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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., 2006; Salamone et al., 2006;...
2005, 1994), and mediates the reinforcing properties of rewards (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 historically been 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 GABA and glutamate 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 neurotensin-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

In order to study the effect of GABAergic LH-VTA activation on behavior, we injected AAV5-DIO-ChR2-eYFP or AAV5-DIO-eYFP into the LH of vesicular GABA transporter (VGAT):Cre mice and placed an optic fiber over the VTA to illuminate LH GABAergic axon terminals (Figure 1A and S1). To test whether stimulating the GABAergic component of the LH-VTA projection (LH\textsuperscript{GABA-VTA}) would support place preference or avoidance, we placed mice into a 3-chamber apparatus where one side of the chamber was paired with optical stimulation (473 nm, 10 Hz, 20 mW, 5 ms pulses; Figure 1B). Surprisingly, we found that LH\textsuperscript{GABA-VTA}:ChR2 mice spent significantly more time in the chamber paired with stimulation than the chamber without stimulation when compared with their eYFP counterparts (Figure 1B and 1C). In addition, to test whether LH\textsuperscript{GABA-VTA} activation could support intracranial self-stimulation (ICSS), we placed mice into an operant chamber with an active and inactive nosepoke operandum. An active nosepoke 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 nosepoke response was paired only with a cue. LH\textsuperscript{GABA-VTA}:ChR2 mice made significantly more responses in the active nosepoke compared with the inactive nosepoke – an effect not observed in the eYFP controls (Figure 1D). These data show that mice prefer LH\textsuperscript{GABA-VTA} stimulation and are willing to perform an instrumental response in order to receive that stimulation.

In order to determine how activation of the glutamatergic component of the LH-VTA projection (LH\textsuperscript{Glut-VTA}) influences motivation, we used the same optogenetic approach and behavioral assays described above in vesicular glutamate transporter 2 (VGLUT2):Cre mice (Figure 1E and S1). In contrast to the robust preference supported by LH\textsuperscript{GABA-VTA} stimulation, activation of the glutamatergic projection was avoided by mice in the real-time place preference/avoidance assay (RTPP/A; Figure 1F and 1G). Consistent with these results, LH\textsuperscript{Glut-VTA}:ChR2 mice did not show a preference for the active nosepoke 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 LH\textsuperscript{GABA-VTA} projection could drive other behaviors in addition to feeding and approach. To assess the effect of LH\textsuperscript{GABA-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 and Supplemental Movie S1 and S2). Time spent engaging in social interaction (e.g. grooming, investigating the face or hind regions, or mounting of the intruder) was measured for three consecutive three-minute epochs, during which blue light (473 nm, 20 Hz, 20 mW, 5 ms pulses) was used to activate LH\textsuperscript{GABA-VTA} projections throughout the second epoch. LH\textsuperscript{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 differences in interaction with juvenile intruders between LH$^{\text{glut}}$-VTA:ChR2 mice and their controls, possibly due to a strong epoch effect (Figure 2D), we did find that LH$^{\text{glut}}$-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 a larger behavioral state in the animal, such as a change in overall motivational level, that can manifest as the investigation of any salient target, regardless of what that target object may be (e.g., food, 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 one hour and were stimulated using blue light (473 nm, 20 Hz, 20 mW, 5 ms pulses) for three-minute epochs at three-minute 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$^{\text{GABA}}$-VTA or LH$^{\text{glut}}$-VTA stimulation. We quantified the time spent investigating the objects and found that LH$^{\text{GABA}}$-VTA:ChR2 mice spent significantly more time investigating the objects during optical stimulation compared with eYFP controls (Figure 2G), while LH$^{\text{glut}}$-VTA:ChR2 mice spent significantly less time investigating objects during optical stimulation compared with their eYFP controls (Figure 2H). Additionally, we quantified the number of zone crossings, defined as transitions between zones, where each zone was the quadrant wherein each novel object was placed. LH$^{\text{GABA}}$-VTA:ChR2 mice made significantly fewer zone crossings during optical stimulation than eYFP controls (Figure 2I), while LH$^{\text{glut}}$-VTA:ChR2 made significantly more zone crossings during optical stimulation than their eYFP controls (Figure 2J). Together, these results suggest that activating the GABAergic LH-VTA projection promotes investigation of the most proximal salient object, while activating the glutamatergic projection reduces investigation of this object and increases exploration of the other chambers.

**Inhibition of the GABAergic LH-VTA Pathway Attenuates Behavioral Responding in Motivated Animals**

We next considered whether inhibiting the GABAergic or glutamatergic LH-VTA projection would be sufficient to produce changes in behavioral responding. In VGAT::Cre and VGLUT2::Cre mice, we bilaterally injected AAV5-DIO-NpHR-eYFP or AAV5-DIO-eYFP into the LH and implanted an optic fiber over the VTA (Figure S3). In the RTPP/A, ICSS, and juvenile/female social interaction assays, we did not detect any significant effects of inhibition of either projection on behavior (Figure S2C–H).

Previously, we demonstrated that activating the LH$^{\text{GABA}}$-VTA projection increased feeding in sated mice (Nieh et al., 2015). 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 x epoch effect (Figure 3B), LH\textsuperscript{GABA}-VTA:NpHR mice showed a significantly greater decrease in time spent feeding during optical inhibition from the baseline epoch compared with eYFP controls (Figure 3C). However, LH\textsuperscript{glut}-VTA:NpHR mice did not show any change in time spent feeding upon optical inhibition compared with their eYFP controls (Figure 3D and 3E). In the four-chamber novel object test (Figure 3F), unrestricted LH\textsuperscript{GABA}-VTA:NpHR mice spent significantly less time investigating the objects (Figure 3G) and made significantly more zone crossings (Figure 3I) during optical inhibition when compared with eYFP controls. No significant differences were found upon LH\textsuperscript{glut}-VTA inhibition (Figure 3H and 3J).

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

We next examined the consequence of LH\textsuperscript{GABA}-VTA and LH\textsuperscript{glut}-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). This revealed that LH\textsuperscript{GABA}-VTA stimulation induced more c-Fos+ DA (TH+) neurons than LH\textsuperscript{glut}-VTA stimulation (Figure 4B), suggesting that stimulation of the LH\textsuperscript{GABA}-VTA pathway enhances the activity of VTA DA neurons.

We next explored how activation of the LH\textsuperscript{GABA}-VTA pathway influences downstream DA signaling in the nucleus accumbens (NAc) using in vivo fast-scan cyclic voltammetry (FSCV) (Figure 4 and S4). We found that LH\textsuperscript{GABA}-VTA activation robustly increased extracellular DA concentration ([DA]) in the NAc (Figure 4C–F). In many subjects, evoked DA release was composed primarily of individual phasic DA release events, or ‘transients’ (Figure 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 D\textsubscript{2} 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 D\textsubscript{2} receptor antagonism, LH\textsuperscript{GABA}-VTA stimulation significantly increased DA neurotransmission in the NAc (Figure 4G–I and S4C).

In contrast, LH\textsuperscript{glut}-VTA activation (Figure 4J) caused a decrease in current at the oxidation potential for DA, indicative of a pause in DA neurotransmission in the NAc, leading to a significant reduction in [DA] at baseline (Figure 4K–M and S4D) and after D\textsubscript{2} receptor blockade (Figure 4N–P and S4E). Consistent with the idea that LH\textsuperscript{glut}-VTA activation results in suppression of activity in NAc-projecting VTA DA neurons, stimulation offset often evoked a phasic DA transient (Figure 4K and S4D) – likely resulting from rebound activity arising from prolonged hyperpolarization of VTA DA cell bodies. Together, these data indicate that GABAergic and glutamatergic LH-VTA projections bidirectionally modulate DA release, with the GABAergic projection increasing DA release and the glutamatergic projection decreasing DA release in the NAc.
Effects of GABAergic LH-VTA Stimulation on Dopamine 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.

In order 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 AAV8-hSyn-FLEX-ChrimsonR-tdTomato into the LH and AAV5-CAG-FLEX-GCaMP6m into the VTA and implanted two optic fibers over the VTA (Figure 5A–C). 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 µW, 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 AAV5-DIO-eYFP into the VTA instead of AAV5-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 LH\textsuperscript{GABA-}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 when compared with pre-stimulation fluorescence and fluorescence from control mice (Figure 5D and 5E). This significant decrease in fluorescence reflects a decrease in VTA GABA neural activity and suggests that LH\textsuperscript{GABA-}VTA stimulation significantly reduces activity in VTA GABA neurons.

Finally, we performed whole-cell patch-clamp recordings from VTA TH+ (dopamine) 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 LH\textsuperscript{GABA-}VTA stimulation were significantly greater in putative GABA neurons compared with DA neurons in the VTA (Figure 6B). Similarly, the amplitudes of excitatory postsynaptic currents (EPSCs) elicited by LH\textsuperscript{Glut-}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 is greater onto putative GABA neurons. Taken together, our data show that 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 (Bielajew and Shizgal, 1986; Hoebel and Teitelbaum, 1962; 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 travelling 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 (Figure 1D and 1H). Furthermore, photostimulation of LH<sup>GABA</sup>-VTA is preferred, while photostimulation of LH<sup>glut</sup>-VTA is avoided (Figure 1B–C and Figure 1F–G).

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 in 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 on positive reinforcement by VTA DA neuron stimulation. 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 upon 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<sub>A</sub> receptors were expressed on both VTA DA neurons (Sugita et al., 1992) and VTA GABA neurons (Rick and Lacey, 1994). Johnson and North first hypothesized that mu-opioid receptor agonists, such as morphine, act in the VTA via disinhibition through GABA neurons (Johnson and North, 1992), while Bocklisch and colleagues showed that cocaine can also 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 between 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 dopamine neurons within the VTA has been shown to indicate a difference in projection target, with dopamine neurons in medial VTA projecting to the NAc medial shell and mPFC and dopamine 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+ with respect to amplitude, and therefore, it does not appear that the GABAergic or glutamatergic LH-VTA projection has preferential input to either population of DA neurons within the VTA.

As a result of the gnawing behavior that occurs in an empty chamber, we conducted the real-time place preference/avoidance and intracranial self-stimulation 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 was much 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 show that LH$_{\text{GABA}}$-VTA or LH$_{\text{glut}}$-VTA stimulation at either 10 Hz or 20 Hz evoke the same pattern of dopamine release and suppression, respectively (Figure 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 can also 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 have also 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 towards saccharin, water, or wood (Navarro et al., 2015). 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 (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 (Supplemental Movies S1 and S2), and in the four-chamber novel object task, wherein the salient stimulus 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 could also be modulating motivation levels in order to promote avoidance. However, because our experiments in this current 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 the current 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, 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 terminalis, 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 release in the dorsal striatum is also critical for feeding (Szczypka et al., 1999, 2001) and compulsive behaviors (Ito et al., 2002; Vanderschuren et al., 2005; Willuhn et al., 2012), 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, thirst, 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 may also be causing peptidergic co-release within the VTA or via axon collaterals, since a subset of GABA-expressing LH neurons also express peptides such as neurotensin ([Leinninger et al., 2009; Opland et al., 2013]).

Conclusion

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 towards 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 Münzberg, 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 dopamine 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 (Betley et al., 2015), LH\textsuperscript{GABA-VTA} stimulation appears to evoke feeding by increasing the motivation for food rewards.

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 induce overeating or compulsive eating and thus elevate food intake to maladaptive levels, but could also potentially lead to compulsive behaviors towards other stimuli as well. This idea that a malfunction in one
neural population may result in compulsive behaviors towards 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 identifies a possible target for therapeutic intervention in compulsive eating and other addictive disorders.

EXPERIMENTAL PROCEDURES

All experiments involving the use of animals were in accordance with NIH guidelines 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.

Fast-Scan Cyclic Voltammetry (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 dopamine release. Recordings were obtained under resting (“baseline”) conditions and after 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 AAV8-hSyn-FLEX-ChrimsonR-tdTomato into the LH and AAV5-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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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 AAV5-DIO-ChR2-eYFP or AAV5-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 LH^GABA-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). (C) LH^GABA-VTA:ChR2 mice had a significantly greater difference score (percentage time spent in stimulation side minus percentage time spent in non-stimulation side) than LH^GABA-VTA:eYFP mice (n=8 ChR2, n=10 eYFP; two-tailed, unpaired Student’s t-test, ****p<0.0001). (D) LH^GABA-VTA:ChR2 mice made significantly more responses at the active nosepoke paired with blue light stimulation (473 nm, 10 Hz, 20 mW, 5 ms pulses, 1 s duration) than the inactive nosepoke as compared with eYFP controls (n=6 ChR2, n=8 eYFP; two-way ANOVA revealed a group x nosepoke interaction, F_1,12=19.40, p=0.0009; Bonferroni post-hoc analysis, ***p<0.001). (E) VGLUT2::Cre mice were injected with AAV5-DIO-ChR2-eYFP or AAV5-DIO-eYFP into the LH, and an optic fiber was implanted over the VTA. (F) Representative track from the RTPP/A assay of an LH^glut-VTA:ChR2 mouse. (G) LH^glut-VTA:ChR2 mice had a significantly lower difference score than LH^glut-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 effects on intracranial self-stimulation in LH^glut-VTA:ChR2 compared with eYFP controls.
(n=7 ChR2, n=6 eYFP; two-way ANOVA: group x nosepoke interaction, $F_{1,11}=0.05$, $p=0.8307$). Error bars indicate ±SEM. See also Figures S1 and S2.
Figure 2. Photostimulation of the GABAergic LH-VTA Projection Promotes Social Interaction and Object Investigation, while Photostimulation of the Glutamatergic LH-VTA Projection Suppresses These Behaviors

(A) To assess social interaction, mice were placed into a cage with a novel juvenile male or an adult female intruder. Time spent interacting was quantified for three consecutive three-minute epochs, with the second epoch paired with blue light stimulation (473 nm, 20 Hz, 20 mW, 5 ms pulses). (B) LH\textsuperscript{GABA-VTA}:ChR2 mice showed increased time spent interacting with juvenile male intruders compared with LH\textsuperscript{GABA-VTA}:eYFP controls during the ON epoch (n=10 ChR2, n=11 eYFP; two-way ANOVA revealed a group x epoch interaction,
F_{2,38}=23.62, p<0.0001; Bonferroni post-hoc analysis, ****p < 0.0001), (C) as well as with female intruders (n=11 ChR2, n=10 eYFP; two-way ANOVA revealed a group x epoch interaction, F_{2,38}=10.05, p=0.0003; Bonferroni post-hoc analysis, ****p<0.0001). (D) LH_{glut}-VTA:ChR2 mice did not show a significant difference in time spent interacting with juvenile male intruders compared with LH_{glut}-VTA:eYFP mice, likely due to a strong epoch effect (n=8 ChR2, n=12 eYFP; two-way ANOVA revealed a significant epoch effect, F_{2,36}=10.05, p=0.0003), (E) but did show a significant decrease in interaction during the ON epoch with female intruders (n=7 ChR2, n=6 eYFP; two-way ANOVA revealed a group x epoch interaction, F_{2,22}=7.45, p=0.0034; Bonferroni post-hoc analysis, **p<0.01). (F) In order 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 one hour while receiving blue light stimulation (473 nm, 20 Hz, 20 mW, 5 ms pulses) for three-minute epochs at three-minute intervals. (G) LH_{GABA}-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), while (H) LH_{glut}-VTA:ChR2 mice had a significantly lower difference score than their respective eYFP counterparts (n=8 ChR2, n=7 eYFP; two-tailed, unpaired Student’s t-test, *p=0.0250). (I) LH_{GABA}-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), while (J) LH_{glut}-VTA:ChR2 mice had a significantly higher difference score (n=8 ChR2, n=7 eYFP; two-tailed, unpaired Student’s t-test, *p=0.0372) than their respective eYFP counterparts. Error bars indicate ±SEM. See also Figures S1 and S2.
Figure 3. Inhibition of the GABAergic LH-VTA Projection of Animals in a Motivated State Suppresses Behavioral Responding

(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 three-minute epochs, with the second epoch paired with yellow light stimulation (589/593 nm, constant, 5 mW). (B) There was a significant interaction of light stimulation on time spent feeding in LH\textsuperscript{GABA-VTA}:NpHR mice relative to eYFP controls (n=8 NpHR, n=9 eYFP; two-way ANOVA revealed a group x epoch interaction, F\textsubscript{2,30}=4.46, p=0.0202). (C) In addition, LH\textsuperscript{GABA-VTA}:NpHR mice had a significantly lower difference...
score in time spent feeding (ON-first OFF) when compared with eYFP controls (n=8 NpHR, n=9 eYFP; two-tailed, unpaired Student’s t-test, *p=0.0210). (D) Meanwhile, no effect was found in LH\textsuperscript{Glu} \text{VTA:NpHR} mice and their controls on the amount of time spent feeding (n=10 NpHR, n=7 eYFP; two-way ANOVA: group x epoch interaction, F\textsubscript{2,30}=0.17, p=0.8484), or (E) in difference score (n=10 NpHR, n=7 eYFP; two-tailed, unpaired Student’s t-test, p=0.5963). (F) In the four-chamber novel object test, (G) LH\textsuperscript{GABA} \text{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) LH\textsuperscript{Glu} \text{VTA:NpHR} mice showed no differences from their eYFP controls (n=10 NpHR, n=7 eYFP; two-tailed, unpaired Student’s t-test, p=0.5358). (I) LH\textsuperscript{GABA} \text{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), while (J) LH\textsuperscript{Glu} \text{VTA:NpHR} mice showed no differences from their eYFP controls (n=10 NpHR, n=7 eYFP; two-tailed, unpaired Student’s t-test, p=0.3247). Error bars indicate ±SEM. See also Figures S2 and S3.
Figure 4. Optogenetic Activation of the GABAergic LH-VTA Projection Increases, while Activation of the Glutamatergic LH-VTA Projection Suppresses, Dopamine Release in the NAc

(A) Representative confocal images from the VTA of LH\textsuperscript{GABA-VTA:ChR2} (top) and LH\textsuperscript{glut-VTA:ChR2} (bottom) mice showing 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- neurons (right) that either co-express or do not co-express c-Fos after LH\textsuperscript{GABA-VTA} or LH\textsuperscript{glut-VTA} photostimulation. Mice receiving LH\textsuperscript{GABA-VTA} stimulation showed a significantly greater proportion of cells co-expressing TH and c-Fos compared with mice receiving LH\textsuperscript{glut-VTA} stimulation (Chi-square=21.77, ****p<0.0001).

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(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, E, F) Optical activation of the LH\textsubscript{GABA}-VTA projection evoked DA release in the NAc. (D) Representative false color plot showing an increase in current at the oxidation potential for DA (~0.65 V) upon LH\textsubscript{GABA}-VTA photostimulation (473 nm, 20 Hz, 20 mW, 5 ms pulses, 10 s duration), (E) which is also evident in the averaged population data after conversion into DA concentration. (F) Quantification of extracellular DA concentration ([DA]) as area under the curve showed that LH\textsubscript{GABA}-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, H, I) Under D\textsubscript{2} receptor blockade (intraperitoneal (IP) raclopride), LH\textsubscript{GABA}-VTA stimulation also increased NAc DA neurotransmission (G) as seen in the representative color plot (H) and averaged population data. (I) Quantification of [DA] as area under the curve revealed a significant increase in DA release under D\textsubscript{2} 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. (K, L, M) LH\textsubscript{glut}-VTA stimulation caused a pause in NAc DA release under resting, baseline conditions. (K) Representative false color plot showing a decrease in current at the oxidation potential for DA in response to LH\textsubscript{glut}-VTA stimulation (473 nm, 20 Hz, 20 mW, 5 ms pulses, 10 s duration). Stimulation offset was accompanied by a “rebound” DA transient, likely caused by rebound firing following hyperpolarization of VTA DA neurons during stimulation, which was also observed in the (L) averaged population data after conversion to [DA]. (M) Quantification of [DA] as area under the curve showed that LH\textsubscript{glut}-VTA stimulation caused a significant decrease in [DA] in the NAc under resting conditions (n=5 mice; two-tailed, paired Student’s t-test, *p=0.0325). (N, O, P) Under the influence of raclopride, LH\textsubscript{glut}-VTA activation robustly inhibited NAc DA release observed in the (N) representative color plot and (O) population average. (P) Quantification of [DA] showed that LH\textsubscript{glut}-VTA activation caused a significant and robust decrease in [DA] under D\textsubscript{2} receptor blockade (n=6 mice; two-tailed, paired Student’s t-test, **p=0.0089). Color plot insets: cyclic voltammograms (CVs) at time-points indicated by the inverted white triangles. Error bars indicate ±SEM. See also Figure S4.
Figure 5. GABAergic LH Inputs Inhibit GABA Neurons in the VTA

(A) In order to activate GABAergic LH-VTA projections and record from GABA neurons in the VTA simultaneously, VGAT::Cre mice were injected with AAV8-hSyn-FLEX-ChrimsonR-tomato into the LH and AAV5-CAG-FLEX-GCaMP6m into the VTA with two optic fibers implanted over the VTA. (B) Confocal image showing ChrimsonR+ cells bodies in the LH (red). (C) Confocal image showing GCaMP6m+ cell bodies in the VTA (green), ChrimsonR+ fibers (red), and TH+ neurons (white). (D) 20 Hz LH$_{GABA}$-VTA photostimulation (593 nm, 5–10 mW, 5 ms pulses, 1 s duration) caused a decrease in

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GCaMP6m fluorescence in VTA GABA neurons, as seen in both population averages for Z-Scores as well as individual heat maps, indicating a decrease in neural activity of VTA GABA neurons. Inset bar graph: the quantification of the area under the curve for stimulation (0–2 s), compared with pre-stimulation (~2–0 s) and eYFP controls (0–2 s) showed that 20 Hz stimulation (593 nm, 5–10 mW, 1 s duration) caused a significant decrease in VTA GABA neural activity (n=6 GCaMP6m, n=5 eYFP; one-way ANOVA, F$_{2,14}$=24.39, ****p<0.0001, Bonferroni post-hoc analysis, **p<0.01, ****p<0.0001) (E) Photostimulation of the LH$^{GABA}$-VTA projection with constant light (593 nm, 5–10 mW, 1 s duration) also caused a significant decrease in GABA neural activity. Inset bar graph (n=6 GCaMP6m, n=5 eYFP; one-way ANOVA, F$_{2,14}$=15.75, ***p<0.0003, Bonferroni post-hoc analysis, **p<0.01, ***p<0.001). Error bars indicate ±SEM.
Figure 6. GABAergic and Glutamatergic LH Projections are Stronger onto Putative GABA Neurons than Dopamine Neurons in the VTA

(A) Whole-cell patch-clamp recordings were made from VTA neurons in brain slices prepared from VGAT::Cre and VGLUT2::Cre mice expressing ChR2 in a Cre-dependent manner in the LH. Neurons were filled with neurobiotin during recording and subsequently processed with immunohistochemistry for TH (red). (B) ChR2-expressing terminals were activated with a 5 ms blue light pulse to elicit inhibitory post-synaptic currents (IPSCs) in VGAT::Cre mice. IPSC amplitude was significantly greater in TH-VTA cells than TH+ cells (n=9 TH+, n=7 TH--; two-tailed, unpaired Student’s t-test, *p=0.0270). (C) Similarly, in
VGLUT2: Cre mice, the amplitude of optically-evoked excitatory post-synaptic currents (EPSCs) was significantly greater in TH- VTA cells than TH+ cells (n=5 TH+, n=5 TH-; two-tailed, unpaired Student’s t-test, *p=0.0464). (D) Model representing the GABAergic projection from the LH onto GABA cells in the VTA. Activation of the GABAergic LH-VTA projection results in disinhibition of VTA DA neurons and therefore increases DA release in the NAc. Error bars indicate ±SEM. See also Figure S5.