currents (IPSCs) in VGAT::Cre mice (blue) and excitatory postsynaptic currents (EPSCs) in VGLUT2::Cre mice (red). (C) No significant differences were found in the onset latencies of optically-evoked IPSCs in TH+ and TH- neurons (n=9 TH+, n=7 TH-; two-tailed, unpaired Student’s t test, p=0.2510) or (D) in the onset latencies of optically-evoked EPSCs in TH+ and TH- neurons (n=5 TH+, n=5 TH-; two-tailed, unpaired Student’s t test, p=0.7289). Error bars indicate ±SEM.
SUPPLEMENTAL MOVIE LEGENDS

Supplemental Movie 1. A male VGAT::Cre mouse expressing ChR2 in GABAergic LH neurons has an optical fiber implanted over the VTA to allow for photostimulation indicated in video as “ON” epoch. Interaction with a juvenile male mouse is quantified in Figure 2.

Supplemental Movie 2. A male VGAT::Cre mouse expressing ChR2 in GABAergic LH neurons has an optical fiber implanted over the VTA to allow for photostimulation indicated in video as “ON” epoch. Interaction with an adult female mouse is quantified in Figure 2.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Animals and Stereotaxic Surgery

Mice were housed in a reverse 12-hour light-dark cycle room with *ad libitum* food and water provided. All procedures involving the handling of animals were in accordance with guidelines from the NIH and approved by the MIT Institutional Animal Care and Use Committee. Surgery was performed on mice under aseptic conditions and body temperature was maintained with a heating pad. Mice were anesthetized with isoflurane (5% for induction, 1-2% for maintenance) and placed in a digital small animal stereotax (David Kopf Instruments, Tujunga, CA, USA). All measurements were made relative to bregma for virus/implant surgeries. Viral injection was performed using a beveled 33 gauge microinjection needle with a 10 µL microsyringe (Nanol; WPI, Sarasota, FL, USA) delivering virus at a rate of 0.1 µL/min with a microsyringe pump (UMP3; WPI, Sarasota, FL, USA) and controller (Micro4; WPI, Sarasota, FL, USA). After the injection was completed, two minutes were allowed to pass before withdrawing the needle 50-100 µm and leaving it for an additional 10 minutes before the needle was then slowly withdrawn completely. After surgery, mice recovered from anesthesia under a heat lamp.

For ChR2 and corresponding control mice used in behavioral, fast-scan cyclic voltammetry (FSCV), and *ex vivo* electrophysiology experiments, 0.3-0.5 µL of an anterogradely travelling adeno-associated virus serotype 5 (AAV5), encoding channelrhodopsin-2 (ChR2)-eYFP, under a double-floxed inverted open-reading frame construct (DIO) (AAV5-Ef1α-DIO-ChR2(H134R)-eYFP) or a null version of the virus only carrying eYFP (AAV5-Ef1α-DIO-eYFP) was injected into the LH (anteroposterior (AP): -0.4 to -0.8 mm; mediolateral (ML): 1.0 mm; dorsoventral (DV): -4.9 to -5.35 mm) in VGAT::IRES-Cre (VGAT::Cre; RRID: IMSR_JAX:016962) or VGLUT2::IRES-Cre (VGLUT2::Cre; RRID: IMSR_JAX:016963) mice. In addition, a manually-constructed optic fiber (300 µm core, 0.37 NA) (Thorlabs, Newton, NJ, USA) held in a 2.5 mm ferrule (Precision Fiber Products, Milpitas, CA, USA) was implanted.
directly above the VTA (AP: -3.1 to -3.6 mm; ML: 0.60 to 0.70 mm; DV: -3.5 to -4.1 mm). For NpHR and corresponding control mice, 0.3-0.5 µL of AAV5, encoding enhanced halorhodopsin 3.0 (NpHR)-eYFP, under a DIO construct (AAV5-Ef1α-DIO-NpHR-eYFP) or AAV5-Ef1α-DIO-eYFP was injected bilaterally into the LH (AP: -0.4 to -0.8 mm; ML: ±1.0 mm; DV: -4.9 to -5.35 mm), and an optic fiber (400 μm core, 0.48 NA) was implanted medially above the VTA between both hemispheres (AP: -3.1 to -3.6 mm; ML: 0.0 mm; DV: -2.5 mm to -3.2 mm). A layer of adhesive cement (C&B Metabond; Parkell, Edgewood, NY, USA) followed by cranioplastic cement (Ortho-Jet; Lang, Wheeling, IL, USA) was used to secure the optic fiber to the skull.

For mice used in photometry experiments, 0.3-0.5 µL of an anterogradely travelling adeno-associated virus serotype 8 (AAV8) encoding ChrimsonR-tomato under a flip-excision (FLEX) switch (AAV8-hSyn-FLEX-ChrimsonR-tomato) was injected into the LH (AP: -0.4 to -0.8 mm; ML: 1.0 mm; DV: -4.9 to -5.35 mm). In addition, an AAV5 carrying the genetically-encoded calcium indicator (GCaMP6m; AAV5-CAG-FLEX-GCaMP6m) was injected into the VTA (AP: -3.1 to -3.6 mm; ML: 0.60 to 0.70 mm; DV: -4.3 to -4.75 mm). One fiber (300 μm core, 0.37 NA or 400 μm core, 0.48 NA) held in a 2.5 mm ferrule was implanted in the VTA (AP: -3.1 to -3.6 mm; ML: 0.60 to 0.70 mm; DV: -4.0 to -4.3 mm). A second fiber (300 μm core, 0.37 NA) held in a 1.25 mm ferrule was implanted in the contralateral hemisphere at a 15º angle to the right targeting the VTA (AP: -3.1 to -3.6 mm; ML: -1.02 to -0.70 mm; DV: -3.0 to -3.5 mm). Adhesive cement and cranioplastic cement were used to secure the optic fibers as above.

**Viral Constructs**

Recombinant AAV vectors were serotyped with AAV5 or AAV8 coat proteins, and those carrying ChR2, NpHR, or ChrimsonR were packaged by the University of North Carolina Vector Core (Chapel Hill, NC, USA). Viruses carrying GCaMP6m were packaged by the University of Pennsylvania Vector Core (Philadelphia, PA, USA).
Behavioral Experiments

Behavioral testing was performed during the active dark phase and at least four weeks following surgery to allow sufficient time for transgene expression. Optic fiber implants were connected to a patch cable with a ceramic sleeve (PFP, Milpitas, CA, USA), which was connected to a commutator (rotary joint; Doric, Québec, Canada) via an FC/PC adapter to allow unrestricted movement. A second patch cable, with a FC/PC connector at either end (Doric, Québec, Canada), was connected to the commutator and then connected to a 473-nm, 589-nm, or 593-nm diode-pumped solid state (DPSS) laser (OEM Laser Systems, Draper, UT, USA). A Master-8 pulse stimulator (A.M.P.I., Jerusalem, Israel) was used to control the output of the 473-nm laser. The 593-nm laser was pulsed using a shutter (SR475; Stanford Research Systems, Sunnyvale, CA, USA) and shutter driver (SR474; Stanford Research Systems, Sunnyvale, CA, USA).

Real-Time Place Preference/Avoidance (RTPP/A)

Mice were placed in an open chamber (57.15 cm x 22.5 cm x 30.5 cm) consisting of left and right chambers (each 24.5 cm x 22.5 cm) and a center compartment (8 cm x 22.5 cm). Mice were allowed to freely move between compartments for 30 minutes, during which entry into one of the two sides was paired with photostimulation (ChR2: 473 nm, 10 Hz, 20 mW, 5-ms pulses; NpHR: 589/593 nm, constant, 5 mW). The side paired with stimulation was counterbalanced between mice. A video camera positioned above the chamber recorded each trial, and mouse locations/velocity were tracked and analyzed using Ethovision XT software (Noldus, Wageningen, Netherlands). Difference scores were calculated by subtracting the percentage of time spent in the non-stimulated side from the percentage of time spent in the stimulated side.
**Intracranial Self-Stimulation (ICSS)**

Mice were removed from *ad libitum* food one day prior to testing to facilitate behavioral responding. Mice were placed into a sound-attenuated operant chamber (Med Associates, Inc., St. Albans, VT, USA) containing two illuminated nose-poke ports (“active” and “inactive”) and speakers to play tones and white noise. A response into either nose-poke port was accompanied by illumination of a cue-light (positioned above the nose-poke port) and a distinct 1-s tone (1 or 1.5 kHz, counterbalanced). A nose-poke response into the “active” port resulted in delivery of photostimulation (ChR2: 473 nm, 10 Hz, 20 mW, 5-ms pulses, 1-s duration; NpHR: 589/593 nm, constant, 5 mW, 1-s duration), while no stimulation was delivered for a nose-poke response in the “inactive” port (counterbalanced between mice). Mice were allowed to explore for one hour. Nose-poke ports were baited with a small amount of crushed sucrose pellets to encourage investigation, and white noise was played throughout the session.

**Social Interaction (Resident-Intruder) Assay**

Mice were placed into a clean cage and given 5 minutes to explore the environment. A juvenile male (3-4 weeks of age, VGAT::Cre or VGLUT2::Cre) or adult female (C57/BL6) mouse was placed into the cage, and a nine minute recording session with three consecutive 3-min epochs was initiated. In the second epoch, mice were photostimulated (ChR2: 473 nm, 20 Hz, 20 mW, 5-ms pulses; NpHR: 589/593 nm, constant, 5 mW). The behavior of the experimental mouse was manually scored by blinded experimenters for social behavior, e.g. grooming, sniffing of the face or hind regions, and mounting of the intruder, using ODLog behavioral analysis software (Macropod Software). Mice that never spent more than 5% of time in any epoch interacting with the intruder mouse were excluded.
**Four-Chamber Novel Object Task**

Mice were placed into an open chamber (50 cm x 53 cm), which was divided into four regions. A distinct novel object was placed into the center of each of the regions. Mice were allowed to explore the chamber for one hour and were stimulated (ChR2: 473 nm, 20 Hz, 20 mW, 5-ms pulses; NpHR: 589/593 nm, constant, 5 mW) for 3-min epochs at 3-min intervals. A video camera positioned above the chamber recorded each session and mouse locations/velocity were tracked and analyzed using Ethovision XT software (Noldus, Wageningen, Netherlands). Zone crossings were identified by Ethovision XT as events where mice crossed from one zone to another. Investigation of objects was manually scored by blinded experimenters using ODLog behavioral analysis software (Macropod Software). Difference scores for investigation time were calculated by subtracting the total amount of time spent investigating objects during OFF epochs from the total amount of time spent investigating objects during ON epochs. Difference scores for the number of zone crossings were calculated by subtracting the total number of zone crossings during OFF epochs from the total number of zone crossings during the ON epochs. One data point was rejected as an outlier using Chauvenet's criterion.

**Feeding Task**

Mice were allowed to explore a chamber with two empty plastic cups placed in opposite corners of the chamber for a period of 5 minutes (habituation). A moist food pellet was then placed into one of the cups (counterbalanced between mice), and a nine minute recording session with three consecutive 3-min epochs was initiated. In the second epoch, mice were photostimulated (NpHR: 589/593 nm, constant, 5 mW). The amount of time spent feeding was manually scored by blinded experimenters using ODLog behavioral analysis software (Macropod Software). Difference scores for feeding were calculated by subtracting the percentage of time spent feeding during the first OFF epoch from the percentage of time spent feeding during the ON epoch.
In Vivo Fast-Scan Cyclic Voltammetry (FSCV)

Anesthetized in vivo FSCV experiments were conducted similarly to those previously described (Matthews et al., 2016). Following behavioral experimentation, mice were anesthetized with 30% urethane (1.5 g/kg, I.P.) diluted in sterile saline and placed in a stereotaxic frame located within a faraday cage. NAc measurements were obtained by using the VTA fiber implant coordinates as reference. Small craniotomies were made above the NAc (~AP: 1.0, ML: 1.0) and contralateral cortex through the existing implant/dental cement. A chlorinated silver (Ag/AgCl) reference electrode was implanted in the contralateral cortex and cemented in place (C&B Metabond; Parkell, Edgewood, NY, USA). A glass-encased carbon fiber electrode (~120-150 µm in length, epoxied seal) was lowered just dorsal of the NAc (DV: -2.9 from brain surface) and was allowed to equilibrate for 20 minutes at 60 Hz and 10 minutes at 10 Hz. Voltammetric recordings were collected using Tarheel CV at 10 Hz by applying a triangular waveform (-0.4 V to +1.3 V to -0.4 V, 400 V/s) to the carbon-fiber electrode versus the Ag/AgCl reference, as has been described previously (Vander Weele et al., 2014). Following cycling, electrodes were lowered into the NAc in 200 µm steps until changes in dopamine release were detected after optical activation of the LH inputs to the VTA using blue light (473 nm, 20Hz, 20 mW, 5-ms pulses, 10-s duration). Data were collected in 30-second files with the stimulation onset occurring ten seconds into the file. 20-25 recordings were collected at 60-second intervals and background subtracted at approximately the lowest current value prior to stimulation onset.

Following completion of baseline recordings, mice were administered the D2 receptor antagonist, raclopride (Sigma-Aldrich, St. Louis, MO, USA, 5.0 mg/kg diluted in sterile saline, I.P.), as a positive control and to enhance background dopamine levels. Raclopride recordings commenced 10 minutes after injection. Carbon-fiber electrodes were pre-calibrated in known concentrations of dopamine (1000, 500, 250 nM) as previously described (Badrinarayan et al., 2012) and calibration data were used to convert in vivo signals to changes in dopamine concentration using chemometric, principal component regression, and residual analyses using
a custom LabView program (Umich CV, Courtesy of Richard Keithley; Keithley et al., 2009). For quantitation of evoked DA, area under the curve was calculated during the 10-s stimulation period (0-10 s) compared with basal fluctuations during the 10-s period prior to stimulation onset (-10-0 s). Following recordings, mice were transcardially perfused, fixed, and processed (as described below) to confirm viral expression and placements of the optic fibers and recording electrode tracks.

Photometry
For the photometry system, 473-nm light from a DPSS laser (30-80 µW; OEM Laser Systems, Draper, UT, USA) was filtered through a neutral density filter (1.0 optical density, Thorlabs, Newton, NJ, USA) held in a filter wheel (FW1A, Thorlabs, Newton, NJ, USA), sent through a chopper (400±10 Hz; SR540 Chopper Controller, Stanford Research Systems, Sunnyvale, CA, USA) through a 473-nm filter (LD01-473, Semrock, Rochester, NY, USA), reflected off a dichroic mirror (FF495, Semrock, Rochester, NY, USA) and coupled through a fiber collimation package (F240FC-A, Thorlabs, Newton, NJ, USA) into a patch cable connected to the ferrule of the upright optic fiber implanted in the mouse via a ceramic sleeve (Precision Fiber Products, Milpitas, CA, USA). GCaMP6m fluorescence emanating through the implanted optic fiber was collected through a 525-nm filter (FF03-525, Semrock, Rochester, NY, USA) into a photodetector (Model 2151, Newport Corporation, Irvine, CA, USA). The signal was passed through a lock-in amplifier (100 ms, 12 dB, 500 mV; SR810, Stanford Research Systems, Sunnyvale, CA, USA) and digitized and collected with a LabJack U6-PRO (250 Hz sampling frequency; LabJack, Lakewood, CO, USA). For stimulation of GABAergic LH terminals in the VTA, mice were placed in their home cage, and 20-Hz stimulation (593 nm, 5-10 mW, 5-ms pulses, 1-s duration) was given every 10 seconds for 30 trials into the angled optic fiber implanted in the mouse. This was then repeated for 30 trials of one second constant stimulation
(593 nm, 5-10 mW). The raw signal was divided by a linear fit to normalize the baseline over the recording session. Z scores were taken using the 5 seconds prior to stimulation as baseline.

**Ex Vivo Electrophysiology**

Brain slices were prepared from VGAT::Cre or VGLUT2::Cre mice which had received an injection of AAV5-DIO-ChR2-eYFP or AAV5-DIO-ChR2-mCherry into the LH at least 7 weeks prior. Mice were deeply anesthetized by IP injection of sodium pentobarbital (200 mg/kg) before transcardial perfusion with 20 mL ice-cold modified ACSF (composition in mM: NaCl 87, KCl 2.5, NaH2PO4*H2O 1.3, MgCl2*6H2O 7, NaHCO3 25, sucrose 75, ascorbate 5, CaCl2*2H2O 0.5, in ddH2O; osmolarity 323-328 mOsm, pH 7.20-7.35) saturated with carbogen gas (95% oxygen, 5% carbon dioxide). The brain was rapidly dissected out of the cranial cavity and 300-μm horizontal slices containing the VTA were prepared on a vibrating-blade microtome (Leica VT1000S, Leica Microsystems, Wetzlar, Germany). Brain slices were then given at least 1 hour to recover in a holding chamber containing ACSF (composition in mM: NaCl 126, KCl 2.5; NaH2PO4*H2O 1.25, MgCl2*6H2O 1, NaHCO3 26, glucose 10, CaCl2*2H2O 2.4, in ddH2O; osmolarity 299-301 mOsm; pH 7.30-7.40) saturated with carbogen gas at 32 °C.

For electrophysiology, slices were transferred to a recording chamber and continuously perfused at a rate of 2 mL/min with fully oxygenated ACSF at 30-32 °C. Electrodes for recording were pulled from thin-walled borosilicate glass capillary tubing on a P-97 puller (Sutter Instrument, Novato, CA, USA) and had resistances of 4-7 MΩ when filled with internal solution (composition in mM: potassium gluconate 125, NaCl 10, HEPES 20, MgATP 3, and 0.1% neurobiotin, in ddH2O; osmolarity 287 mOsm; pH 7.30). Whole-cell patch-clamp recordings were performed using a Multiclamp 700B amplifier and Clampex 10.4 software (Molecular Devices, Sunnyvale, CA, USA). Signals were low-pass filtered at 1 kHz and digitized at 10 kHz using a Digidata 1550 (Molecular Devices, Sunnyvale, CA, USA). Cell capacitance, series resistance, and input resistance were frequently measured during recordings to monitor cell health.
Neurons were visualized via a 40X water-immersion objective on an upright microscope (Scientifica, Uckfield, UK) equipped with IR-DIC optics and a QImaging Retiga EXi camera (QImaging, Surrey, BC, Canada). The region containing ChR2-expressing terminals in the VTA was identified by brief illumination through a 470 nm LED light source (pE-100; CoolLED, River Way, UK). A subset of VGAT::Cre mice received an injection of AAV₅-DIO-ChR2-mCherry into the LH and AAV₅-DIO-eYFP into the VTA in order to identify GABA neurons. In these brain slices, ChR2-expressing terminals were visualized by illumination through a 595 nm LED light source (pE-100; CoolLED, River Way, UK) and GABA neurons in the VTA by brief illumination through the 470 nm LED light source (pE-100; CoolLED, River Way, UK).

Neurons were recorded in voltage-clamp mode at a holding potential of -70 mV in VGLUT2::Cre mice to elicit glutamatergic excitatory postsynaptic currents and at 0 mV in VGAT::Cre mice to elicit GABAergic inhibitory postsynaptic currents. ChR2-expressing terminals were activated by a 5-ms pulse of 470 nm LED light, delivered through the objective, every 20 s. Analysis was subsequently performed in Clampfit 10.4 software (Molecular Devices, Sunnyvale, CA, USA). The average light-evoked current was calculated using at least 12 stable sweeps, from which peak current amplitude and onset latency were measured.

To determine the TH content of recorded neurons, brain slices were subsequently processed with immunohistochemistry. Recorded slices were fixed in 4% paraformaldehyde (PFA) overnight at 4 °C, then washed four times in phosphate-buffered saline (PBS) for 10 minutes each wash. Slices were then blocked in 1x PBS containing 0.3% Triton X-100 (PBS-T 0.3%) with 5% normal donkey serum (NDS) (Jackson ImmunoResearch Labs, West Grove, PA, USA) for 1 hour at room temperature followed by incubation in primary antibody solution: chicken anti-TH (1:1000; Millipore Cat# AB9702, RRID: AB_570923; Millipore, Billerica, MA, USA) in 1x PBS-T 0.3% with 3% NDS for 18-24 hours at 4 °C. Slices were subsequently washed four times in 1x PBS (for 10 minutes each) and then transferred to secondary antibody solution: Alexa Fluor 647-conjugated donkey anti-chicken (1:1000; Jackson ImmunoResearch
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Labs Cat# 703-605-155, RRID: AB_2340379; Jackson ImmunoResearch Labs, West Grove, PA, USA) and 405-conjugated streptavidin (1:1000; Biotium, Hayward, CA, USA) in 1x PBS-T 0.1% with 3% NDS for 2 hours at room temperature. After a further four washes in 1x PBS (for 10 minutes each), slices were mounted onto glass slides and cover-slipped using polyvinyl alcohol (PVA) mounting medium with DABCO (Sigma-Aldrich, St. Louis, MO, USA).

Histology

Perfusion and Storage

Subjects were deeply anesthetized with sodium pentobarbital (200 mg/kg; I.P.) and transcardially perfused with 20 mL of Ringer’s solution followed by 20 mL of cold 4% PFA dissolved in 1x PBS. The brain was extracted and placed in 4% PFA solution and stored at 4 °C for at least 24 hours. Brains were then transferred to a 30% sucrose solution in 1x PBS for 24 hours at room temperature. Brains were sectioned into 40-60 µm slices on a sliding microtome (HM420; Thermo Fischer Scientific, Waltham, MA, USA). Sections were stored in 1x PBS at 4 °C until immunohistochemical processing.

Immunohistochemistry

Sections were blocked in 1x PBS-T 0.3% with 3% NDS (Jackson ImmunoResearch Labs, West Grove, PA, USA), for one hour at room temperature. LH sections were incubated in a DNA-specific fluorescent probe (DAPI: 4’,6-Diamidino-2-Phenylindole; 1:50,000 in 1x PBS) for 30 minutes, washed four times for 10 minutes each in 1x PBS, mounted on glass microscope slides, and cover-slipped using PVA mounting medium with DABCO (Sigma-Aldrich, St. Louis, MO, USA). VTA sections were incubated in a solution containing chicken anti-TH (1:500; Millipore Cat# AB9702, RRID: AB_570923; Millipore, Billerica, MA, USA) and rabbit anti-c-Fos (1:500; Santa Cruz Biotechnology Cat# sc-52, RRID: AB_2106783; Santa Cruz Biotechnology, Dallas, TX, USA) in 1x PBS-T 0.1% (or 1x PBS-T 0.3%) with 3% NDS for 24 hours at room
temperature or 24-48 hours at 4 °C. Sections were then washed four times (10 minutes each) in 1x PBS and immediately transferred to secondary antibody solution containing Alexa Fluor 647-conjugated donkey or goat anti-chicken (1:500; Jackson ImmunoResearch Labs Cat# 703-605-155, RRID: AB_2340379; Jackson ImmunoResearch Labs Cat# 103-605-155, RRID: AB_2337392; Jackson ImmunoResearch Labs, West Grove, PA, USA), Cy3-conjugated donkey anti-rabbit (1:500; Jackson ImmunoResearch Labs Cat# 711-165-152, RRID: AB_2307443; Jackson ImmunoResearch Labs, West Grove, PA, USA), and DAPI (1:50,000) in 1x PBS containing 3% NDS for two hours at room temperature. In some animals in which c-Fos was not analyzed, Cy3-conjugated donkey anti-chicken (1:500; Jackson ImmunoResearch Labs Cat# 703-165-155, RRID: AB_2340363; Jackson ImmunoResearch Labs, West Grove, PA, USA) was used. Sections were washed four times (10 minutes each) in 1x PBS, mounted on glass microscope slides, and cover-slipped with PVA-DABCO.

Confocal Microscopy

Fluorescent images were captured using a confocal scanning microscope (Olympus FV1000, Olympus, Center Valley, PA, USA) with FluoView software (Olympus, Center Valley, PA, USA) under a 10x/0.40 NA dry objective or a 40x/1.30 NA oil immersion objective. The locations of the virus injection sites were estimated by comparing the surgical injection coordinates and the presence of dense eYFP-expressing cell bodies. The locations of optic fiber tips and carbon-fiber recording electrodes were determined by the presence of a lesion in the slices.

Cell Counting

For c-Fos cell counting, following behavioral experiments, VGLUT2::Cre and VGAT::Cre mice were stimulated for ten minutes in a dark, sound-attenuating room (473 nm, 20 Hz, 20 mW, 5-ms pulses). Eighty minutes later, mice were anesthetized and transferred to the lab. Approximately 5-10 minutes later, mice were transcardially perfused and the brains processed
and imaged as described above. Two blinded experimenters counted 400-500 DAPI+ cells randomly distributed throughout the VTA and then identified cells for their co-expression with TH and c-Fos, or lack thereof.

For cell counting to quantify the proportion of ChR2+ or NpHR+ cells throughout the LH, ~100-200 random DAPI+ cells were identified (ChR2: right hemisphere; NpHR: both hemispheres) in the LH, and then the number of those cells that were also co-expressing ChR2 or NpHR were counted to generate a relative proportion of LH neurons that were ChR2+ or NpHR+. Counting was done in 40x z-stacks (8 slices in 3 µm steps) taken in the LH at -0.6 AP just lateral/ventral to the fornix, and at -1.0 and -1.4 AP lateral to the fornix.

Statistics
Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA), OriginPro 8.6 (OriginLab, Northampton, MA, USA), and MATLAB (Mathworks, Natick, MA, USA). Group comparisons were made using two-way ANOVA followed by Bonferroni post-hoc tests to control for multiple comparisons. Paired and unpaired Student’s t tests, as well as one-way ANOVA were used to make single-variable comparisons, and Chi-squared tests were used to compare populations.
SUPPLEMENTAL REFERENCES


