Excitatory VTA to DH projections provide a valence signal to memory circuits

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The positive or negative value (valence) of past experiences is normally integrated into neuronal circuits that encode episodic memories and plays an important role in guiding behavior. Here, we show, using mouse behavioral models, that glutamatergic afferents from the ventral tegmental area to the dorsal hippocampus (VTA→DH) signal negative valence to memory circuits, leading to the formation of fear-inducing context memories and to context-specific reinstatement of fear. To a lesser extent, these projections also contributed to opioid-induced place preference, suggesting a role in signaling positive valence as well, and thus a lack of dedicated polarity. Manipulations of VTA terminal activity were more effective in females and paralleled by sex differences in glutamatergic signaling. By prioritizing retrieval of negative and positive over neutral memories, the VTA→DH circuit can facilitate the selection of adaptive behaviors when current and past experiences are valence congruent.
Positive or negative experiences usually lead to the formation of memories carrying similar valence1. Such memories (henceforth referred to as “positively or negatively valenced”) can impact threat avoidance and reward approach behaviors long after the original experience, not the least because their retrieval is often prioritized relative to neutral memories, especially in affective states that are congruent with their valence5. However, persistent affective memory bias can also have maladaptive consequences: negatively valenced memories contribute to anxiety disorders by triggering reinstatement of fear in situations that no longer predict danger1, while positively valenced memories contribute to addiction by triggering relapse of drug seeking despite an individual’s awareness of the drug’s life-threatening consequences4,5. It is yet to be established whether sex differences in processing memory valence contribute to the heightened propensity of women to develop anxiety disorders6,7 and their higher susceptibility to drug abuse relapse8,9.

The dorsal hippocampus (DH) plays a well-established role in the formation and retrieval of episodic memories9, that is, memories of personal experiences of events, the places in which they happened, and the times of their occurrence10. Our understanding of the cortical inputs to DH neurons, required for the formation of the sensory, spatial, and temporal components of episodic memories has significantly advanced in recent years11–13, particularly with the delineation of the roles of discrete entorhinal cortical projections to dentate gyrus (DG) granule cells14,15. Importantly, these cells also encode, in a flexible manner, the valence of memories of environmental contexts in which reinforcing events have taken place16. However, the circuits that signal valence to DG neurons have not been identified.

The activity of midbrain dopamine neurons in the ventral tegmental area (VTA) has long been implicated in signaling reward (both natural rewards and reward associated with drugs of abuse)17,18, as well as aversive outcomes19,20. However, a proposed role of DH-projecting VTA dopamine neurons in memory21–23, has recently been challenged because of the finding that dopaminergic projections from VTA to DH are sparse and that dopaminergic mechanisms of memory primarily involve projections from the locus coeruleus24,25. Apart from dopaminergic neuronal populations, VTA contains glutamatergic and GABAergic neurons26–28 that could contribute to DG granule cell activity29. We therefore sought to determine the extent of DH innervation provided by these projections and their role in driving behaviors based on valenced memories. We demonstrated a significant contribution of glutamatergic VTA→DH projections to the encoding of negative valence of context memories and to the reinstatement of fear after extinction. These projections also contributed to behavior guided by positively valenced memories, such as opioid-seeking following abstinence. The observed effects were especially strong in females, who showed higher density of VTA→DH terminals than males after reinstatement of fear or opioid-induced place preference, as well as faster decay of glutamate currents in response to activation of VTA→DH terminals. Together, these findings showed that excitatory VTA to DH projections provide a valence signal to memory circuits.

Chemogenetic inactivation of vGlut2-positive VTA→DH terminals impairs contextual fear conditioning. We recently demonstrated that local application of clozapine-N-oxide (CNO) in the brain effectively inhibits terminals arising from long-range DH-projecting neurons expressing AA8-hM4(Gi)-mCherry31, and confirmed these observations in DH slices (Supplementary Fig. 6a). We therefore used this approach to determine whether VTA→DH projections contribute to contextual fear conditioning. Thirty minutes before training, mice expressing mCherry in VTA-originating terminals were injected with either vehicle (VEH) or CNO into DH (Fig. 2a). Injection of CNO did not affect activity to the context or shock at training (Supplementary Fig. 6b), but significantly impaired freezing behavior at test performed a day later. CNO was effective in both males and females (Fig. 2b). To determine whether the observed effect was specific for the encoding of negative contextual valence rather than the contextual representation, we also used a paradigm based on the context pre-exposure facilitation effect (CPFE)32, which allows delineating drug effects on context memory formation from context-shock associations33. In this paradigm, mice are pre-exposed to the to-be-conditioned context 1 day before exposure to an immediate shock, resulting in context-specific fear conditioning34 (Supplementary Fig. 6c, left). Injections of CNO before context pre-exposure were ineffective, demonstrating that silencing of VTA→DH did not affect the formation of context representations (Fig. 2c). Thus, the effects of CNO on context fear conditioning were most likely due to the association of context
with the aversive footshock, resulting in a negatively valenced context memory.

Silencing vGlut2-positive VTA→DH terminals abolishes reinstatement of fear. Fear conditioning followed by extinction typically results in the formation of negatively valenced (fear-inducing) and neutral (extinction) memories, with predominant retrieval of the neutral memory following sequential extinction trials. To determine whether excitatory VTA→DH afferents bias retrieval towards fear-inducing context memories, we examined their potential contribution to fear reinstatement after extinction. Following shock delivery (Supplementary Fig. 6c, right). One hour before the shock reminder, mice were injected with either VEH or CNO into DH. Following fear extinction (Fig. 3c, left) and shock reminder (Fig. 3c middle), vGlut2-Cre mice injected with VEH showed robust reinstatement of freezing. Compared with the last extinction test, reinstatement of freezing was reduced in CNO-treated mice and the reinstatement index was lower when compared with the VEH-treated group (Fig. 3c, right). We used the same chemogenetic approaches to silence SUM→DH terminals and examine the neuroanatomical specificity of the observed effects. In these experiments, CNO did not affect fear conditioning or reinstatement of fear after extinction (Supplementary Fig. 6d, e), which is consistent with the small contribution of SUM to hippocampus-dependent memory processes despite the abundance of SUM afferents in DH.

We subsequently performed a similar experiment in vGlut2-Cre females. Following extinction (Fig. 3d, left) and shock reminder (Fig. 3d, middle), the freezing response showed reinstatement in VEH-treated mice but was completely abolished in CNO-injected females, as revealed both by the lack of increase relative to the last extinction test \(P = 0.4890\) and between group differences of the reinstatement index \(P < 0.01\) (Fig. 3d, right). To directly examine the role of sex in the effects of VTA→DH terminals in fear extinction, we also investigated the effects of CNO in a separate experiment that included male and female...
mice (Fig. 3e). While replicating the main finding, this study also demonstrated a significant effect of sex, revealed by a sex × treatment interaction (P < 0.05).

Optogenetic stimulation of vGlut2-positive VTA→DH projections trigger reinstatement of fear mediated by NMDA receptors. We next sought to determine whether replacing the reminder shock with unilateral optogenetic stimulation of vGlut2-positive VTA→DH projections would affect fear reinstatement. Mice were infused with AAV8-Ef1a-DIO hChR2-EYFP or control virus AAV-Ef1a-DIO-EGFP and 3 weeks later implanted with optofluidic devices (Fig. 4a). Based on pilot data involving different protocols (including 20 Hz stimulation shown below), we injected the mice with VEH or APV, and 20 min later applied tonic, 4 Hz blue light stimulation over 3 min (Fig. 4b). The unilateral design enabled to determine the effects of APV on optogenetic stimulation without interfering with fear reinstatement independently (Supplementary Fig. 7a). After fear conditioning, extinction (Fig. 4c, left), and 4 Hz tonic optogenetic stimulation in the presence of VEH or APV, vGlut2-Cre mice were placed in the conditioning context to record freezing behavior. Mice expressing hChR2 showed significant reinstatement of freezing after optogenetic stimulation performed in the presence of VEH (P < 0.001), whereas mice expressing a control virus and mice expressing hChR2 but stimulated in the presence of APV did not show reinstatement (P = 0.4947). This was revealed both by two-way ANOVA of freezing behavior (Fig. 4c, middle) and by between group comparison of the reinstatement index (Fig. 4c, right). Importantly, unilateral optogenetic stimulation itself was devoid of aversive reinforcing properties, as indicated by lack of freezing behavior after pairing of a conditioning context with optogenetic stimulation instead of footshock (Supplementary Fig. 7b).

To determine how optogenetic stimulation affected neuronal activity within the DH, we quantified cFos responses 1 h after stimulation or reminder shock. Exposure to a reminder shock resulted in a significant upregulation of cFos-positive neurons in the CA3 subfield and in the DG (Fig. 4d, e, Supplementary Fig. 8). When compared with a reminder shock, optogenetic 4 Hz stimulation did not significantly induce cFos activity in CA3 (P = 0.4113), however levels in DG were similar to those induced by the shock reminder and significantly higher than in naïve (P < 0.01) controls and APV-injected mice (P < 0.01).

Similar to males, females implanted with devices for optogenetic stimulation end expressing ChR2 in their VTA→DH terminals (Fig. 4f), were presented with tonic 4 Hz light pulses in the DH instead of exposure to shock reminder (Fig. 4g). Addition of the 20 Hz stimulation protocol served to further control for the specificity of stimulation effects. Following fear conditioning, extinction (Fig. 4h, left), and optogenetic stimulation, females stimulated with 4 Hz but not 20 Hz showed significant reinstatement of freezing behavior (P < 0.001, Fig. 4h, middle and right). Thus, in males and in females, activity of vGlut2-positive VTA→DH terminals was both necessary and sufficient to trigger fear reinstatement.

Chemogenetic inactivation of vGlut2-positive VTA→DH terminals also abolishes reinstatement of morphine-induced conditioned place preference (M-CPP). We next examined whether the role of VTA→DH projections extends to the retrieval of positively valenced memories under valence congruent conditions. To ensure that behavior was primarily driven by the rewarding properties of morphine rather than contextual cues, we used a place preference paradigm paradigm that results in sub-threshold CPP (about 10%, not significant from chance) but robust (about 30% or more) M-CPP accompanied by increased total locomotion. We also used a priming dose of morphine that was ineffective in the absence of previously conditioned M-CPP memory (P = 0.9848, Supplementary Fig. 9a, b). Also, this paradigm is not sensitive to stress-induced CPP (S-CPP) (Supplementary Fig. 9c, d), which rules out a confounding effect of negatively valenced information as potential trigger of CPP. Surgeries and virus infusions were performed as described above, and 5 weeks later mice were trained in M- CPP as described in
the Methods and schematically shown in Fig. 5a, b. vGlut2-Cre mice injected with vehicle displayed significant M-CPP (main effect of test: $F(1, 22) = 10.98, P = 0.0032$, post hoc comparison with off drug CPP, $P = 0.0099$), an effect that was found in vGlut2-Cre mice injected with CNO (post hoc comparison with off drug CPP, $P = 0.2727$) (Fig. 5c). However, there were no significant group differences of M-CPP [vGlut2-Cre: $F(1, 22) = 0.5639$, $P = 0.4607$] nor changes of morphine-induced total activity in the CPP apparatus [vGlut2-Cre: $F(1, 22) = 0.04847$, $P = 0.8278$] relative to controls. In contrast to the partial effect observed in males, chemogenetic silencing of vGlut2-positive VTA→DH terminals completely abolished M-CPP ($P = 0.9916$) in females, indicating the lack of place preference, without interfering with total locomotion (Fig. 5d) or with CPP in the absence of morphine (Supplementary Fig. 10a). This sex difference was replicated in a separate experiment performed with both males and females (Fig. 5e), and was revealed, using three-way ANOVA as significant sex × treatment interaction ($P < 0.05$). We next examined, using vGlut2-Cre females, whether M-CPP can be induced using optogenetic stimulation of VTA→DH terminals. However, three different protocols, involving stimulation with 4 Hz, 8 Hz, or 20 Hz between two off drug tests (Fig. 5f), or during CPP or M-CPP proved ineffective (Supplementary Fig. 10b, c). These results showed a complex contribution of VTA→DH projections to behavior based on positively valenced memories, with greater overall involvement in females than in males. However, even though activation of these projections was both necessary and sufficient for fear reinstatement, it was necessary but not sufficient for M-CPP.
Fig. 4 Optogenetic stimulation of vGlut2 VTA→DH terminals triggers fear reinstatement. a Virus infusion into VTA and implantation of optofluidic device in DH of vGlut2-Cre males. b After extinction, mice were injected with VEH (n = 8) or APV (n = 8) 20 min before receiving tonic 4 Hz stimulation.

c Left, extinction [RM one-way ANOVA for all mice, F(3,68, 77.45) = 43.19, ***P < 0.0001]. Middle, fear reinstatement after optogenetic stimulation. Two-way ANOVA revealed significant Stimulation effect [F(2, 57) = 11.17, P < 0.0001], and reinstatement in the VEH-4 Hz group (**P < 0.0001). Right, increased reinstatement index in VEH but not APV or control virus (n = 6) groups [two-way ANOVA, F(2, 19) = 21.94, P < 0.0001, **P < 0.0001 VEH-4 Hz vs. AAV-GFP-4 Hz, **P = 0.0029 VEH-4 Hz vs. APV-4 Hz].

d Upregulation of cFos in DG [n = 6/group, two-way ANOVA, F(3, 28) = 13.16, P < 0.0001, ***P < 0.0001 naïve vs. reminder shock] and CA3 [two-way ANOVA, F(3, 28) = 13.16, P < 0.0001, ***P < 0.0001 naïve vs. reminder shock]. e Illustration of cFos labeling.

f Virus infusions and implantation of optostimulator devices in vGlut2-Cre females. g DH stimulation with 4 (n = 6) or 20 (n = 6) Hz for 1 min. h Left, extinction [RM one-way ANOVA for all mice, F(2,86, 48.72) = 20.11, ***P < 0.0001]. Middle, fear reinstatement after stimulation of vGlut2 VTA→DH terminals with 4 (n = 6) but not 20 Hz (n = 6) in ChR2 but not GFP (n = 6) groups [one-way ANOVA, F(2,15) = 11.27, P = 0.0010, **P = 0.0011 AAV-ChR2-4 Hz vs. AAV-GFP-4 Hz, **P = 0.0001 AAV-ChR2-4 Hz vs. AAV-ChR2-20 Hz]. Right, reinstatement index [one-way ANOVA, F(2, 15) = 19.32, P < 0.0001, *P = 0.023 AAV-ChR2-4 Hz vs. AAV-GFP-4 Hz, ***P < 0.0001 AAV-ChR2-4 Hz vs. AAV-ChR2-20 Hz].
Chemogenetic inactivation of GAD2-positive VTA→DH terminals does not affect fear reinstatement or M-CPP. We next examined whether the effects observed with chemogenetic approaches were restricted to vGlut2-positive VTA→DH terminals or also occur after silencing of GAD2-containing projections. We performed similar experiments as described above with GAD2-Cre male and female mice (Fig. 6), however the only detectable effect was on total activity in the M-CPP paradigm in males (Fig. 6b) but not in females (Fig. 6f). Overall, freezing after fear conditioning was lower in this mouse strain relative to vGlut2-Cre mice (~40–50% vs. 60–70%). We therefore performed an additional experiment with GAD2-Cre male mice using three shock presentations that resulted in similar freezing level as shown for vGlut2-Cre males (Fig. 3c). However, CNO remained ineffective (Supplementary Fig. 11). Together, the findings indicated a lack of significant contribution of GAD2-Cre VTA→DH afferents to the retrieval of fear-inducing or M-CPP memories.

vGlut2-positive VTA→DH terminals are more abundant in females and show sex-specific differences in glutamate current kinetics. To gain first insight into the stronger involvement of vGlut2-positive VTA→DH projections in behavioral regulation in females, we compared the density of their DH terminals in males and females and found a significantly denser innervation of the stratum lacunosum moleculare, CA3 and especially in the DG (F (1, 24) = 20.89, P < 0.001) of females (Supplementary Fig. 12). These differences were significant after fear reinstatement and M-CPP (Fig. 7a). Two-way ANOVA revealed significant effects of sex [F (1, 30) = 30.61, P < 0.001] and Group [F (2, 30) = 17.75, P < 0.001]. This was not due to the greater labeling of VTA neurons in females because, when compared with males, females had similar numbers of VTA neurons expressing mCherry [F (1, 4) = 2.167, 8.66] = 16.36, P = 0.0009, however, while morphine-induced place preference (M-CPP vs. CPP, *P = 0.0465), stimulations were ineffective (4 Hz vs. CPP F = 0.5198, 8 Hz vs. CPP P = 0.2307, 20 Hz vs. CPP P = 0.4556).

**Fig. 5 Silencing VTA→DH terminals impairs reinstatement of M-CPP.** a Virus infusion into VTA and cannula implantation in DH. b Mice were trained in M-CPP triggered by a priming dose of morphine. One hour before M-CPP, VEH (n = 11) or CNO (n = 13) were injected into DH. In vGlut2-Cre males, M-CPP was detected in VEH but not CNO group (left, two-way ANOVA, effect of CPP (F (1, 44) = 7.909, P = 0.0073, *P = 0.0498 VEH-CPP vs. VEH-M-CPP, P = 0.5198 CNO-CPP vs. CNO-M-CPP), but there was no group effect (left, two-tailed unpaired t test, t(12) = 1.054, P = 0.3035). Enhanced of locomotion was similar in both groups (right, two-way ANOVA, F(1, 44) = 30.28, P < 0.0001, *P = 0.0027, VEH CPP vs. M-CPP, **P = 0.0012, CNO CPP vs. M-CPP). c In vGlut2-Cre females, CNO (n = 6) prevented M-CPP, as revealed by within subject (two-way ANOVA, F(1, 40) = 4.3, P = 0.0497, *P = 0.0383 VEH CPP vs. M-CPP), and group effects (unpaired two-tailed t test, t(10) = 2.25, *P = 0.0482 vs. VEH (n = 6)). Locomotor activity was not affected (two-way ANOVA, F(1, 10) = 2.26, P = 0.1484), showing increases in both groups (*P < 0.0252 VEH CPP vs. M-CPP, **P = 0.0328 CNO CPP vs. M-CPP). d In vGlut2-Cre females, CNO (n = 6) prevented M-CPP, as revealed by within subject (two-way ANOVA, F(1, 40) = 5.276, P = 0.0269), with M-CPP in VEH groups only (***P = 0.0099 males and **P = 0.0018 females), and similar locomotion (F(1, 40) = 3.366, P = 0.0740). e Females (n = 5) received optogenetic stimulation on consecutive days followed by a priming dose of morphine. RM one-way ANOVA revealed significant effects of treatment, F(2,167, 8.66) = 16.36, P = 0.0009, however, while morphine-induced place preference (M-CPP vs. CPP, *P = 0.0465), stimulations were ineffective (4 Hz vs. CPP F = 0.5198, 8 Hz vs. CPP P = 0.2307, 20 Hz vs. CPP P = 0.4556).
conditions that allow recording glutamate and GABA-induced currents from DH slices collected 4 weeks after infusion of AAV2-DIO-Chr2 into the VTA. As expected, optogenetic stimulation triggered both types of currents with similar amplitudes in males and females (Fig. 7c, top), but with significantly faster decay of the glutamate current in females (Fig. 7c, bottom). These findings suggest that in addition to the greater abundance of terminals innervating granule cells, differences in the biophysical properties of the glutamate current may also contribute to the stronger involvement of glutamergic

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**Fig. 6 Silencing VTA-DH neurons in male and female GAD2-Cre did not affect fear reinstatement or M-CPP.**

a Virus infusion into VTA and cannulation in DH. b Fear reinstatement procedure. c Left, extinction in GAD2-Cre males [RM one-way ANOVA, $F_{(3,049, 54,88)} = 18.5, P < 0.0001$]. Middle, reinstatement of freezing behavior in VEH ($n = 9, ***P < 0.0001$) and CNO ($n = 10, ***P < 0.0001$) groups without treatment × test interaction [two-way ANOVA, $F_{(2, 31)} = 1.259, P = 0.2925$]. Right, CNO did not affect the fear reinstatement index (right, two-tailed unpaired $t$-test, $t_{17} = 1.178, P = 0.2550$).

d Extinction in GAD2-Cre females [left, $F_{(2,666, 26,86)} = 15.17, P < 0.0001$]. CNO did not affect fear reinstatement [middle, two-way ANOVA revealed effects for Test: $F_{(2,27)} = 19.95, P < 0.0001$, VEH ($n = 5$) $*P = 0.0339$, CNO ($n = 6$) $*P = 0.0189$ but not treatment $F_{(1, 9)} = 0.2262, P = 0.6642$ or treatment × test interaction: $F_{(2, 27)} = 0.0, P = 0.9956$] or the reinstatement index (right, two-tailed unpaired $t$-test, $t_{9} = 0.9585, P = 0.3629$).

e M-CPP procedure. f Chemogenetic inactivation of GAD2 terminals in males did not affect M-CPP [two-way ANOVA $F_{(1, 24)} = 0.05301, P = 0.8199$, VEH ($n = 7$) $*P = 0.0143$, CNO ($n = 7$) $*P = 0.0035$] but enhanced morphine-induced locomotion [two-way ANOVA treatment effect $F_{(1, 24)} = 8.048, P = 0.0091$, VEH $**P = 0.0008$, CNO $***P < 0.0001$, two-tailed unpaired $t$-test $t_{12} = 2.483, P = 0.0288$]. g In GAD2-Cre females, chemogenetic silencing did not affect M-CPP [left, two-way ANOVA, $F_{(1, 18)} = 0.001841, P = 0.9662$, two-tailed unpaired $t$-test $t_{9} = 0.01115, P = 0.9921$, CNO ($n = 6$) vs. VEH ($n = 5$)]. Locomotor activity was also not affected [right, two-way ANOVA $F_{(1, 18)} = 2.381, P = 0.1402$, two-tailed unpaired $t$-test $t_{9} = 0.5042, P = 0.6642$]. Significant increases in M-CPP (VEH $P = 0.0255$, CNO $*P = 0.0385$) and activity (VEH $P = 0.0433$, CNO $*P = 0.0213$) were found in all groups.
Female fear appears to drive both fear reinstatement and morphine seeking, as input for valence signals to the DH. Surprisingly, this circuit projections often fail to report the presence of VTA negatively, and, to a lesser extent, positively valenced memories.

In this comparative study of the distribution and function of VTA→DH projections in females. We also recorded in the presence of the μ opioid receptor (MOP) agonist DAMGO, to examine the functioning of VTA→DH projections under conditions relevant for M-CPP. In the presence of DAMGO, all current amplitudes were significantly reduced in both sexes (changes of individual amplitudes and representative traces are shown in Fig. 7c, middle and right, respectively). Thus, differences in glutamatergic signaling might contribute to the sex-specific roles of vGlut2-positive VTA→DH projections in the retrieval of valenced memories.

**Discussion**

In this comparative study of the distribution and function of VTA→DH projections, we identified a glutamatergic VTA→DH circuit that impacts the activity of DG cells and provides key input for valence signals to the DH. Surprisingly, this circuit appears to drive both fear reinstatement and morphine seeking, especially in females. These findings provide a long-sought link between VTA and DH that drives motivated behavior rooted in negatively, and, to a lesser extent, positively valenced memories.

Although studies that have focused on the most abundant VTA projections often fail to report the presence of VTA→DH efferents, the identification of rostral VTA neurons as the primary source of DH efferents in this work is consistent with tracing studies focusing on hippocampal VTA projections. Despite their sparsity relative to other VTA projections, vGlut2-containing VTA→DH terminals proved to be of high functional significance for the formation and retrieval of negatively valenced relative to neutral context memories. The chemogenetic and optogenetic approaches used throughout the experiments were selected based on their superior efficacy in silencing (chemogenetic) and activating (optogenetic) terminals relative to cell bodies. Using these approaches, we demonstrated that these afferents are both necessary and sufficient for the reinstatement of freezing even after extensive extinction. It was recently demonstrated that optogenetic 4 Hz stimulation of the prefrontal cortex coordinates activity of prefrontal-amygdala circuits and elicits freezing behavior. A similar mechanism, synchronization of hippocampus-amygdala circuits at 4 Hz, which is known to contribute to freezing behavior, might account for fear reinstatement induced by 4 Hz stimulation of VTA→DH afferents in our study. We did not observe effects with 20 Hz stimulation that was previously effective in inducing reinstatement of avoidance behavior, which could be due the fact that we stimulated terminals of long-range projections rather than DG cells. The sufficiency of our protocol to trigger fear reinstatement enabled us to demonstrate, by using wireless optofluidic devices, that...
NMDA receptors are the mediators of this effect. This is in agreement with previous work\textsuperscript{15,46}, showing that, as in fear conditioning\textsuperscript{47}, glutamatergic-induced plasticity mediates fear reinstatement, and provides new evidence that such plasticity is facilitated by excitatory VTA→DH afferents.

Contrary to other VTA projections (e.g., to the nucleus accumbens or lateral habenula)\textsuperscript{38,49}, activation of vGlut2 VTA→DH projections under the conditions used in our study did not induce behavioral changes consistent with the primary aversive or appetitive reinforcement of footshock and priming dose of morphine, respectively. This was concluded from the findings that chemogenetic silencing or optogenetic stimulation of vGlut2 VTA→DH terminals did not affect locomotion and shock responses during fear conditioning and could not substitute for the shock input. Although we cannot rule out the possibility that reinforcing effects might occur with stronger stimulation or with other behaviors, our findings show that negative valence signals provided by VTA→DH afferents are not sufficient to drive unconditional freezing behavior, and suggest that integration of VTA signals with inputs from other afferents may be required (e.g., sensory information from the entorhinal cortex for context/place representations\textsuperscript{15}) for fear conditioning. Nevertheless, stimulation of these afferents was sufficient for reinstatement of freezing behavior, suggesting that they provided negative valence-congruent signals facilitating retrieval of fear-inducing over neutral memories. VTA→DH projections also contributed to M-CPP although their stimulation could not substitute for morphine, nor affected locomotion or side preference in M-CPP. This suggests that in addition to VTA→DH input, DG neurons might need to integrate other inputs, as discussed above, or act in synergy with primary reinforcement circuits, such as amygdala and nucleus accumbens, to induce positive valence congruent signals needed for retrieval of opioid-related memories. The former possibility is supported by observations that direct activation of DG cells, simulating responses to multiple inputs, can induce approach toward natural rewards\textsuperscript{16}.

Unlike the typically differentiated roles of glutamatergic, dopaminergic, and GABAergic projections in aversion and reward (e.g., to the nucleus accumbens, lateral habenula, and medial prefrontal cortex)\textsuperscript{28}, such differentiation was less evident in vGlut2-positive VTA→DH projections. Clearly, under drug-free conditions, these projections contributed to fear-motivated behavior. However, under morphine, the same projections contributed, especially in females, to the preference for a context previously associated with reward. Given the multiplexed neurochemical composition of VTA neurons\textsuperscript{50}, it is possible that there is a further, so far unknown, differentiation within vGlut2-positive VTA→DH projections that signal valence information. In view of recent findings, demonstrating that the same glutamatergic VTA neurons respond to aversion and reward\textsuperscript{51}, it is more likely that the activity of these projections could be interpreted state-dependently under conditions that trigger aversion (e.g., shock reminder or 4 Hz stimulation) versus reward (e.g., morphine). Accordingly, in response to VTA terminal activation, DG cell current properties differed substantially depending on mu opioid receptor activation, raising the interesting possibility that DG cell current amplitudes might be related to the polarity of valence (e.g., high amplitude currents signaling negative, and low amplitude currents signaling positive memory valence). This is consistent with findings showing that the same excitatory VTA neurons respond with increased firing to aversive and decreased firing to rewarding stimuli\textsuperscript{31}. This possibility is also in line with emerging support for state-dependent rather than dedicated processing of positive and negative valence\textsuperscript{25,53}.

The abundance of vGlut2-positive terminals and kinetics of glutamate currents might have contributed to the stronger impact of excitatory VTA→DH projections in fear reinstatement and M-CPP in females than in males. Interestingly, a similar, female-specific increase of vGlut2-positive terminals was found in the nucleus accumbens in response to stress\textsuperscript{35}, however, it is not known whether this increase was restricted to VTA afferents or occurs more generally as a reflection of enhanced stress sensitivity in females. Given that the total number of VTA neurons was similar across conditions, it is likely that the causes for these differences are to be found in mechanisms underlying activity-dependent axonal branching or synaptic connectivity of VTA→DH terminals\textsuperscript{55–57}. In addition, the observed sex differences could be due, at least in part, to the faster decay of glutamatergic (but not GABAergic) currents in females than in males, which might indicate the engagement of different glutamate receptor types or NMDAR subunits in driving fear reinstatement and M-CPP in females and males\textsuperscript{68,69}.

It was recently suggested that GAD2-Cre and vGlut2-positive VTA→DH terminals similarly regulate the activity of DG granule cells through GABA and glutamate co-release\textsuperscript{29,60}. However, we found that these projections markedly differ in abundance, localization and functionality, and this could account for the profound impact of chemogenetic inactivation of vGlut2-positive, but not GAD2-positive VTA→DH projections, on fear reinstatement and M-CPP. Replication of these findings with additional mouse lines, such as vGat-Cre\textsuperscript{61}, is yet to be performed to strengthen this view. The only behavioral phenotype found by silencing GABAergic VTA afferents in DH was a male specific contribution to morphine-induced locomotion. Because place preference and locomotion are viewed as competing behaviors\textsuperscript{62}, the role of the inhibitory VTA→DH projections in constraining locomotion could synergize with the enhancement of place preference by excitatory VTA→DH projections, and thus coordinate M-CPP in males.

Overall, our findings show that excitatory, non-dopaminergic VTA neurons signal negative and positive valence to DH memory circuits. This could prioritize the retrieval of negative and positive over neutral memories, but also ensure flexible interpretation of contextual stimuli with changing experiences. While such circuit mechanisms have an important adaptive value in organizing threat avoidance and reward approach behaviors under valence congruent conditions, it is likely that excessive bottom-up control of DH by VTA could also drive persistent reinstatement of fear and opioid seeking, which, in humans, might contribute to the development of anxiety disorders and opioid addiction. Moreover, the observed sex differences might prove relevant to the heightened vulnerability of females to these disorders.

**Methods**

**Mice.** We performed the experiments using male and female C57BL/6J mice, vGlut2-Cre, Gad2-Cre, and DAT-Cre mice. Wild type C57BL/6J mice were purchased from Harlan, Indianapolis, IN). All other mouse lines were obtained from the Jackson Laboratory (Bar Harbor, ME). The vGlut2-Cre knockin mice, also known as Slc17a6tm2(cre) and Lowl or VGlut2-ires-Cre, express Cre recombinase in excitatory glutamatergic neuron cell bodies containing vGlut2 (ref.\textsuperscript{83}). The Gad2-Cre mice (Gad2tm2(cre/2j2f);), developed and characterized by Taniguchi et al.\textsuperscript{64}, express Cre in GABAergic neurons, whereas DAT-Cre mice [B6.SJL-Skfs6tm3.1(cre)Bckmn/j], developed and characterized by Backman et al.\textsuperscript{65}, express Cre in dopaminergic neurons containing the DAT.

Heterozygous mice were backcrossed with wild-type C57BL/6J for six generations in our facility to achieve offspring with a genetic identity which is closer to the C57BL/6J strain. The colony was subsequently expanded by homzygous breeding and confirmed by genotyping using the primers and protocols posted on the Jackson Laboratory website (vGlut2: common, CCG TAC CAC AAA ATC GTA CGG, mutant reverse ATC GAC CAA TAA TGG ACC; wild type reverse CAT GGT CGT TTT TTA TAG ATT CAG; Gad2-Cre: common AAC AGT TTT GTG AGT CAG GTG A, mutant forward CAC TGC ATT CTA ATT GTG TGG TGT GTG, wild type forward CCT TGG CAC TGA CGT TCT; DAT-Cre: commonTGG CTC TGG TGT GAA AGT; mutant reverse CCA AAA GAC GGC AAT ATG GT, wild type reverse GGA CAG GGA CAT GGT TGA CT. Typically, we obtained 4–6/l breeding cycle with 5–8 mice/l with similar...
distribution of males and females. All mice were 8 weeks of age at the beginning of the experiments. The mice were maintained under standard housing conditions (12/12 h light/dark cycle) in our satellite behavioral facility. All behavioral experiments were performed between 9 a.m. and 5 p.m. Randomization was performed by assigning similar numbers of littermates to the different treatment conditions. The behavioral experiments were performed by two experimenters of which one was blind to genotypes and drug treatments. All animal procedures used in this study were approved by the Northwestern University IACUC and complied with federal regulations set forth by the National Institutes of Health.

**Contextual fear conditioning, extinction, and reinstatement.** Contextual fear conditioning was performed in an automated system (TSE Systems)\(^6\). Mice were exposed for 3 min to a novel context, followed by a footshock (2 s, 0.7 mA, constant current), and tested for memory retrieval 24 h later by returning them to the conditioning context for 3 min. Freezing was scored every 10 s during all exposures and expressed as a percentage of the total number of observations during which the mice were motionless. Activity was recorded automatically by an infrared beam system and expressed as cm/s. Extinction trials consisted of daily exposures of mice to the conditioning context for 3 min without footshock. Criteria for successful extinction of fear was significant decrease of average group freezing during three successive exposures to the context when compared with the first extinction trial, as determined by repeated measures ANOVA. After the last extinction trial, the mice were split into experimental and control groups so that the mean % freezing/group would be similar while randomizing the distribution of littermates. Fear extinction tests were performed 1 week after the last extinction trial, at a rate of 5 s per footshock. A small amount of medical device glue was applied to the needle’s holder on the skin and the body of the device to connect to the arm of a stereotaxic stage for the device was used to connect to the arm of a stereotaxic stage for holding the needle and the body of the device. A small amount of medical device glue was applied to the skin over and closing it with Kwik-sil tissue glue. The opto-stimulation device was affixed to the skull using dental cement (Stoelting). For optogenetic fear reinstatement, instead of exposure to a reminder shock, mice were exposed to a wirelessly powered blue light photostimulation (473 nm, 3 W, 20 Hz) using 2 ms 4 Hz or 20 Hz pulses. In one experiment, 4 Hz stimulation was performed using optofluidic wireless devices allowing for delivery of an N-methyl-D-aspartate receptor antagonist 2-amino-5-phosphonopentonic acid (APV) or VEH (artificial cerebrospinal fluid).

**Implantation of devices for optogenetic stimulation and optofluidic devices.** All hardware and wireless devices for optogenetic stimulation with or without drug treatment were developed by and obtained from Neuroplus (Urbana, IL)\(^1\). The implantation procedure was performed 1 week before optogenetic stimulation and drug treatment. Mice were anesthetized using isoflurane (2% in O2) for sub-cutaneous implantation of the device. The device was endoscopically implanted into the skull after verifying the device would be positioned to the skull after placing the skin over and closing it with Kwik-sil tissue glue. The optofluidic device was affixed to the skull using dental cement (Stoelting). For optogenetic reinstatement, instead of exposure to a reminder shock, mice were exposed to a wirelessly powered blue light photostimulation (473 nm, 3 W, 20 Hz) using 2 ms 4 Hz or 20 Hz pulses. In one experiment, 4 Hz stimulation was performed using optofluidic wireless devices allowing for delivery of an N-methyl-D-aspartate receptor antagonist 2-amino-5-phosphonopentonic acid (APV) or VEH (artificial cerebrospinal fluid).

**Image analysis and quantification.** For cFos analyses, flat images were obtained using a 10× objective on a Leica microscope with a Leica DFC450 C digital camera. Leica Application Suite software was used for image capturing. Equal light exposure was applied to all images. Images were converted to binary format, and watershed segmentation was used to separate overlapping nuclei. For each section, background was calculated as a difference image. Cell counts from hippocampal subregions were performed using a confocal laser-scanning microscope (Olympus Fluoview FV10). For light microscopy, signals were visualized with diaminobenzidine (Sigma).
above 0.47 (arbitrary units). Counts were then normalized to area. Quantification of VTA-infected neurons was performed using the same approach and included the rostral VTA. Quantification of DH terminals in the hilus of the DG was performed with densitometry using the same area size for each section. For each section, background was calculated as a difference between signals from unlabelled tissue relative to corpus callosum and subtracted from the optical density. All quantifications were completed by an experimenter blind to treatment condition.

### Electrophysiological slice recordings

Four weeks after viral injection into the VTA, male and female mice were anesthetized with isoflurane gas in a desiccated chamber. The brain was removed from the skull in ice-cold ACSF solution (containing (in mM): 125 NaCl, 25 NaHCO3, 2.5 KCl, 1.25 NaH2PO4, 25 glucose, 2 CaCl2, 1 MgCl2, saturated with 95% O2 and 5% CO2) with 3 mM kynurenic acid and cut just caudal to bregma. A total of 300 μm thick coronal slices were cut using a vibro-slicer (Leica VT-1200), stored for ~20 min at 35°C and allowed to recover at room temperature (22–24°C) for at least 30 min in the same solution, but without kynurenic acid.

For recordings, slices were transferred to a recording chamber and continuously superfused with ACSF (without kynurenic acid). Bath temperature was kept between 31 and 32°C using a TC-324B control unit (Warner Instruments). DG granule cells of the DH were visualized using an upright microscope with oblique illumination and video microscopy using a digital camera (DVC). Cells were identified according to location, size, and shape. All recordings were performed using an Axopatch 200B amplifier. Signals were filtered at 2 kHz and sampled at 5 kHz, and all recordings were averaged using pClamp software running on a Mac. Traces were pulled from thick wall borosilicate glass with filament (1.5 mm OD; Sutter Instrument) using a horizontal puller (P97; Sutter Instruments) and were filled with a KCl internal solution consisting of (in mM): 148 KCl, 6 NaCl, 2 MgATP, 0.2 Na2GTP, 10 Hepes, 0.1 EGTA, 10 QX-314, pH 7.3 (with KOH). Pipette resistances of the working solutions ranged from 4 to 6 MΩ, yielding series resistances of 20–30 MΩ.

Cells were voltage-clamped at −70 mV. CH2R stimulation was achieved delivering 1 ms light pulses (using a 450 nm LED light source; Prizmatix) directed to the slice through a 60x, 0.99 NA water immersion objective. The light intensity was normalized in the different recordings. For each recorded cell, Ca2+ current was first established the lowest intensity that would produce a maximal (saturating) response using a 1 ms pulse. This light intensity was then used throughout the experiment. In each cell we first recorded the synaptic response in absence of any synaptic blocker, and then in the presence of either picrotoxin (to measure glutamatergic currents) or kynurenic acid (to measure GABA currents). DAMGO was then added to the bath. In a subset of recordings picrotoxin (for GABA currents) or kynurenic acid (for glutamatergic currents) was finally added to confirm the identity of the recorded current. Current amplitudes were measured as the average (4–7 sweeps) peak response to a single light pulse. A 20–30 s-interval between each stimulation was used to allow for presynaptic recovery and to avoid inducing long lasting synaptic plasticity. Current amplitudes were measured as the peak to baseline immediately preceding the stimulus from the average of 5–7 consecutive sweeps. Traces presented in the figures also represent the average of up to 5–7 sweeps. Decay time constant was measured fitting a single exponential function over a 70 ms time interval following the last stimulation of the peak of the Ca2+ current.

To test the effectiveness of hM4D(Gi) in silencing synaptic transmission in slices, AA8-V10-DIO-hM4D(Gi), and AA5-V5-DIO- hChR2 were infused into DH of either vGlut1- or vGlut2-Cre mice via cannula. The infusion of hM4D(Gi) preceded that of hChR2 by 3 weeks, and 3 more weeks were then allowed for viral expression; thus, hM4D(Gi) was expressed for 6 weeks to be consistent with expression time used in behavioral experiments. DH slices were then prepared, and whole-cell recordings were made from DH terminals, evoking long-lasting synaptic plasticity. Current amplitudes were measured as the peak to baseline immediately preceding the stimulus from the average of 5–7 consecutive sweeps.

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### Data availability

All source data for the preparation of graphs (Figs. 2–7 and Supplementary Figs. 6, 7, 9–11) are provided in a linked Source Data Excel file.

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Author contributions
Y.H. partly and V.S.G., M.A.A.M., and V.J., performed the behavioral analyses, circuit
tracings and manipulations, and immunohistochemical analyses. Y.Z., Y.W., P.G., and J.
A.R. designed the wireless implantable devices for optogenetic stimulation and opto-
fluidic devices, and helped with their in vivo implementation. W.R. and D.J.S. helped
with VTA surgeries and analyses of DAT-Cre mice. M.V.C., A.V.A., and C.G. helped
with the design and performance of CPP experiments. A.G. helped with the animal
breeding and genotyping, behavioral experiments, and tissue processing. H.K. performed
the in vitro electrophysiological recordings. MM designed and analyzed the in vitro
electrophysiological experiments. J.R. designed most of the experiments, helped with the
data analysis, and wrote the manuscript. All authors discussed the findings and com-
mented on the manuscript.

Competing interests
The authors declare no competing interests.

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