

Stimulation of VTA dopamine inputs to LH upregulates orexin neuronal activity in a DRD2-dependent manner

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Abstract

Highlights

- Optical VTA DA neuron stimulation is sufficient to elicit a Pavlovian-like dopamine transient in the NAc
- Dopamine in the LH encodes both negative and positive reward prediction errors
- Dopamine in the LH positively modulates orexin neuronal activity locally in a D2R dependent way

Dopamine and orexins (hypocretins) play important roles in regulating reward-seeking behaviors. It is known that hypothalamic orexinergic neurons project to dopamine neurons in the ventral tegmental area (VTA), where they can stimulate dopaminergic neuronal activity. Although there are reciprocal connections between dopaminergic and orexinergic systems, whether and how dopamine regulates the activity of orexin neurons is currently not known. Here we implemented an opto-Pavlovian task in which mice learn to associate a sensory cue with optogenetic dopamine neuron stimulation to investigate the relationship between dopamine release and orexin neuron activity in the LH. We found that dopamine release can be evoked in LH upon optogenetic stimulation of VTA dopamine neurons, and is also naturally evoked by cue presentation after opto-Pavlovian learning. Furthermore, orexin neuron activity could also be upregulated by local stimulation of dopaminergic terminals in the LH in a way that is partially dependent on dopamine D2 receptors (DRD2). Our results reveal previously unknown orexinergic coding of reward expectation and unveil an orexin-regulatory axis mediated by local dopamine inputs in the LH.

eLife assessment

This study presents **valuable** findings that expand our view of dopamine release in different brain regions and show that dopamine release in the lateral hypothalamus is related to the activity of orexin neurons. The evidence supporting the claims of the authors is **solid**, although inclusion of tests that directly assess causality of the noble pathways would have been even more conclusive. The work will be of interest of neuroscientists who study the neural basis of motivation.

Introduction

Dopamine in the ventral and dorsal striatum shapes reward-related behaviors^{1–5}; its dysregulation has been associated with several psychiatric disorders, including addiction^{6–8} and depression^{9–11}. It is known that rewarding stimuli evoke dopamine transients both in the ventral^{12,13} and dorsal striatum¹⁴, and that the stimulation of dopaminergic neurons^{15,16} or terminals³ in the striatum is sufficient to trigger operant or Pavlovian conditioning¹⁷ as well as conditioned place preference. Instead, aversive stimuli or omission of expected reward delivery cause a decrease in dopamine in the ventral striatum, resulting in negative reinforcement learning^{18,19} via D2 receptors^{20,21}.

Although the role of the dopaminergic projections to the striatum or mesolimbic dopamine pathway has been investigated extensively^{12,22} – their role in encoding reward prediction errors (RPE) in particular has been a point of focus^{12,23} – the role of dopamine in other brain regions is relatively understudied^{24–27}. The lateral hypothalamus (LH) plays a pivotal role in reward-seeking behavior^{28–32} and feeding^{33–36}, and several dopamine receptors are reported to be expressed in the LH³⁷. The mechanism through which dopamine modulates neuronal activity in the LH, resulting in the modulation of behaviors, has not been established. To the best of our knowledge, there have been no measurements of dopamine transients in the LH during reward-associated behaviors.

The LH is a heterogeneous structure containing glutamatergic and GABAergic neurons, as well as several neuropeptidergic neurons, such as melanin-concentrating hormone positive and orexin-positive neurons^{38,39}. Like dopamine, orexins (also known as hypocretins) are reported to play a pivotal role in reward-seeking behavior^{29,40,41}. Orexinergic and dopaminergic systems are known to have reciprocal connections with each other, and some orexinergic neurons project to dopaminergic neurons in the ventral tegmental area (VTA), positively modulating their activity^{42,43}. While there has been extensive investigation into how dopamine modulates orexinergic neuronal activity *ex vivo* (i.e. acute brain slices)^{44–47}, it remains unclear whether and how dopamine transients modulate orexin neuronal activity *in vivo*⁴⁷. Advancements in optical tools, such as optogenetics for manipulating dopamine neurons and genetically encoded dopamine sensors for monitoring dopamine transients, have made it possible to precisely control and observe the dynamics of dopamine in neural systems^{13,48–52}. Here, we implemented an ‘opto-Pavlovian task’¹⁷, in which mice learn to associate a sensory cue with optogenetic dopamine neuron stimulation. Using this task we measured dopamine transients in the nucleus accumbens (NAc), finding that dopamine activity patterns are consistent with previous reports of RPE-encoding dopaminergic neuron activity²². Using the same paradigm, we found that optical stimulation of dopaminergic neurons in the VTA evokes an increase of extra-synaptic dopamine in the LH, where the delivery of a cue preceding a reward also triggers dopamine transients in a way that is consistent with RPEs²³. Furthermore, we investigated the regulation of LH orexinergic neurons by VTA dopaminergic neurons, and observed a dopamine transient in the LH and an

increase in orexinergic neuronal activity during both predictive cue and the delivery of laser stimulation, indicating that the concentration of extra-synaptic dopamine in the LH and orexinergic neuronal activity are positively correlated. Finally, by stimulating dopaminergic terminals in the LH combined with pharmacological intervention, we found that dopamine in the LH positively modulates orexinergic neurons via the type 2 dopamine receptor (D2).

Overall, our study sheds light on the meso-hypothalamic dopaminergic pathway, and its impact on orexinergic neurons.

Results

RPE-like dopamine transient in the NAc in response to VTA dopamine neuron stimulation

Previous work established an optogenetics-powered Pavlovian conditioning task (hereon called opto-Pavlovian) wherein animals learn to associate the delivery of a cue with optogenetic activation of their midbrain dopamine neurons¹⁷. This previous study determines that dopaminergic neuron responses to optical stimulation-predictive cues become established over multiple learning sessions. However, in light of recent evidence demonstrating that dopamine release in the mesolimbic system and dopamine neuron activity can be uncoupled we sought out to determine whether dopamine release would also follow the same patterns of dopamine somatic activity during this task^{53,54}. To selectively stimulate and monitor dopamine release from ventral tegmental area (VTA) dopaminergic neurons in the nucleus accumbens (NAc), we injected a cre-dependent ChrimsonR AAV in the VTA as well as dLight1.3b¹³, a genetically encoded dopamine sensor AAV, in the NAc of DAT-cre mice. The recording optic fiber was placed directly above the NAc injection site (**Fig. 1A**). Mice then underwent the ‘opto-Pavlovian task’¹⁷, where one cue (tone+light, 7s) was paired with the optogenetic stimulation of dopamine neurons in the VTA (**Fig 1D**), while the other cue was not (**Fig. 1B**). We observed a gradual increase of dopamine transients in response to the delivery of the laser-associated cue (**Fig. 1C,E and F**). In contrast, the change of response to the non-laser-paired cue was smaller (**Fig. 1C,E and F**), suggesting that mice discriminated between the two cues. After 10 sessions of the opto-Pavlovian task, mice were exposed to omission sessions (**Fig 2A**), in which one-third of the laser-paired cues failed to trigger laser stimulation and the other two-thirds were followed by laser stimulation of VTA dopamine neurons (**Fig 2A, B and C**). The omission of the laser stimulation triggered a dip of dLight signal (**Fig 2D**). We also observed a small dip of dLight signal during non-laser paired cue delivery (**Figure 1 – Supplement 1**). Overall, the dopamine transient observed during the opto-Pavlovian task was consistent with classical Pavlovian conditioning^{17,22}, indicating that mice engage similar learning processes whether the reward consists of an edible entity or of optogenetic stimulation of VTA dopamine neurons.

Dopamine transients in the LH follow the same rules as in the NAc

Given the involvement of the lateral hypothalamus (LH) in reward-seeking behaviors^{28,55}, we next asked whether a similar neuromodulatory coding of predictive cues could take place in the hypothalamus, outside of the mesolimbic dopamine system. To answer this question, we followed the same procedure as for the NAc, except injecting dLight1.3 and positioning the optic fiber for photometry recordings in the LH (**Fig 3A**). We observed Chrimson positive fibers in the LH originating from the VTA (**Fig 3A**) and found that the stimulation of VTA dopamine neurons reliably evoked dopamine transients in the LH (**Fig 3B**). The injected mice expressing dLight1.3b in the LH then underwent the opto-Pavlovian task (**Fig 3C-G**). On session 1 of the task, we observed dopamine transients neither around laser-paired cue nor around non-laser-paired cue presentation (**Fig 3C and D**). However, in the LH as in the NAc, there was a gradual increase of

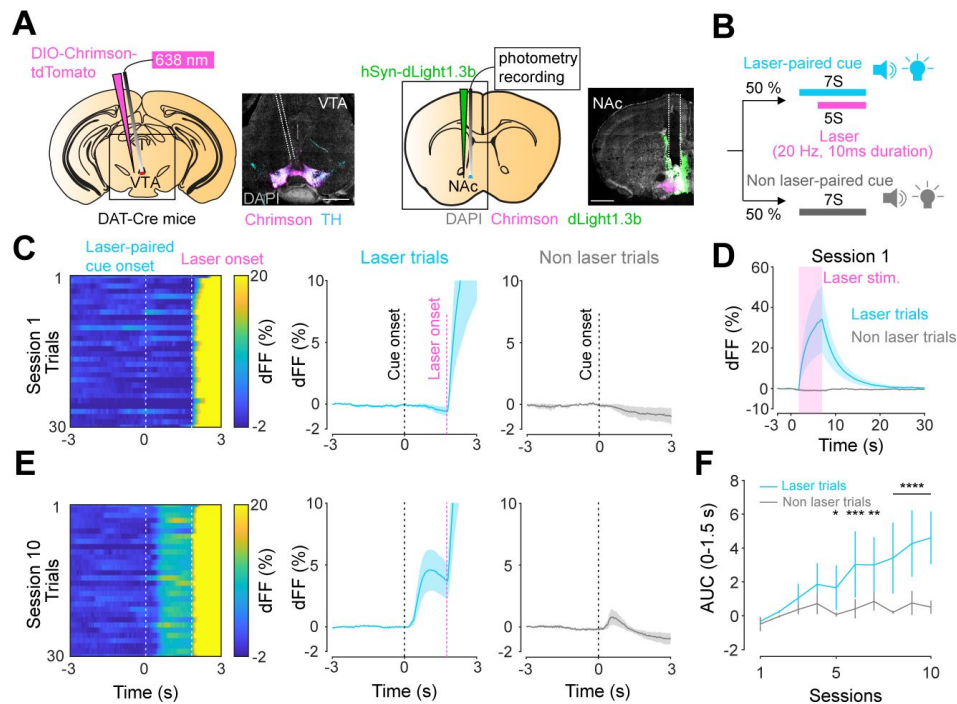


Figure 1.

A. Preparation for opto-Pavlovian task combined with dLight recordings in the NAc. Scale bar; 1mm. White dashed lines indicate fiber tracts. **B.** Schematic for opto-Pavlovian task. One cue was associated with the laser delivery while the other cue was not. **C.** dLight recordings in the NAc of a representative mouse around the laser-paired cue presentation at session (left) and grouped data (middle). dLight recordings of non laser-paired trials are also shown (right) at session 1. **D.** dLight signals at session 1 during laser stimulation. The signals during non-laser trials are shown also. **E.** The signals of a representative mice around laser-paired cue (left), grouped data (middle) and signals around non-laser paired cue presentation (right) at session 10. **F.** Area under the curve (AUC) of dLight signal in the NAc around the cue presentations (0-1.5 seconds) across sessions. Laser-paired cue triggered bigger transient than non-laser paired cue. 2-way repeated measures ANOVA. Session, $F_{9, 27} = 3.339$, $P = 0.0072$. Cue, $F_{1, 3} = 3.997$, $P = 0.139$. Interaction, $F_{9, 27} = 5.287$, $P = 0.0003$. Tukey's multiple comparison, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$. $n = 4$ mice.

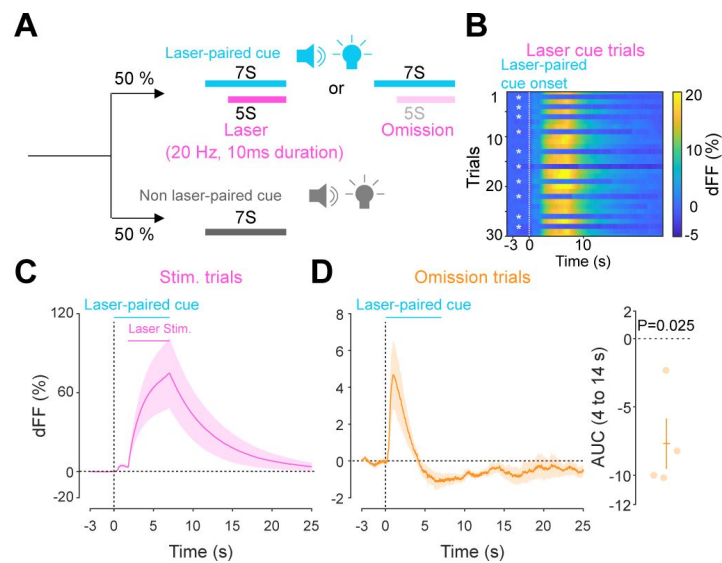


Figure 2.

A. Schematic for the omission sessions. Two thirds of laser associated cue was followed by the laser stimulation while the other one third of the laser associated cue failed to trigger the laser stimulation. **B.** dLight recordings of a representative mouse during omission sessions. dLight signal around the laser-paired cue presentation is shown here. White asterisks indicate omission trials, while in the other trials, the laser stimulation was delivered. **D.** dLight recordings in the NAc during stimulation trials and during omission trials(**C**). A dip of dLight signals was observed. One sample t test; $t=4.176$, $df=3$. $P=0.0250$. $n=4$ mice.

dopamine transients around the laser-paired cue delivery (**Fig 3 E,F and G**), consistent with RPE-like dopamine transients. Omission sessions after 10 sessions of the task (**Fig 3H**) showed a dip of dopamine signal during omission trials (**Fig 3H**). These results are indicative of the presence of a certain amount of tonic dopamine in the LH under unstimulated conditions and that negative RPEs can induce a decrease in the concentration of LH dopamine. Interestingly, the dopamine transients in the LH observed in these experiments mirrored the RPE-encoding dopamine responses we observed in the NAc.

Different kinetics of dopamine in the NAc and LH

After conducting dLight recordings in the NAc and LH during the opto-pavlovian task, we observed distinct kinetics of dopamine in these two brain regions. First we compared the dopamine transient during stimulation trials of omission sessions, where mice already learned the association between the cue and the laser stimulation (**Figure 3 - Supplement 1A**). In the NAc, the dLight signal continued to increase until the laser was turned off, while in the LH, the dLight signal plateaued shortly after the initiation of the laser stimulation (**Figure 3 - Supplement 1A**). To precisely assess the kinetics of the dLight signals, we calculated their derivatives (**Figure 3 - Supplement 1B**). In the NAc, the derivative crossed zero shortly after the termination of the laser stimulation, while in the LH, the zero-crossing point was observed during the laser stimulation (**Figure 3 - Supplement 1B and C**), indicating a different timing of direction change in the dLight signal. We applied the same analysis to the omission trials (**Figure 3 - Supplement 1D, E, and F**). Following the initiation of the laser-paired cue, two zero-crossing points of the derivative of the dLight signal were identified. The first one corresponded to the maximum of the dLight signal, and the second one corresponded to the minimum of the dLight signal. In the LH, both zero-crossing points were smaller than in the NAc, suggesting that LH dopamine exhibits faster kinetics.

Orexin neuron dynamics during the opto-Pavlovian task

We next addressed the hypothesis positing that dopamine in the LH can modulate orexinergic neuronal activity. We injected DAT-cre mice with an orexin promoter-driven GCaMP6s^{56,59}, which has been reported to target orexin neurons with >96% specificity⁵⁹, in the LH and used fiber photometry to monitor the calcium transients of LH orexinergic neurons while optically controlling dopamine release via ChrimsonR expressed in the VTA (**Fig 4A and B**). After the mice fully recovered from the surgery, they underwent the opto-Pavlovian task. On session 1, calcium transients in orexin neurons were not modulated by the presentation of laser-paired or non-laser-paired cues (**Fig 4C**), although laser stimulation triggered the increase of calcium signal (**Figure 4 - Supplement 1**). As we observed with dLight recordings in the NAc and LH, the orexin-specific GCaMP signal increased across sessions around the presentation of the laser-paired cue (**Fig 4D and E**), therefore following a similar time course to the evolution of dopamine release in the LH. After mice learned the association, we tested the omission of laser stimulation (**Fig 4F**). Unlike dopamine signals, we did not observe a dip in orexin activity during omission trials (**Fig 4F**). Orexin neuron activity is known to be associated with animal locomotion^{60,61}. To exclude the possibility that the increase in calcium signaling during laser-paired cue trials is an indirect effect of stimulation-induced locomotion^{60,61}, we performed photometry recordings and optogenetic stimulation of VTA dopaminergic terminals in the LH both in freely-moving or in isoflurane-anesthetized conditions (**Fig 5A**). In both conditions we observed an increased orexinergic neuron activity after the onset of laser stimulation (**Fig 5B and C**), suggesting that the observed upregulation in orexinergic neuronal activity is independent from animal locomotion. Finally, to identify which dopamine receptor is responsible for this increase in orexinergic calcium, we systemically (I.P.) injected a D1 (SCH 23390) or D2 (raclopride) receptor antagonist, and optically stimulated dopaminergic terminals in the LH (**Fig 5E** and **Figure 5 - Supplement 1**). Raclopride largely reduced the observed orexin neuronal activity increases while SCH 23390 did not, indicating that the signal is at least in part mediated by the D2

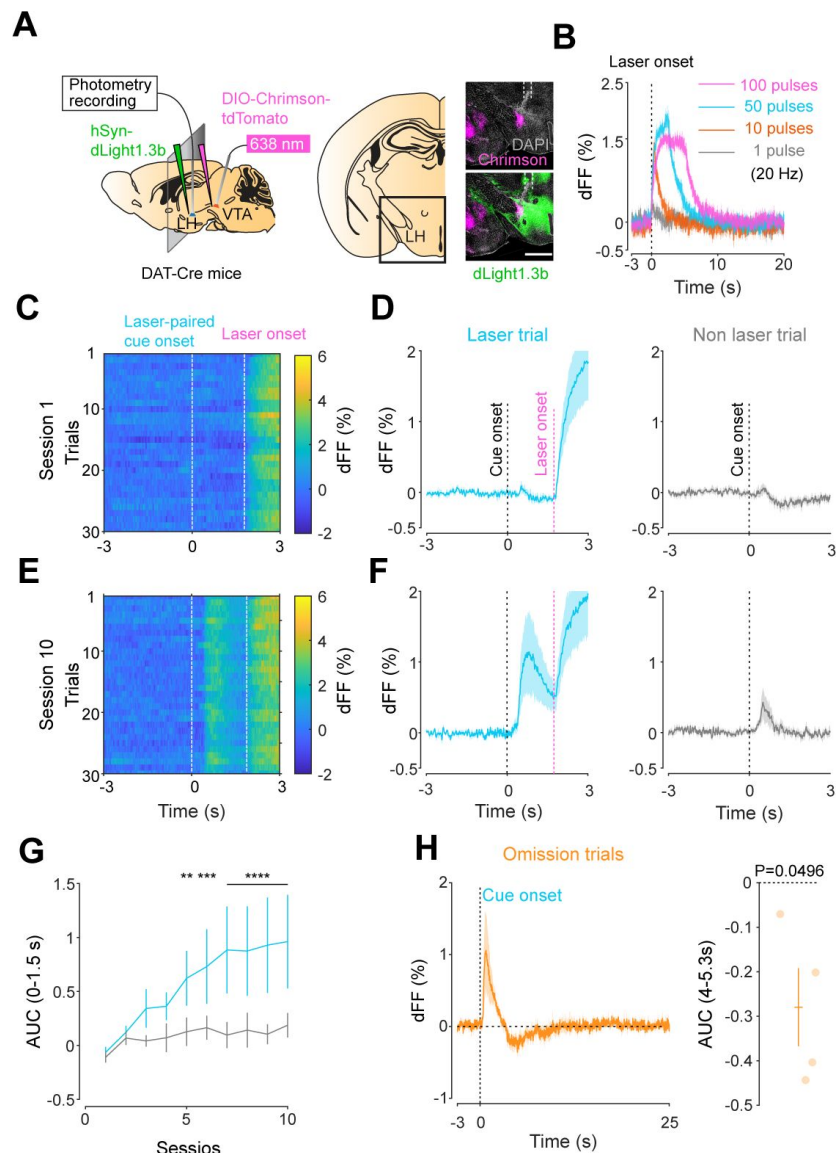


Figure 3.

A. Schematic for the dLight recording in the LH while stimulating dopamine neurons in the VTA (left). Coronal image of the LH of a mouse infected with AAV-hSyn-DIO-Chrimson-tdTomato in the VTA and AAV-hSyn-dLight1.3b in the LH (right). White dashed lines indicate fiber tracts. Scale bar; 1mm. **B.** dLight signal in the LH during dopaminergic stimulation in the VTA at several number of pulses (20Hz, 10ms duration for each pulse). **C.** dLight recordings during the laser-paired cue presentation of a representative mouse at session 1. **D.** dLight recordings around the laser-paired cue presentation (left) and non-laser-paired cue presentation (right) at session 1. **E.** dLight recordings during the laser-paired cue presentation of a representative mouse at session 10. **F.** dLight recordings around the laser-paired cue presentation (left) and non-laser-paired cue presentation (right) at session 10. **G.** Area under the curve (AUC) of dLight signal in the LH around the cue presentations (0-1.5 seconds) across sessions. Laser-paired cue triggered bigger transient than non-laser paired cue. 2-way repeated measures ANOVA. Session, $F_{9, 27} = 3.814$, $P = 0.0033$. Cue, $F_{1, 3} = 5.818$, $P = 0.0948$. Interaction, $F_{9, 27} = 3.923$, $P = 0.0027$. Tukey's multiple comparison, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$. **H.** dLight recordings in the LH during omission trials. A dip of dLight signals was observed. One sample t test; $t = 3.193$, $df = 3$, $P = 0.0496$. $n = 4$ mice.

receptor (Fig 5F). Our experiments suggest that LH orexin neurons participate in the LH response to VTA dopamine, and that D2 receptors play an important role locally in the LH in regulating orexin neuron activity evoked by dopamine release.

Discussion

The mesolimbic dopamine system has been proposed to encode reward prediction errors (RPEs)^{12,22,23}, which signal a discrepancy between expected and experienced rewards. Recently, it has been demonstrated that the optical stimulation of midbrain dopamine neurons is sufficient to create Pavlovian conditioning¹⁷. While it is known that cells within the LH express several different dopamine receptor subtypes³⁷, and microinjection of D1 and D2 receptor agonists have been shown to decrease food intake in rodents⁶², before our study, dopamine transients in the LH during reward associated tasks had not been reported. Here, we used an opto-Pavlovian task that echoed, with NAc dopamine measurements, already reported findings on the midbrain dopamine neurons' RPE-encoding role¹⁷. Then, we determined that VTA dopaminergic neurons release dopamine in the LH and found that dopamine transients in the LH in response to the same opto-Pavlovian task were qualitatively similar to those observed in the mesolimbic dopamine system.

Recent findings suggest that dopaminergic transients in the dorsal bed nucleus of the stria terminalis (dBNST) encode RPE²⁶, indicating qualitative similarities in dopamine activity within this brain region compared to what we observed in the LH and NAc. Conversely, dopamine responses in other brain regions, such as the medial prefrontal cortex (mPFC)^{25,63} and amygdala^{50,64}, predominantly react to aversive stimuli. The dopamine transients in the LH during aversive stimuli, with their robust orexinergic responses to aversiveness⁶⁵, warrant careful characterization.

Indeed, during the opto-Pavlovian task, in which we stimulated VTA dopamine neurons and measured dopamine, we observed dopamine transients around a Pavlovian laser-paired cue presentation. We also observed a dip of dLight signal during omission trials, suggesting that a detectable concentration of dopamine is at extra-synaptic space in the LH at basal condition and that at the moment of omission, the concentration of extra-synaptic dopamine decreases. These data indicate that dopamine transients in the LH, as in the NAc, could be encoding reward prediction error.

While smaller than the response to the laser-paired cue, we observed modulation of the dLight signal in the NAc during the presentation of the non-laser paired cue. In Session 1, the cue presentation immediately triggered a dip, whereas in Session 10, it evoked a slight increase in the signal, followed by a dip. Our hypothesis suggests that two components contribute to the dip in the signal. The first is the aversiveness of the cue; the relatively loud sound (90dB) used for the cue could be mildly aversive to the experimental animals. Previous studies have shown that aversive stimuli induce a dip in dopamine levels in the NAc, although this effect varies across subregions^{3,63}. The second component is related to reward prediction error. While the non-laser paired cue never elicited the laser stimulation, it shares similarities with the laser-paired cue in terms of a loud tone and the same color of the visual cue (albeit spatially different). We posit that it is possible that the reward-related neuronal circuit was slightly activated by the non-laser paired cue. Indeed, a small increase in the signal was observed on day 10 but not on day 1. If our hypothesis holds true, as this signal is induced by two components, further analysis unfortunately becomes challenging.

While dopaminergic transients in the NAc and LH share qualitative similarities, the kinetics of dopamine differs between these two brain regions. Under optical stimulation, the dLight signal in the NAc exhibited a continuous increase, never reaching a plateau until the laser was turned off.

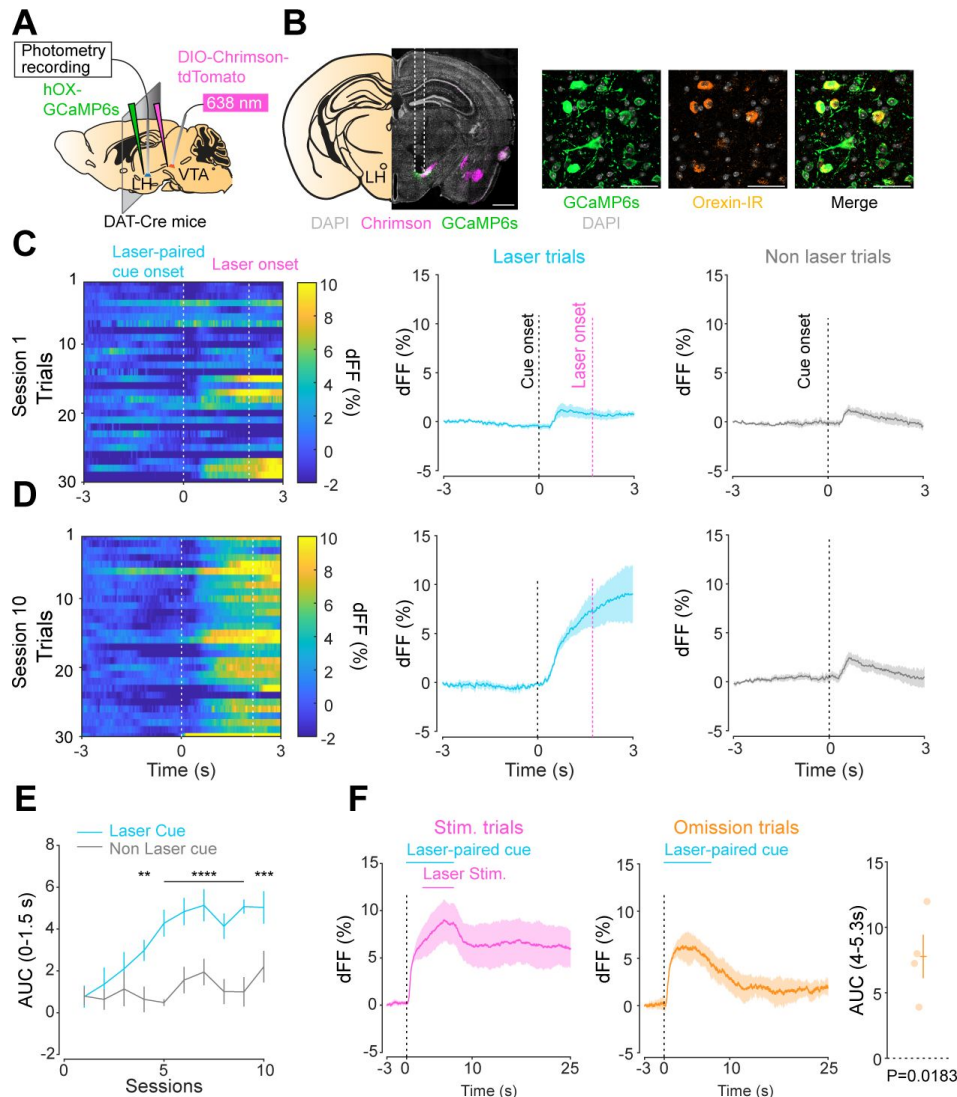


Figure 4.

A. Schematic of the preparation for opto-Pavlovian task combined with orexin promoter GCaMP recordings in the LH. **B.** Coronal image of a mouse brain slice infected with AAV-hSyn-DIO-ChrimsonR-tdTomato in the VTA and AAV1-hOX-GCaMP6s in the LH (left. Scale Bar; 1mm). White dashed lines indicate fiber tracts. Zoom of infected LH with AAV1-hOX-GCaMP6s and co-localization orexin IR and GCaMP6s (right. Scale Bars; 50 μ m). **C.** Orexin promoter GCaMP recordings in the LH of a representative mouse around the laser-paired cue presentation at session 1 (left), grouped data (middle) and recordings during non laser-paired trial (right). **D.** Orexin promoter GCaMP recordings in the LH of a representative mouse around the laser-paired cue presentation at session 10 (left), grouped data (middle) and recordings during non laser trial (right). **E.** Area under the curve (AUC) of hOX-GCaMP signal in the LH around the cue presentations (0-1.5 seconds) across sessions. Laser-paired cue triggered bigger transient than non-laser paired cue. 2-way repeated measures ANOVA. Session, $F_{9,27} = 4.438$, $P = 0.0012$. Cue, $F_{1,3} = 25.41$, $P = 0.0151$. Interaction, $F_{9,27} = 4.125$, $P = 0.0020$. Tukey's multiple comparison, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$. **F.** Orexin promoter GCaMP recordings during stimulation trials (left) and omission trials (middle and right). AUC around the omission was higher than baseline. One sample t test; $t = 4.693$, $df = 3$, $P = 0.0183$. $n = 4$ mice.

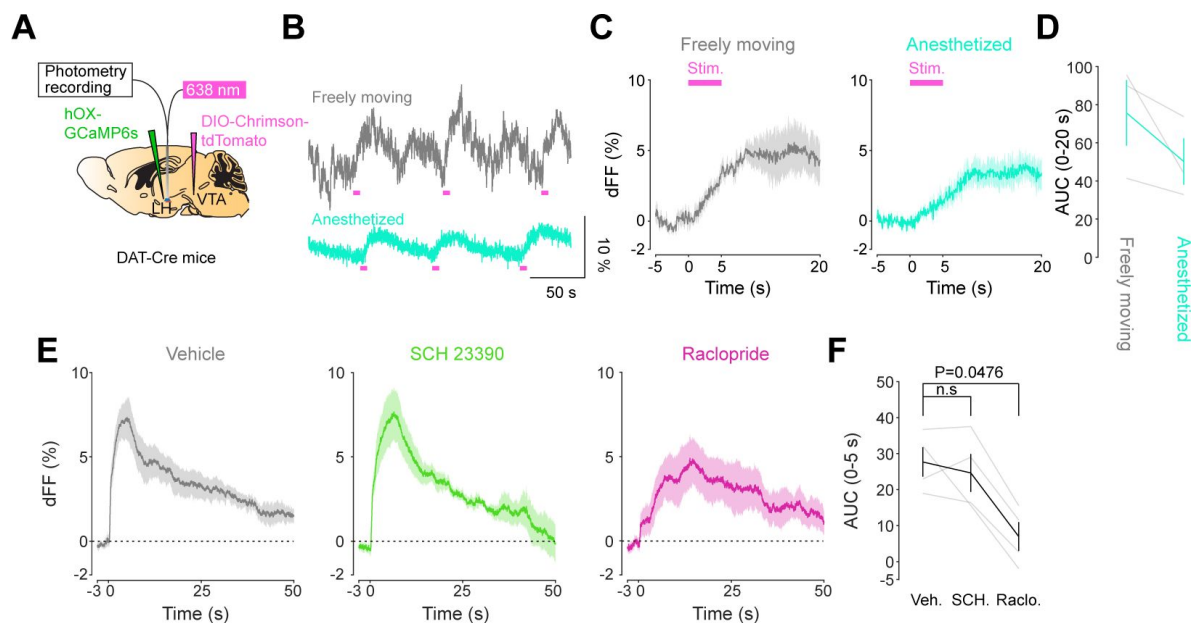


Figure 5.

A. Schematic for the orexin promoter GCaMP recording in the LH while stimulating dopamine terminals in the LH. **B.** Orexin promoter GCaMP signals of a representative mouse. Recordings were performed while mice were freely moving (top) and anesthetized with isoflurane (bottom). Red bars indicate the stimulation. (20Hz, 100 pulses, 10 ms duration). **C.** Orexin promoter GCaMP signals around the stimulation of dopamine terminals in the LH while animals were freely moving (left) and anesthetized (right). **D.** AUC at 0 to 20 seconds was not significantly different between freely moving and anesthetized conditions. Paired t test, $t=1.923$ $df=2$, $P=0.1944$, $n=3$ mice. **E.** In freely moving condition, recordings were performed after mice received the intraperitoneal injection of vehicle (left), SCH 23390 (1mg/kg, middle), and raclopride (1mg/kg, right). **F.** Area under the curve (AUC) at 0-5 seconds. Black line indicates the mean for each condition and grey lines show individual mice. The administration of raclopride decreased the AUC significantly while SCH 23390 did not change the AUC. One-way analysis of variance; $F(3, 6) = 5.305$, $P=0.04$. Tukey's multiple comparison test. vehicle vs. SCH 23390; $P=0.8145$. vehicle vs. raclopride; $P=0.0476$. $n=4$ mice.

In contrast, in the LH, the dLight signal reached a plateau shortly after the initiation of the laser stimulation. The distinction in dopamine kinetics was also evident during omission trials, where the dopamine kinetics in the LH were faster than those in the NAc. The molecular mechanisms underlying this difference in kinetics and its impact on behavior remain to be elucidated. Due to this kinetic difference, we employed distinct time windows to capture the dip in the dLight signal during omission trials.

Previous work indicates that orexin neurons project to VTA dopamine neurons^{40,42,43}, facilitating dopamine release in the NAc and promoting reward-seeking behavior. However, while it has been demonstrated that systemic injection of dopamine receptor agonists activates orexin neurons⁴¹, their reciprocal connection with dopaminergic neurons had not yet been investigated *in vivo*⁴⁷. Here, we studied the relationship between orexinergic and dopaminergic activity in the LH and found that LH dopamine transients and orexinergic neuronal activities are positively correlated. Seeing as dopamine-related orexinergic activity was reduced by systemic injections of raclopride, we postulate that dopamine in the LH activates orexin neurons via D2R. D2R couples to Gi proteins⁶⁶, so it is unlikely that dopamine directly activates orexin neurons. Our testable hypothesis is that dopamine modulates orexin neuron activation via a disinhibitory mechanism; for example, GABA interneurons could be inhibited by the activation of D2R, consequently disinhibiting orexin neurons^{67,68}. It has been established that D1 receptor expressing medium spiny neurons (D1-MSNs) in the NAc densely project to the LH, especially to GABAergic neurons^{33,69}, raising a possibility that dopamine in the LH modulates the presynaptic terminals of D1-MSNs. However, administration of D1R antagonist (SCH 23390) did not block the calcium transient in orexin neurons evoked by the dopaminergic terminal stimulation in the LH, implying that the contribution of D1-MSNs to orexin neuronal activity is minimal in our experimental design. While systemic injections of raclopride effectively reduced dopaminergic terminal stimulation-evoked orexinergic activity, the long-lasting calcium signal remained unaltered (**Fig. 5E**). This discrepancy could arise from an insufficient blockade of dopamine receptors. For D1R blockade, we administered 1 mg/kg of SCH-23390 5 minutes before recordings. This dose is adequate to induce behavioral phenotypes⁷⁰ and block D1R-based dopamine sensors¹³, although higher doses have been used in some studies⁵⁰. To block D2R, we injected 1 mg/kg of raclopride, a dose known to induce hypo-locomotion⁷¹, indicating effective modification of the neuronal circuit. However, these data do not guarantee complete receptor blockade, and it is possible that optical stimulation resulted in high extra-synaptic dopamine concentration, leading to partial receptor binding. Alternatively, this component might be mediated by other neurotransmitters, such as glutamate^{72–74} or GABA⁷⁵, which are known to be co-released from dopaminergic terminals.

Several *ex vivo* experiments suggest that dopamine, particularly at high concentrations (50 μ M or higher), reduces the firing rate of orexin neurons, albeit with a potency significantly lower than that of norepinephrine^{44,45} through both direct and indirect mechanisms^{46,47}. This apparent discrepancy with our results could be attributed to a different time course of dopamine transients. In slice experiments, the concentration of dopamine is determined by the experimenter and often maintained at high levels for minutes. In contrast, in our experimental setup, dopamine evoked by laser stimulation is reuptaken as soon as the laser is turned off. This variation in the time course of dopamine transients could contribute to the observed differences in responses to dopamine. Another plausible explanation for this discrepancy is the difference in dopamine concentration. Modulations of synaptic transmission to orexinergic neurons by dopamine are reported to be concentration-dependent⁴⁶. The relationship between the response of genetically encoded dopamine sensors and extracellular dopamine concentrations is non-linear¹³, which makes estimating dopamine concentrations *in vivo* based on the sensor's brightness not technically possible. Therefore, it is challenging to determine the exact dopamine concentration achieved by laser stimulation, and it is possible that this concentration differs from the one that triggers the reduction in the firing rate of orexin neurons.

Although presentation of laser-paired cue and laser stimulation of VTA dopamine neurons evoked dopamine transient in the LH and an increase of calcium signals of orexin neurons, we did not observe a dip of calcium signal of orexin neurons during omission trials. This lack of a dip could be due to 1) slow sensor kinetics⁷⁶ – since the pre-omission cue triggers LH dopamine release, and increases the calcium transient in orexin neurons, if the kinetics of GCaMP6s expressed in orexin neurons were too slow, we would not be able to observe an omission-related orexin activity dip – 2) dopamine signaling properties. Dopamine receptors couple to G proteins⁷⁷, which act relatively slowly, potentially preventing us from seeing an omission-related signaling dip. Both theories are compatible with our observation that orexinergic activity increases over time during the presentation of our laser-paired cue, as our observed increases are not sporadic but developed over time. Recent studies indicate that orexin neurons respond to cues associated with reward delivery. However, unlike dopaminergic responses, which linearly correlate with the probability of reward delivery, the orexin response plateaus at around 50% probability of reward delivery⁵⁶. This observation indicates that orexin neurons encode multiplexed cognitive information rather than merely signaling reward prediction error. Our data indicate a direct conveyance of dopaminergic information, specifically reward prediction error, to orexinergic neurons. However, the mechanism by which orexinergic neurons process and convey this information to downstream pathways remains an open question.

The silencing of orexinergic neurons induces conditioned place preference⁷⁸, suggesting that the silencing of orexin neurons is positively reinforcing. Considering that the stimulation of VTA dopamine neurons^{15,16} and dopaminergic terminals in the LH⁷⁹ is generally considered to be positively reinforcing, the activation of orexin neurons by dopaminergic activity might be competing with dopamine's own positive reinforcing effect. At the moment of omission, we observed a dopamine dip both in the NAc and LH, while orexin neurons were still activated. These data suggest that there is a dissociation between dopamine concentration and orexin neuronal activity at the moment of omission. This raises the intriguing possibility that this dissociation - the activation of orexin neurons during a quiet state of dopamine neurons – could be highly aversive to the mice, therefore could be playing a role in negative reinforcement^{20,22,39}.

It has been demonstrated that the orexin system plays a critical role in motivated learning⁸⁰. Blocking orexin receptors impairs Pavlovian conditioning⁸¹, operant behavior⁸², and synaptic plasticity induced by cocaine administration⁴⁰. Additionally, dopamine in the LH is essential for model-based learning, and the stimulation of dopaminergic terminals in the LH is sufficient to trigger reinforcement learning⁷⁹. These collective findings strongly suggest that the activation of orexin neurons, evoked by dopamine transients, is crucial for reinforcement learning. Our data indicate that dopamine in both the NAc and LH encodes reward prediction error (RPE). One open question is the existence of such a redundant mechanism. We hypothesize that dopamine in the LH boosts dopamine release via a positive feedback loop between the orexin and dopamine systems. It has already been established that some orexin neurons project to dopaminergic neurons in the VTA, positively modulating firing⁴². On the other hand, our data indicate that dopamine in the LH stimulates orexinergic neurons. These collective findings suggest that when either the orexin or dopamine system is activated, the other system is also activated consequently, followed by further activation of those systems. Although the current findings align with this idea, the hypothesis should be carefully challenged and scrutinized.

In summary, by implementing an opto-Pavlovian task combined with fiber photometry recordings, we found evidence that the meso-hypothalamic dopamine system exhibits features qualitatively similar to those observed in the mesolimbic dopamine system – where dopamine is thought to encode RPEs. Furthermore, our findings show that dopamine in the LH positively modulates the neuronal activities of orexin neurons via D2 receptors. These findings give us new insights into the reciprocal connections between the orexin and dopamine systems and shed light on the previously overlooked direction of dopamine to orexin signaling, which might be key for understanding negative reinforcement and its dysregulation.

Methods

Animals

All animal procedures were performed in accordance to the Animal Welfare Ordinance (TSchV 455.1) of the Swiss Federal Food Safety and Veterinary Office and were approved by the Zurich Cantonal Veterinary Office. Adult DAT-IRES-cre (B6.SJL-Slc6a3tm1.1(cre)Bkmn/J; Jackson Labs) mice of both sexes were used in this study. Mice were kept in a temperature- and humidity-controlled environment with ad libitum access to chow and water on 12-h/12-h light/dark cycle.

Animal surgeries and viral injections

Surgeries were conducted on adult anesthetized mice (males and females, age > 6 weeks). AAV5-hSyn-FLEX-ChrimsonR-tdTomato (UNC Vector Core, 7.8×10^{12} vg/ml) was injected in the VTA (−3.3 mm AP, 0.9 mm ML, −4.28 mm DV, with 10 degrees angle, volume: 600 nL). Above the injection site, a single optic fiber cannula (diameter: 200 μ m) was chronically implanted (−3.3 mm AP, 0.9 mm ML, −4.18 mm DV). In the NAc (1.5 mm AP, 0.7 mm ML, −4.5 mm DV), AAV9-hSyn1-dLight1.3b-WPRE-bGHp (Viral Vector Facility, 7.9×10^{12} vg/ml) was injected and an optic fiber (diameter: 400 μ m) was implanted (1.5 mm AP, 0.7 mm ML, −4.4 mm DV) for photometry recordings. In some mice, dLight virus or AAV1.pORX.GCaMP6s.hGH⁵⁶ was injected in the LH (−1.4 mm AP, 1.1 mm ML, −5.0 mm DV), followed by an optic fiber implantation (−1.4 mm AP, 1.1 mm ML, −4.8 mm DV).

Opto-Pavlovian task

Dat-cre mice infected with AAV5-hSyn-FLEX-ChrimsonR-tdTomato in the VTA were placed in an operant chamber inside a sound-attenuating box with low illumination (30 Lux). Chamber functions synchronized with laser light deliveries were controlled by custom-written Matlab scripts via a National Instrument board (NI USB 6001). The optic fiber implanted above the VTA was connected to a red laser (638 nm, Doric Lenses; CLDM_638/120) via an FC/PC fiber cable (M72L02; Thorlabs) and a simple rotary joint (RJ1; Thorlabs). Power at the exit of the patch cord was set to 15 ± 1 mW. Two visual cues were in the operant chamber and a speaker was placed inside the sound-attenuating box. The laser-predictive cue was composed of the illumination of one visual stimulus (7 seconds continuous) and a tone (5kHz, 7 seconds continuous, 90dB), while the non-laser-paired cue was composed of a second visual stimulus (7 seconds continuous) and a different tone (12kHz, 7 seconds continuous, 90dB). Each cue was presented for 7 seconds. Two seconds after the onset of the laser-predictive cue, the red laser was applied for 5 seconds (20Hz, 10ms pulse duration). The presentation of the non-laser cue was followed by no stimuli. In random interval 60 seconds (45–75 seconds), one cue was presented in a pseudorandom sequence (avoiding the presentation of the same trials more than three times in a row). Mice were exposed to 30 laser cues and 30 Non-laser-paired cues in each session. Mice were trained 5 days per week. After 10 sessions of opto-Pavlovian training, mice underwent 2 sessions of omission. In the omission sessions, two thirds of laser-paired cue presentation was followed by the delivery of the laser stimulation (laser trial), and one third of laser-paired cue presentation didn't lead to laser stimulation (omission trial). The laser-paired cue was kept the same for laser and non-laser trials. Each omission session was composed of 20 laser trials, 10 omission trials, and 30 non-laser trials.

Photometry recordings

Fiber photometry recordings were performed in all the sessions. Dat-cre mice injected with AAV9-hSyn1-dLight1.3b-WPRE-bGHp in the NAc or LH, or AAV1.pORX.GCaMP6s.hGH in the LH were used. All the mice were infected with AAV5-hSyn-FLEX-ChrimsonR-tdTomato in the VTA. iFMC6_IE(400–410)_E1(460–490)_F1(500–540)_E2(555–570)_F2(580–680)_S photometry system (Doric Lenses) was controlled by the Doric Neuroscience Studio software in all the photometry experiments except for Fig. 5's anesthesia experiment. In Fig 5's experiment, a 2-color +

optogenetic stimulation rig (Tucker-Davis Technologies, TDT) was used. Mice were exposed to 5% isoflurane for anaesthesia induction, and were kept anesthetized at 2 % isoflurane through the rest of the experiment. The recordings started 10min after the induction of anesthesia. A low-autofluorescence patch cord (400 μm , 0.57 N.A., Doric Lenses) was connected to the optic fiber implanted above the NAc or LH. The NAc or LH was illuminated with blue (465 nm, Doric) and violet (405 nm, Doric) filtered excitation LED lights, which were sinusoidally modulated at 208 Hz and 572 Hz (405nm and 465nm, respectively) via lock-in amplification, then demodulated on-line and low-passed filtered at 12 Hz in the Doric system. In the TDT system, signals were sinusoidally modulated, using the TDT Synapse® software and a RX8 Multi I/O Processor at 210 Hz and 330 Hz (405nm and 465nm, respectively) via a lock-in amplification detector, then demodulated on-line and low-passed filtered at 6 Hz. Analysis was performed offline in MATLAB. To calculate $\Delta F/F_0$, a linear fit was applied to the 405 nm control signal to align it to the 470 nm signal. This fitted 405 nm signal was used as F_0 in standard $\Delta F/F_0$ normalization $\{F(t) - F_0(t)\}/F_0(t)$. For antagonist experiments in **Figure 5**, SCH-23390 (1mg/kg in saline) or raclopride (1mg/kg in saline) was injected (I.P.) 5 minutes before recordings.

Immunohistochemistry

Perfused brains were fixed with 4% Paraformaldehyde (Sigma-Aldrich) overnight (room temperature) and stored in PBS at 4°C for a maximum of one month. Brains were sliced with a Vibratome (Leica VT1200S; feed=60 μm , freq=0.5, ampl=1.5), and brain slices near the fiber tracts were subsequently selected for staining. These slices were permeabilized with 0.3% Triton X-100 for 10 min (room temperature). Next, they were incubated with blocking buffer for 1 h (5% bovine serum albumin, BSA; 0.3% Triton x-100) before staining with the respective primary antibodies (NAc and LH with αGFP chicken 1:1000, Aves Labs ref GFP-1010; amCherry rabbit, 1:1000, abcam ab167453; and αOrexin goat, 1:500, Santa Cruz Biotech, C-19; VTA with amCherry rabbit, 1:1000, abcam, ab167453; and αTH chicken, 1:500, TYH0020) overnight. After three washes with 0.15% Triton, samples were incubated with the respective secondary antibodies and DAPI (for GFP donkey- $\alpha\text{chicken}$, 1:1000, AlexaFluor 488, 703-545-155; for mCherry donkey- αrabbit 1:67, Cy3, Jackson, 711-165-152; for orexin donkey- αgoat , 1:500, Cy5; for TH donkey- $\alpha\text{chicken}$, 1:67, AlexaFluor647, 703-605-155; for DAPI 1:2000, Thermofisher, 62248) for 1h. Finally, samples were washed three times with PBS and mounted on microscope slides with a mounting medium (VectaShield® HardSet™ with DAPI, H-1500-10). Image acquisition was performed with a ZEISS LSM 800 with Airyscan confocal microscope equipped with a Colibri 7 light source (Zeiss Apochromat).

Statistical Analysis

Statistical analysis was performed in Graphpad Prism9. For all tests, the threshold of statistical significance was placed at 0.05. For experiments involving one subject, one sample t-test was used. For experiments involving two independent subjects or the same subjects at two different time points, two tailed Student's unpaired or paired t-test was used, respectively. For experiments involving more than two groups, one-way or two-way ANOVA was performed and followed by Tukey's multiple comparison test. All data are shown as mean $\pm$ SEM.

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Author contributions

MH performed and analyzed all behavioral and optogenetic experiments as well as fiber photometry recordings. MW prepared and programmed the automated operant chamber. LSC performed immunohistochemistry. MH, TP, MW, LSC and DB contributed to writing.

Competing financial interests

The authors have nothing to disclose.

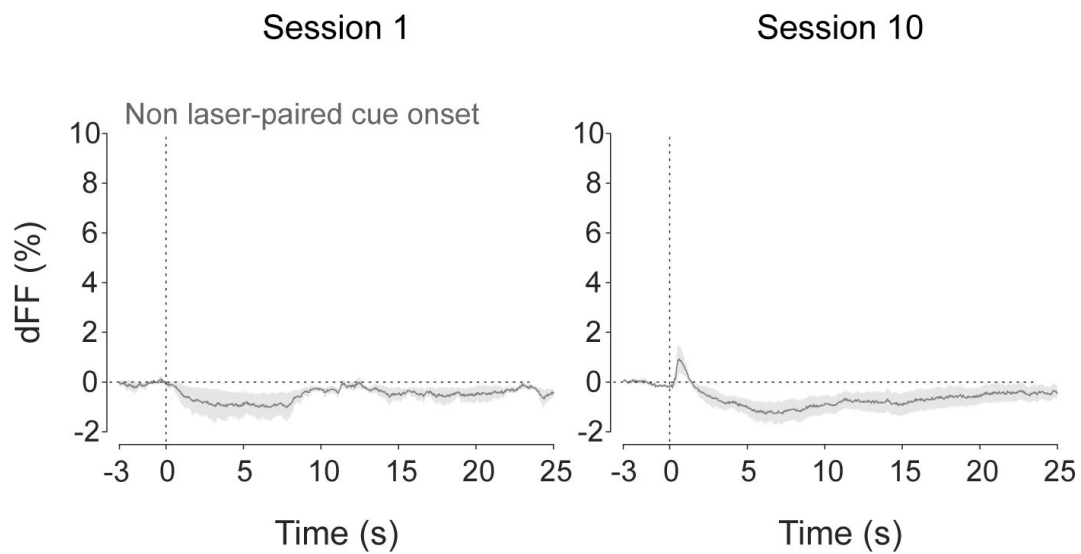


Figure 1 - Supplement 1.

dLight recordings in the NAc during non-laser-paired cue delivery at session 1 (left) and 10(right).

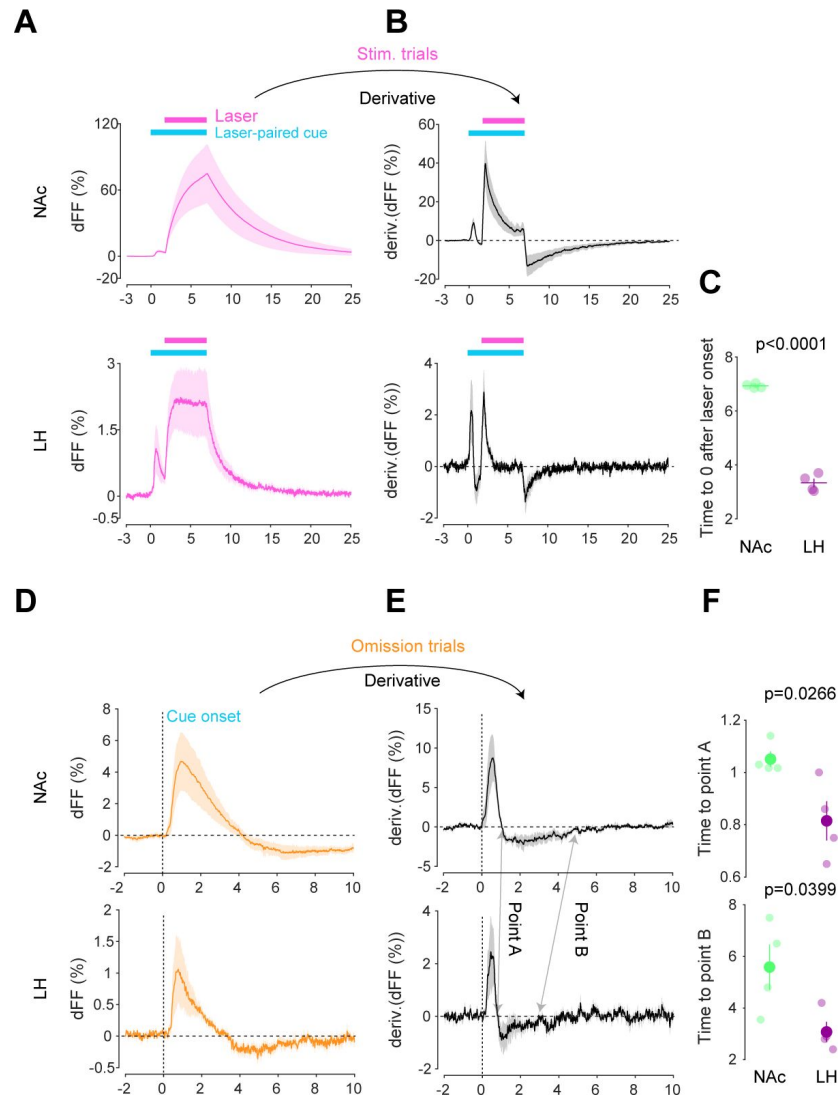


Figure 3 - Supplement 1.

A

dLight recordings in the NAc (top) and LH (bottom) during optical stimulation of VTA dopamine neurons. **B.** Derivative of panel A. **C.** quantification of zero-crossing point in panel B after the initiation of laser stimulation. Unpaired t-test; $t=21.69$, $df=6$. $p<0.0001$. **D.** dLight recordings in the NAc (top) and LH (bottom) during omission trials. **E.** Derivative of panel D. **F.** Quantification of first (top, point A) and second (bottom, point B) zero-crossing points after the initiation of the cue in panel E. Top, unpaired t-test. $T=2.920$, $df=6$. $p=0.0266$. bottom, unpaired t-test. $T=2.614$, $df=6$. $p=0.0399$. Note that panels A and D are shown in the main figures also. They are displayed for comparison purposes.

Opto-Pavlovian day 1

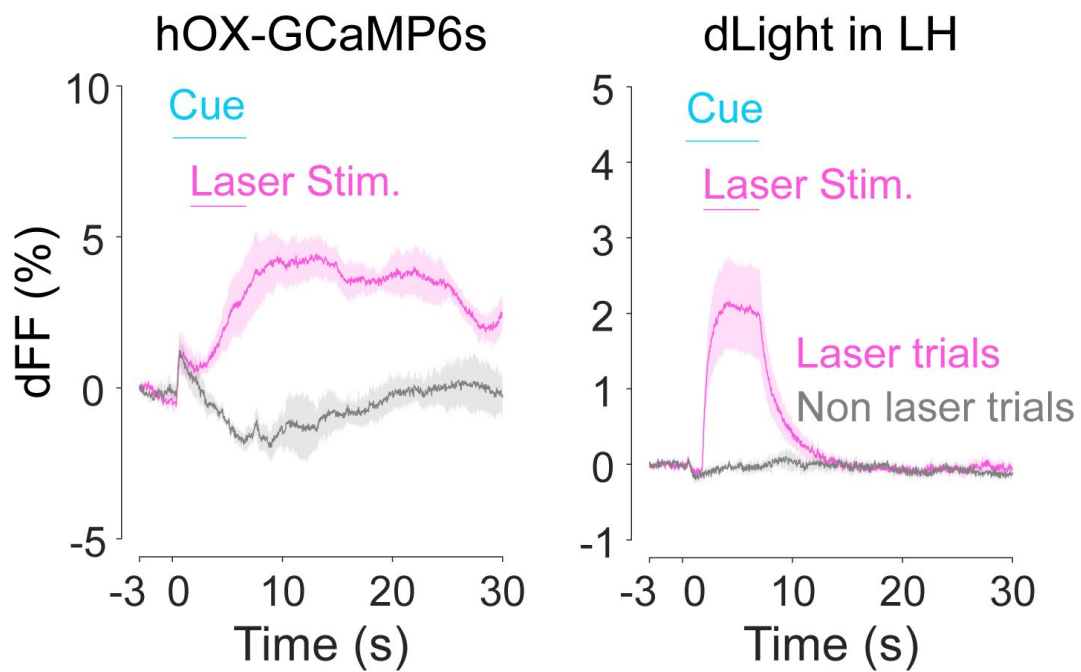


Figure 4 – Supplement 1.

Orexin-promoter GCaMP recording (left) and dLight recording during stimulation at session 1.

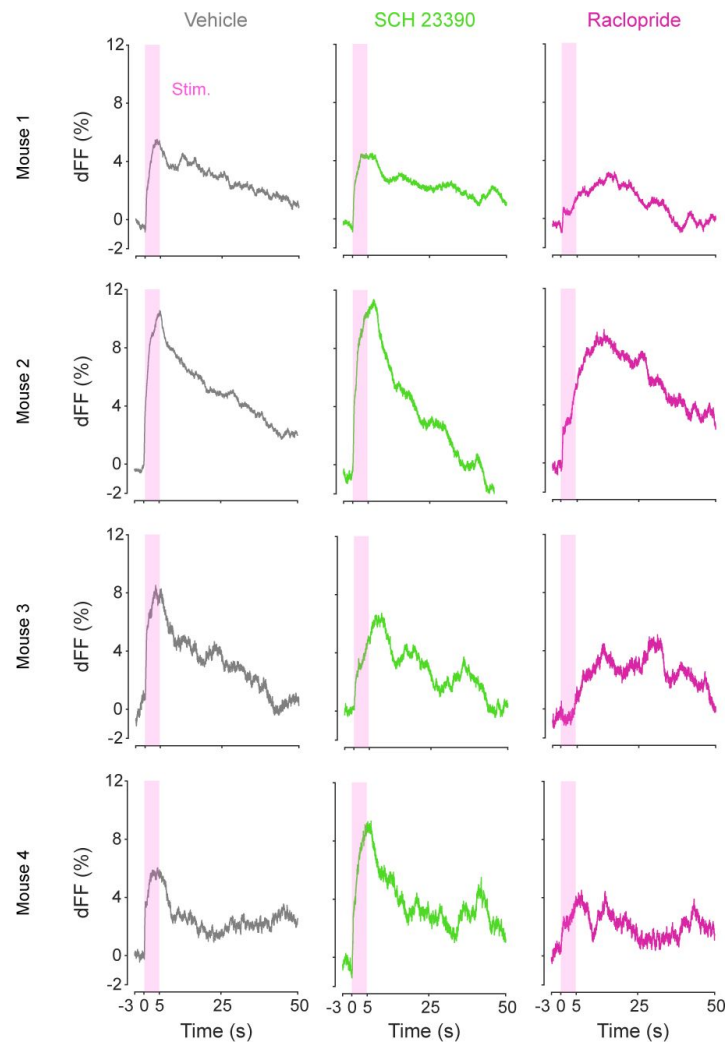


Figure 5 – Supplement 1.

Individual traces for **Figure 5E**.

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Reviewer #1 (Public Review):

Summary:

Mice can learn to associate sensory cues (sound and light) with a reward or activation of dopamine neurons in the ventral tegmental area (VTA), and then anticipate the reward from the sensory cue only. Using this paradigm, Harada et al. showed that after learning, the cue is able to induce dopamine release in the projection targets of the VTA, namely the nucleus accumbens and lateral hypothalamus (LH). Within the LH, dopamine release from VTA neurons (either by presentation of the cue or direct optical stimulation of VTA neurons) activates orexin neurons, measured as an increase in intracellular calcium levels.

Strengths:

This study utilized genetically encoded optical tools to selectively stimulate dopamine neurons and to monitor dopamine release in target brain areas and calcium response of orexin neurons. This allowed a direct assessment of the relationship between the behavioral response of the animals, release of a key neurotransmitter in select brain areas and its effect on target cells with the precision previously not possible. The results shed light onto the mechanism underlying reward-related learning and expectation.

Weaknesses:

Supplementary Fig.2: While the differences in time course are analyzed and extensively discussed, there is also a large discrepancy in the magnitude of change in DA levels in the two areas that is not mentioned. Specifically, DA increases is about 90-fold of baseline in NAc while it is about 2-fold in the LH. This could be because the DA level is either higher during baseline or lower during peak in the LH. Is there a known difference in the DA fiber density or extracellular DA levels in the two areas?

The DA antagonist i.p. study (Fig.5E and suppl fig 4) appears to be repeated measurements in same animals. If so, is it possible that repeated opto-sessions result in desensitization of the response, and therefore the smaller response is not due to the antagonist? Ideally, the order of experiments (i.e. vehicle, SCH23390 and raclopride) would be randomized, and a control group should be shown where DA terminal-stimulation induces consistent response in orexin neurons when applied three times without any antagonists. The result should be assessed using one-way repeated measures ANOVA.

Importantly, only 5 minutes were allowed for i.p. injected drugs to be absorbed and distributed to the brain before DA release was evoked and ORX neuron activity were monitored. Unfortunately, this is too short (In Ref 13, ip injection of SCH 23390 was 30 minutes prior to optogenetics/photometry experiments. In Ref 70, no effect on behavior was detected at 10 min post-i.p. injection of SCH 23390; In Ref 71, the effect of raclopride on behavior was measured 30 min post-ip injection).

Overall, it seems premature to make a conclusion about a role for D2 receptors or lack of involvement of D1 receptors in the observed phenomenon.

Reciprocal activation of VTA DA neurons and LH orexin neurons is an interesting idea. However, if this is the case, the activity of these two types of cells should show similar pattern and time course. This manuscript shows that extracellular DA levels decays quickly following the cessation of optical stimulation (Fig. 3B) whereas orexin neuron activity is long-lasting (Fig. 5). Thus, the hypothesis does not seem to be fully supported by experimental data.

<https://doi.org/10.7554/eLife.90158.2.sa1>

Reviewer #3 (Public Review):

Summary:

Harada and colleagues describe an interesting set of experiments characterizing the relationship between dopamine cell activity in ventral tegmental area (VTA) and orexin neuron activity in lateral hypothalamus (LH). All experiments are conducted in the context of an opto-Pavlovian learning task, in which a cue predicts optogenetic stimulation of VTA dopamine neurons. With training, cues that predict DA stimulation come to elicit dopamine release in LH (a similar effect is seen in accumbens). After training, omission trials (cue followed by no laser) result in a dip (inhibition) of dopamine release in LH, characteristic of reward prediction error observed in striatum. Across cue training, the activity pattern of orexin neurons in LH mirrors that of LH DA levels. However, unlike the DA signal, orexin neurons do not exhibit a decrease in activity in omission trials. Systemic blockade of D2 but not D1 receptors blocked DA release in LH following VTA DA cell stimulation.

Strengths:

Although much work has been dedicated to examining projections from orexin cells to VTA, less has been done to characterize reciprocal projections and their function. In this way, this paper is a very important addition to the literature. The experiments are technically sound (with some limitations, below) and utilize sophisticated approaches, the manuscript is nicely written, and the conclusions are mostly reasonable based on the data collected.

Weaknesses:

I believe the impact of the paper could be enhanced by considering and/or addressing the following:

Major

- I encourage the authors to discuss in the Introduction previous work on DA regulation of orexin neurons. In particular, the authors cite, but do not describe in any detail, the very relevant Linehan paper (2019; *Am J Physiol Regul*) which shows that DA differentially alters excitatory/inhibitory input onto orexin neurons and that these actions are reversed by D1 vs D2 receptor antagonists. Another paper (Bubser, 2005, *EJN*) showed that dopamine agonists increase activity of orexin neurons and that these effects are blocked by D1/D2 antagonists. The current findings should be discussed in the context of these (and any other relevant) papers in the Discussion, too.

The revised manuscript addresses these concerns.

- In the Discussion, the authors provide 2 (plausible) explanations for why they did not observe a dip in calcium signal of orexin neurons during omission trials. Is it not possible that these cells do not encode for this type of RPE?

The revised manuscript addresses these concerns.

- Related to the above - I am curious about the authors' thoughts on why there is such redundancy in the system. i.e. why is dopamine doing the same thing in NAC and LH in the context of cue-reward learning?

The revised manuscript addresses these concerns.

- The data, as they stand, are largely correlative and do not indicate that DA recruitment of orexin neurons is necessary for learning to occur. It would be compelling if blocking the orexin cell recruitment affected some behavioral outcome of learning. Similarly - does raclopride treatment across training prevent learning?

I maintain that experiments testing the causality of these effects on learning/behavior would enhance the impact of the paper. However, I recognize that this would require substantial additional experimentation and the data here are interesting regardless.

- Only single doses of SCH23390 and raclopride were used. How were these selected? It would be nice to use more of a dose range to show that 1) an effect of D1R blockade was not missed, and 2) that the reduction in orexin signal with raclopride was dose-dependent.

Additional information on dose selection has been included - thank you. Again, these data might be more impactful if the effects of antagonists were found to be dose-dependent.

- Fig 1C, could the effect the authors observed due to movement? Relatedly, what was the behavior like when the cue was on? Did mice orient/approach the cue? Also, when does the learning about the cue occur? Does it take all 10 days of learning or does this learning/cue-induced increase in dopamine signaling occur in less than 10 days?

These have been addressed in the revised manuscript

- Also related to above, could the observed dopamine signal be a result of just the laser turning on? It would seem important to include mice with a control sensor.

The authors note that a control channel was recorded. I agree this is useful, but my concern is that the illumination of laser itself might startle the animal (promote movement), resulting in dopamine release. Showing this does not occur with the same laser in *chr2*-lacking *vta* neurons would help resolve this issue.

- Fig 1E, the effect seems to be driven by one mouse which looks like it could be a statistical outlier. Inclusion of additional animals would make these data more compelling.

I would still argue that these data could be strengthened by the addition of more mice. I note that the graph depicting individual data points has been removed from the revised manuscript - I would recommend re-including this figure.

- For Fig 1C, 3D, 3F, and 4D, could the authors please show the traces for the entire length of laser onset? It would be helpful to see both the rise and the fall of dopamine signals.
- Fig 2C, could the authors comment on how they compared the AUC to baseline? Was this comparison against zero? Because of natural hills and troughs during signals prior to cue (which may not equate to a zero), comparing the omission-induced dip to a zero may not be appropriate. A better baseline might be using the signals prior to the cue.
- Could the authors comment on how they came up with the 4-5.3s window to observe the AUC in Fig 3H?

These have all been addressed.

Minor

- When discussing the understudied role of dopamine in brain regions other than the striatum in the Introduction, it might be helpful to cite this article: <https://elifesciences.org/articles/81980> where the authors characterize dopamine in the bed nucleus of stria terminalis in associative behaviors and reward prediction error.
- In Discussion, it might be better to refrain from describing the results as 'measuring dopamine release' in the LH. Since there was no direct detection of dopamine release, rather dopamine binding to the dLight receptors, referring to the detection as dopamine signaling/binding/transients is a better alternative.
- In Discussion, without measuring tonic dopamine release, it is difficult to say that there was a tonic dopamine release in the LH prior to negative RPE. In addition, I wouldn't describe the negative RPE as silencing of dopamine neurons projecting to the LH since this was not directly measured and it is hard to say for sure if the dip in dopamine is caused by silencing of the neurons. There certainly seems to be a reduction in extrasynaptic dopamine signaling in LH, however what occurs upstream is unknown.
- Typo at multiple places: 'Tekey's multiple comparison test'.

These have been addressed.

<https://doi.org/10.7554/eLife.90158.2.sa0>

Author Response

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

Summary:

Mice can learn to associate sensory cues (sound and light) with a reward or activation of dopamine neurons in the ventral tegmental area (VTA), and then anticipate the reward from the sensory cue only. Using this paradigm, Harada et al. showed that after learning, the cue is able to induce dopamine release in the projection targets of the VTA, namely the nucleus accumbens and lateral hypothalamus (LH). Within the LH, dopamine release from VTA neurons (either by presentation of the cue or direct optical stimulation of VTA neurons) activates orexin neurons, measured as an increase in intracellular calcium levels.

Strengths:

This study utilized genetically encoded optical tools to selectively stimulate dopamine neurons and to monitor dopamine release in target brain areas and the calcium response of orexin neurons. This allowed a direct assessment of the relationship between the behavioral response of the animals, the release of a key neurotransmitter in select brain areas, and its effect on target cells, with a precision previously not possible. The results shed light on the mechanism underlying reward-related learning and expectation.

Weaknesses:

- The Ca increase in orexin neurons in response to optical stimulation of VTA DA neurons is convincing. However, there is an accumulated body of literature indicating that dopamine inhibits orexin neurons through D2 receptors, particularly at high concentrations both directly and indirectly (PMID 15634779, 16611835, 26036709, 30462527; but note that synaptic effects at low conc are excitatory - PMID 30462527, 26036709). There should be a clear acknowledgment of these previous studies and a discussion directly addressing the discrepancy. Furthermore, there are in-vivo studies that investigated the role of dopamine in the LH involving orexin neurons in different behavioral contexts (e.g. PMID 24236888). The statement found in the introduction "whether and how dopamine release modulates orexin neuronal activity has not been investigated vigorously" (3rd para of Introduction) is an understatement of these previous reports.*

We thank the Reviewer for pointing out that we missed several important citations. We added the references mentioned and the discrepancy of concern is addressed in the discussion section

- Along these lines, previous reports of concentration-dependent bidirectional dopaminergic modulation of orexin neurons suggest that high and low levels of DA would affect orexin neurons differently. Is there any way to estimate the local concentration of DA released by the laser stimulation protocol used in this study?*

Could there be a dose dependency in the Intensity of laser stimulation and orexin neuron response?

We agree that this is an interesting point. However, one limitation of our study, and of intensity-based genetically-encoded sensors in general, is that the estimation of the concentration is technically difficult. The sensor effectively reports changes in extra-synaptic levels of neurotransmitters, but to get the absolute value other modalities would be needed such as fast scan voltammetry. This limitation is now included in the discussion section.

- *The transient dip in DA signal during omission sessions in Fig2C (approx 1% decrease from baseline) is similar in amplitude compared to the decrease seen in non-laser trials shown in Fig 1C right panel (although the time course of the latter is unknown as the data is truncated). The authors should clarify whether those dips are a direct effect of the cue itself or indeed reward prediction error.*

Thanks for raising this important point. Indeed, there is a dip of the signal during non-stimulation trials. At day 1, the delivery of the cue triggered a dip and at day 10, there was a slight increase of the signal and followed by the dip. The data is difficult to interpret but our hypothesis is that two components trigger this dip of the signal. One is the aversiveness of the cue. Because a relatively loud sound (90dB) was used for the cue, it would not be surprising if the auditory cue was slightly aversive to the experimental animals. It has been shown that aversive stimuli induce a dip of dopamine in the NAc, although it is specific to NAc subregions. The second component is reward prediction error. Although the non-laser paired cue never triggered the laser stimulation, it is similar to the laser paired one. In a way both are composed of loud tone and same color of the visual cue (spatially different). We think it is possible that reward-related neuronal circuit was slightly activated by the non-laser paired cue. In line with this interpretation, a small increase of the signal was observed at day 10 but not day 1. If our hypothesis is true, since this signal was induced by two components, further analysis is unfortunately difficult.

- *There seem to be orexin-negative-GCaMP6 positive cells (Fig. 4B), suggesting that not all cells were phenotypically orexin+ at the time of imaging. The proportion of GCaMP6 cells that were ORX+ or negative and whether they responded differently to the stimuli should be indicated.*

While we acknowledge the observation of orexin-negative-GCaMP6 positive cells in Figure 4B, it's important to note that this phenomenon is consistent with the characteristics of the hOX-GCaMP virus used in prior experiments. The virus has undergone thorough characterization, and it has been reported to exhibit over 90% specificity, as demonstrated in prior work conducted in the laboratory of one of our contributing authors (PMID: 27546579). To address the concern raised by the reviewer, we have included Supplemental Figure 4 confirming that all mice consistently exhibited qualitatively similar hOX-GCaMP transients upon dopaminergic terminal stimulation. This additional evidence supports the reliability and specificity of our experimental approach.

- *Laser stimulation of DA neurons at the level of cell bodies (in VTA) induces an increase in DA release within the LH (Fig. 3C, D), however, there is no corresponding Ca signal in orexin neurons (Fig.4C).*

We realized that the figures were not clear and we understood that the reviewer did not see any corresponding Ca signal, but this description is not true. We now added Supplemental Figure 3 to show that there is Ca signal at day 1 already.

In contrast, stimulating DA terminals within the LH induces a robust, long-lasting Ca signal (> 30s) in orexin neurons (Fig. 5). The initial peak is blocked by raclopride but the majority of Ca signal is insensitive to DA antagonists (please add a positive control or cite references indicating that the dose of antagonists used was sufficient; also the timing of antagonist administration should be indicated).

This is now included in the discussion section. Also, the timing and dose of the antagonist is now described in the method section.

Taken together, these results seem to suggest that DA does not directly increase Ca signal in orexin neurons. What could be mediating the remaining component?

This point has been included in the discussion section.

- Similarly, there is an elevation of Ca signal in orexin neurons that remains significantly higher after the cue/laser stimulation (Fig. 4F). It appears that it is this sustained component that is missing in omission trials. This can be analyzed further.*

It is true that there is a sustained component in stimulation trials, that is missing in omission trials. Most likely that is evoked by the stimulation of dopamine neurons. We argue that this component is isolated in Fig 5 and analyzed as much as we can.

- Mice of both sexes were used in this study; it would be interesting to know whether sex differences were observed or not.*

We agree that this is an important point. However, our sample number is not high enough to make a meaningful comparison between male and female.

Reviewer #2 (Public Review):

Summary:

This is an interesting and well-written study assessing the role of dopaminergic inputs from the VTA on orexin cell responses in an opto-pavlovian conditioning task. These data are consistent with a possible role of this system in reward expectation and are surprisingly one of the first demonstrations of a role for dopamine in this phenomenon.

Strengths:

The study has used an interesting opto-Pavlovian approach combined with fibre photometry.

Weaknesses:

It is unclear what n size was used or analysed, particularly for AUC measures e.g. Figures 1 D/E and 3 G. The number of trials reflected and the animal numbers need clarification.

The sample size is indicated in the legend section.

The study focused on opto-stim omissions - this work would be significantly strengthened by a comparison to a real-world examination where animals are trained for a radiation reward (food pellet).

We agree that this would be an important experiment. This experiment is partially done in one of the contributing authors laboratories (doi.org/10.1101/2022.04.13.488195) and would be one of our follow up study.

Have the authors considered the role of orexin in the opposing situation i.e. a surprise addition of reward?

That would be an interesting experiment. To do that, natural reward, not optical stimulation, should be used as a reinforcer. This could be part of our follow up study.

Similarly, there remains some conjecture regarding the role of these systems in reward and aversion - have the authors considered aversive learning paradigms - fear, or fear extinction - to further explore the roles of this system? There are some (important) discussions about the possible role of orexin in negative reinforcement. Further studies to address this could be warranted.

It is true that dopamine also plays a significant role in aversive learning. Therefore, this would be an interesting experiment. The discussion section now includes this point.

I think some further discussion of the work by Lineman concerning the interesting bidirectional actions of d1/d2 r signalling on glutamatergic transmission onto orexin neurons is worthwhile. While this work is currently cited, the nuance and perhaps relevance to d1 and d2 signalling could be contextualised a little more (<https://doi.org/10.1152/ajpregu.00150.2018>).

Thanks for the suggestion. The discussion has been expanded.

Reviewer #3 (Public Review):

Summary:

Harada and colleagues describe an interesting set of experiments characterizing the relationship between dopamine cell activity in the ventral tegmental area (VTA) and orexin neuron activity in the lateral hypothalamus (LH). All experiments are conducted in the context of an opto-Pavlovian learning task, in which a cue predicts optogenetic stimulation of VTA dopamine neurons. With training, cues that predict DA stimulation come to elicit dopamine release in LH (a similar effect is seen in accumbens). After training, omission trials (cue followed by no laser) result in a dip (inhibition) of dopamine release in LH, characteristic of reward prediction error observed in the striatum. Across cue training, the activity pattern of orexin neurons in LH mirrors that of LH DA levels. However, unlike the DA signal, orexin neurons do not exhibit a decrease in activity in omission trials. Systemic blockade of D2 but not D1 receptors blocked DA release in LH following VTA DA cell stimulation.

Strengths: Although much work has been dedicated to examining projections from orexin cells to VTA, less has been done to characterize reciprocal projections and their function. In this way, this paper is a very important addition to the literature. The experiments are technically sound (with some limitations, below) and utilize sophisticated approaches, the manuscript is nicely written, and the conclusions are mostly reasonable based on the data collected.

Weaknesses:

I believe the impact of the paper could be enhanced by considering and/or addressing the following:

Major:

- *I encourage the authors to discuss in the Introduction previous work on DA regulation of orexin neurons. In particular, the authors cite, but do not describe in any detail, the very relevant Linehan paper (2019; Am J Physiol Regul) which shows that DA differentially alters excitatory/inhibitory input onto orexin neurons and that these actions are reversed by D1 vs D2 receptor antagonists. Another paper (Bubser, 2005, EJN) showed that dopamine agonists increase the activity of orexin neurons and that these effects are blocked by D1/D2 antagonists. The current findings should be discussed in the context of these (and any other relevant) papers in the Discussion, too.*

Thanks for the valuable suggestion. This point has been integrated and the introduction and discussion sections have been revised carefully.

- *In the Discussion, the authors provide two (plausible) explanations for why they did not observe a dip in the calcium signal of orexin neurons during omission trials. Is it not possible that these cells do not encode for this type of RPE?*

We completely agree that it is possible. Now our current hypothesis is that dopamine in the LH encodes RPE and that information is transmitted to orexin neurons. Orexin neurons integrate other information and encode something else, we call it ‘multiplexed cognitive information’. It is still open question what this means exactly. This point is now mentioned in the discussion section.

- *Related to the above - I am curious about the authors' thoughts on why there is such redundancy in the system. i.e. why is dopamine doing the same thing in NAC and LH in the context of cue-reward learning?*

Thank you for the question. This is an important point, indeed. Our current hypothesis is described in the discussion section.

‘Our data indicate that dopamine in both the NAC and LH encodes reward prediction error (RPE). One open question is the existence of such a redundant mechanism. We hypothesize that dopamine in the LH boosts dopamine release via a positive feedback loop between the orexin and dopamine systems. It has already been established that some orexin neurons project to dopaminergic neurons in the VTA, positively modulating firing. On the other hand, our data indicate that dopamine in the LH stimulates orexinergic neurons. These collective findings suggest that when either the orexin or dopamine system is activated, the other system is also activated consequently. Although the current findings align with this idea, the hypothesis should be carefully challenged and scrutinized.’

- *The data, as they stand, are largely correlative and do not indicate that DA recruitment of orexin neurons is necessary for learning to occur. It would be compelling if blocking the orexin cell recruitment affected some behavioral outcomes of learning. Similarly - does raclopride treatment across training prevent learning?*

We appreciate the insightful comment. It is indeed a limitation of our study that we lack behavioral data. However, given the extensive previous research on the crucial role of orexin in motivated behavior, we argue that establishing dopaminergic regulation of the orexin system itself is a valuable contribution. This perspective is thoroughly discussed in the

dedicated section of our paper. It's important to note that the injection of D2 antagonists, including raclopride, is known to induce significant sedation. Due to this sedative effect, combining behavioral experiments with these drugs poses considerable challenges.

- *Only single doses of SCH23390 and raclopride were used. How were these selected? It would be nice to use more of a dose range to show that 1) and effect of D1R blockade was not missed, and 2) that the reduction in orexin signal with raclopride was dose-dependent.*

The rationale of the dose has been added to the discussion section. It is reported that these doses block dopamine receptors. We agree that it would be nice to have a dose-response curve, we are reluctant to increase the doses to avoid adverse effect to the experimental animals. The doses we used effectively induced hypo-locomotion, although data is not shown.

- *Fig 1C, could the effect the authors observed be due to movement?*

We argue this is unlikely. We recorded two channels one for the control and the other one for the signal. The motion-related artifact is corrected based on the control channel. One example trace around the laser stimulation is shown below. Please note that a typical motion-related artifact is a fast dip of the signal, normally observed in both 405 and 465 nm channels.

Relatedly, what was the behavior like when the cue was on? Did mice orient/approach the cue?

Although it has been reported that rats approach the cue (PMID: 30038277) in a similar task, it was not obvious in our case. It could be because we used both visual and auditory cues. Mice showed a general increase of locomotion during the cue and the stimulation but the direction was not clear to the experimenter.

Also, when does the learning about the cue occur? Does it take all 10 days of learning or does this learning/cue-induced increase in dopamine signaling occur in less than 10 days?

It is hard to say when the learning occurs. When we look at the learning curve of Figures 1,3 and 4, it seems the response to the cue plateaus at day 5 but since we don't have behavioral data, the assessment is relayed only on the neuronal signal.

- *Also related to the above, could the observed dopamine signal be a result of just the laser turning on? It would seem important to include mice with a control sensor.*

We recorded two channels, 405 nm and 465 nm wavelength. 405 nm signal did not show increase of the signal while 465 nm signal did. The example trace is shown. Besides, the sensor has been characterized by the corresponding author already so we argue that this is unlikely.

Author response image 1.



Fig 1E, the effect seems to be driven by one mouse which looks like it could be a statistical outlier. The inclusion of additional animals would make these data more compelling.

We agree that adding more mice would make data more compelling. However, considering the fact that dopamine in the accumbens has been investigated vigorously and our data is in line with the prior studies, we argue that we have enough data to claim our conclusion.

- For Fig 1C, 3D, 3F, and 4D, could the authors please show the traces for the entire length of laser onset? It would be helpful to see both the rise and the fall of dopamine signals.*

For Fig 1C, one panel has been added. For fig 3, 4, supplemental figure was created to show the signal around laser stimulation.

- Fig 2C, could the authors comment on how they compared the AUC to baseline? Was this comparison against zero? Because of natural hills and troughs during signals prior to cue (which may not equate to a zero), comparing the omission-induced dip to a zero may not be appropriate. A better baseline might be using the signals prior to the cue.*

The signal immediately before the cue onset was considered as a baseline, and baseline was subtracted. This means zero and baseline would be the same in our way of analysis.

- Could the authors comment on how they came up with the 4-5.3s window to observe the AUC in Fig 3H?*

Since the kinetic of dopamine in the NAc and LH is different, different time windows have been used to observed a dip of dopamine. The analysis of the kinetics has been added.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

Specific feedback to the authors

- Sample size for each experiment/group could not be found.*

The sample size is now included in the legends.

- In most figures, the timing of onset for the cue and laser stimulation is unclear. This makes the data interpretation difficult. They should be labeled as in Fig. 3C, for example.*

Panels have been updated to address this point.

- Please provide the rationale for selecting the time range for the measurement of AUC for different experiments (e.g. Fig. 2C, 3H, 4A, 5F).*

The kinetics of dopamine in NAc and LH are different. This is now shown in the new Supplemental Figure 2. Based on this difference, the different window was chosen.

- *Fig. 1E, 3G right, 4E right: statistical analysis should use two-way repeated measures ANOVA rather than one-way ANOVA. Fig 1D, 3G left and 4E left panels can also be analyzed by two-way repeated measures ANOVA.*

We realized that those panels were redundant. Some panels have been removed and the analysis has been conducted according to this point.

Minor comments:

Fig. 2C can also show non-omission trials as a comparison.

The panel has been updated.

- *The term "laser cue" is confusing, as the cue itself does not involve a laser.*

'Laser-paired cue' is used instead.

- *Color contrast can be improved for some figures, including Fig. 2C right, Fig. 3H right, and green and blue fluorescent fonts.*

The panels have been updated.

- *Figure legends: Tukey's test, rather than Tekey's test.*

This has been fixed.

- *There are some long-winded sentences that are hard to follow.*

Edited.

- *p.2, line 11 from bottom: should read ...the VTA evokes the release of dopamine.*

Edited

- *p.3, line 9: remove e from release.*

This has been addressed.

Reviewer #3 (Recommendations For The Authors):

Minor:

- *When discussing the understudied role of dopamine in brain regions other than the striatum in the Introduction, it might be helpful to cite this article: <https://elifesciences.org/articles/81980> where the authors characterize dopamine in the bed nucleus of stria terminalis in associative behaviors and reward prediction error.*

The discussion session has been updated accordingly.

- *In the Discussion, it might be better to refrain from describing the results as 'measuring dopamine release' in the LH. Since there was no direct detection of dopamine release, rather a dopamine binding to the dLight receptors, referring to the detection as dopamine signaling/binding/transients is a better alternative.*

This point has been addressed.

- *In the Discussion, without measuring tonic dopamine release, it is difficult to say that there was a tonic dopamine release in the LH prior to negative RPE. In addition, I wouldn't describe the negative RPE as silencing of dopamine neurons projecting to the LH since this was not directly measured and it is hard to say for sure if the dip in dopamine is caused by silencing of the neurons. There certainly seems to be a reduction in extra-synaptic dopamine signaling in LH, however, what occurs upstream is unknown.*

We respectfully disagree with this point. In our opinion, the dopamine transient is more important than the firing of dopamine neurons because what matters for downstream neurons is dopamine concentration. For example, administration of cocaine increases the dopamine concentration extra-synaptically via blockade of DAT, while the firing of dopamine neurons go down via activation of D2 receptors expressed in dopamine neurons. Administration of cocaine is not known to induce negative RPE.

- *Typo at multiple places: 'Tekey's multiple comparison test'.*

This has been fixed.