VTA glutamatergic inputs to nucleus accumbens drive aversion by acting on GABAergic interneurons

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The ventral tegmental area (VTA) is best known for its dopamine neurons, some of which project to nucleus accumbens (nAcc). However, the VTA also has glutamatergic neurons that project to nAcc. The function of the mesoaccumbens glutamatergic pathway remains unknown. Here we report that nAcc photoactivation of mesoaccumbens glutamatergic fibers promotes aversion. Although we found that these mesoaccumbens glutamatergic fibers lack GABA, the aversion evoked by their photoactivation depended on glutamate- and GABA-receptor signaling, and not on dopamine-receptor signaling. We found that mesoaccumbens glutamatergic fibers established multiple asymmetric synapses on single parvalbumin GABAergic interneurons and that nAcc photoactivation of these fibers drove AMPA-mediated cellular firing of parvalbumin GABAergic interneurons. These parvalbumin GABAergic interneurons in turn inhibited nAcc medium spiny output neurons, thereby controlling inhibitory neurotransmission in nAcc.

To our knowledge, the mesoaccumbens glutamatergic pathway is the first glutamatergic input to nAcc shown to mediate aversion instead of reward, and the first pathway shown to establish excitatory synapses on nAcc parvalbumin GABAergic interneurons.

RESULTS

VGLUT2-expressing mesoaccumbens fibers synapsing in the nAcc

We first targeted VTA glutamatergic neurons and their axons by injecting a Cre-inducible adeno-associated virus (AAV) with a double-floxed inverted open reading frame (DIO) expressing ChR2 fused with enhanced yellow fluorescent protein (eYFP; VGlut2-ChR2-eYFP mice) or mCherry into the VTA of VGlut2::Cre transgenic mice (Fig. 1a). Previously we showed the VTA-selective expression of Cre-inducible reporter genes under the vglut2 promoter in neurons expressing Vglut2 (Slc17a6) mRNA27. Here we targeted viral injections into VTA (Fig. 1b), but because numerous VGlut2 neurons surround the mouse VTA18,28, we first verified that infected neurons were confined to the VTA by analyzing the rostro-caudal expression of eYFP or mCherry (Supplementary Fig. 1). Within nAcc, we found that the fibers expressing eYFP or mCherry under the regulation of the vglut2 promoter were localized predominantly in mShell (Fig. 1c,d) and sometimes extended into the ventromedial shell and olfactory tubercle (Fig. 1c and Supplementary Fig. 2a).

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We next analyzed the characteristics of VTA VGluT2 inputs in nAcc mShell by immunoelectron microscopy. We detected mCherry (from infected VTA VGluT2 neurons) confined to axons and axon terminals. The mCherry axon terminals established asymmetric (excitatory-type) synapses on dendrites (Fig. 1e). These mCherry axon terminals were found to coexpress VGluT2 immunolabel (Fig. 1e), indicating that the VGluT2 fibers from the VTA establish excitatory-type synapses in nAcc mShell.

Behaviors promoted by VGluT2-expressing mesoaccumbens fibers
To assess the behavioral consequence of activation of VTA VGluT2 fibers innervating nAcc mShell (mesoaccumbens glutamatergic inputs), we implanted optical probes bilaterally in nAcc mShell to photostimulate VGluT2-expressing mesoaccumbens fibers in behaving mice (Fig. 2 and Supplementary Fig. 2). VGluT2-ChR2-eYFP and VGluT2-eYFP (control) mice were tested in a three-chamber apparatus and given continuous trains of photostimulation whenever they entered and for as long as they remained in the single photostimulation-paired chamber. VGlut2-eYFP mice spent similar amounts of time in the two chambers, whereas VGluT2-ChR2-eYFP mice spent significantly less time in the photostimulation-paired chamber (Fig. 2c). After 2 d of testing under conditions where the VGluT2-ChR2-eYFP mice controlled the stimulation, the mice were tested in the absence of stimulation; on that day the VGluT2-ChR2-eYFP mice avoided the chamber where they had previously received photostimulation (Fig. 2c and Supplementary Fig. 2c). Thus, VGluT2-ChR2-eYFP mice avoided the photostimulation and acquired a conditioned aversion to the stimulation-paired chamber as reflected by their avoidance of that chamber during the stimulation-free test day. However, the conditioned aversion observed 24 h after the last photostimulation (test 1; Supplementary Fig. 2e) was no longer observed after an additional 24 h (test 2; Supplementary Fig. 2e).

We further determined whether the photostimulation of VGluT2-expressing mesoaccumbens fibers would establish an escape-avoidance response in a negative-reinforcement task. We placed mice in an apparatus featuring two wheels for operant responding. The mice were required to turn either the right wheel (days 1–6) or the left wheel (reversal days 1–4) to terminate (for 2 s) ongoing photostimulation (20 Hz, 0.5 s on/off) (Fig. 2d). VGluT2-ChR2-eYFP mice rotated the ‘active’ wheel (which resulted in photostimulation timeout) significantly more than they did the ‘inactive’ wheel (Fig. 2e) and significantly more than the VGluT2-eYFP mice did during both initial training and reversal training (Fig. 2e). Thus VGluT2-ChR2-eYFP mice discriminated the photostimulation as an aversive stimulus and learned to avoid the aversive stimulation by rotating the appropriate wheel.

We next tested whether photoactivation of VGluT2-expressing mesoaccumbens fibers would disrupt positive reinforcement by a natural reward. Using a two-lever chamber, we trained mice to press either lever to earn food pellets. After a period of stable responses (days 1–3), we changed the conditions so that pressing one of the two levers resulted in nAcc mShell photostimulation of VGluT2-expressing mesoaccumbens fibers (1 s, 20 Hz) along with delivery of a food pellet (Fig. 2f). VGluT2-ChR2-eYFP mice pressed the photostimulation-paired lever significantly less than they pressed the unpaired lever (Fig. 2g). To gain the food pellets, the VGluT2-ChR2-eYFP mice pressed the stimulation-unpaired lever (right lever on days 4 and 5, left lever on days 7 and 8) more often. The VGluT2-ChR2-eYFP mice quickly learned not to press the lever associated with photostimulation.

To determine the conditioned response to the photoinhibition of VGluT2-expressing mesoaccumbens fibers, we delivered an AAV-DIO viral vector expressing halorhodopsin (eNpHR) fused with eYFP into the VTA of VGluT2::Cre mice (VGluT2-eNpHR-eYFP mice). The VGluT2-eNpHR-eYFP and control (VGluT2-eYFP) mice were tested under the place-conditioning paradigm (Fig. 3a,b). We found that neither VGluT2-eNpHR-eYFP mice nor VGluT2-eYFP mice showed preference for or aversion to the photoinhibition-associated chamber (Fig. 3c). To test whether photoinhibition of VGluT2-expressing mesoaccumbens fibers was able to affect aversion resulting from unpredictable foot shock, which is known to induce learned helplessness, we evaluated immobility scores in a forced swim test before and after sessions of inescapable-foot-shock delivery (Fig. 3d). Whereas the VGluT2-eYFP mice showed increased immobility in the forced swim test, VGluT2-eNpHR-eYFP mice subjected to photoinhibition of VGluT2-expressing mesoaccumbens fibers did not, suggesting that the photoinhibition prevented increased immobility of those mice in the forced swim test (Fig. 3e).

Receptors participating in VGluT2 mesoaccumbens aversion
Given that some VTA VGluT2 neurons corelease glutamate and DA, whereas others corelease glutamate and GABA, we next evaluated the effects of intra-nAcc injections of glutamate-receptor, GABA-receptor or DA-receptor antagonists on the aversion driven by photooactivation of VGluT2-expressing mesoaccumbens fibers (Fig. 4).
Ten minutes before being tested in the three-chamber apparatus, different groups of mice received intra-nAcc injections of a mix of NMDA-receptor antagonist (MK-801) and AMPA-receptor antagonist (CNQX), a mix of GABAA-receptor antagonist (bicuculline) and GABAB-receptor antagonist (saclofen), a dopamine D1-receptor antagonist (SCH23390), a D2-receptor antagonist (eticlopride), or a mix of SCH23390 and eticlopride (Fig. 4b). Control mice that received only artificial cerebrospinal fluid (ACSF) avoided the photostimulation-paired chamber on the conditioning day (Fig. 4c). In contrast, mice that received MK-801–CNQX or bicuculline–saclofen did not avoid the chamber where they received photostimulation of VGluT2-expressing mesoaccumbens fibers (Fig. 4c). Thus the aversive effects of activation of the mesoaccumbens glutamatergic pathway were mediated by nAcc signaling of both glutamate and GABA receptors.

We next used a combination of anatomical approaches to explore the possible mechanism by which signaling of glutamate and GABA receptors may contribute to the aversion induced by activation of the mesoaccumbens glutamatergic pathway. Knowing that most VTA VGluT2 neurons projecting to lateral habenula coexpress glutamate and GABA receptors, we determined whether any such neurons project to nAcc mShell. Using a combination of FluoroGold (FG) retrograde tract tracing, immunohistochemistry and dual *in situ* hybridization, we found that most VTA VGluT2 neurons projecting to nAcc mShell lacked Gad65 and Gad67 mRNA (90%...
conditioned place aversion between mice that received intra-nAcc microinjections of VGluT2-eNpHR-eYFP and VGluT2-eYFP mice received nAcc mShell photoinhibition of mesoaccumbens fibers during conditioning days 1 and 2. (c) Neither VGluT2-eNpHR-eYFP mice (n = 11) nor VGluT2-eYFP mice (n = 9) showed preference for or aversion to the chamber where photoinhibition was applied (group × day × chamber interaction: F_{1,108} = 1.072, P = 0.384, three-way ANOVA). Data are presented as mean ± s.e.m. Green bars indicate that photoinhibition was applied in the photoinhibition-associated chamber on those days. (d) Learned helplessness timeline: the forced swim protocol was performed before and after the foot-shock training session, in which mice were subjected to 30 unpredictable shocks (5 s, 0.35 mA; 55-s inter-shock interval) over a period of 30 min in each of five daily sessions. (e) nAcc mShell photoinhibition of mesoaccumbens fibers attenuated foot-shock-induced learned helplessness. Foot shock significantly increased immobility during the forced swim test (compared with pretest values) in VGluT2-eYFP mice (n = 8), but not in VGluT2-eNpHR-eYFP mice (n = 9) (group × day interaction: F_{1,15} = 9.85, P = 0.007, two-way ANOVA; *P < 0.05, Newman–Keuls post hoc test). In box plots, center lines denote medians, edges represent upper and lower quartiles, and whiskers indicate minimum to maximum values.

172 out of 192 VGluT2 neurons; Fig. 5 and Supplementary Fig. 4). The lack of coexpression of Gad65 and Gad67 mRNA in the majority of VGluT2-expressing mesoaccumbens neurons suggests that these neurons do not have the capability to synthesize GABA, and that they might not corelease glutamate and GABA in nAcc mShell. However, recent electrophysiological studies suggest that DA-axon terminals may release GABA in the striatum, even in the absence of Gad65 and Gad67 (ref. 30). Because we found that more than half of the VGluT2-expressing mesoaccumbens neurons coexpressed TH (64%; 122 out of 192; Fig. 5 and Supplementary Fig. 4), we sought to determine the extent to which these glutamatergic-dopaminergic (VGluT2-TM) mesoaccumbens neurons contribute to aversion. We compared conditioned place aversion between mice that received intra-nAcc injections of vehicle and those injected with the dopaminergic neurotoxin 6-hydroxodopamine (6-OHDA). We confirmed degeneration of DA axons by post hoc brain analysis after behavioral testing. Animals treated with 6-OHDA showed loss of TH immunoreactivity in nAcc mShell without effects on aversion induced by activation of the mesoaccumbens glutamatergic pathway (Supplementary Fig. 5). These results suggest a lack of a major participation of VTA VGluT2-TH neurons in mediating aversion induced by photoactivation of VGluT2-expressing mesoaccumbens fibers.

To directly test the possible involvement of mesoaccumbens GABA neurons in place aversion, we inducedChr2-eYFP expression in VTA neurons under the regulation of the vesicular GABA transporter (vgat) promoter (VGAT-Chr2-eYFP mice; Supplementary Fig. 6a,b). In contrast to the high number of VGluT2-expressing mesoaccumbens fibers observed in nAcc mShell (Fig. 1c and Supplementary Fig. 2a), VGAT mesoaccumbens fibers were sparse in both the shell and the core of nAcc (Supplementary Fig. 6c–e), as recently reported31.

### Figure 4 Photostimulation of nAcc mShell fibers from VTA VGluT2 neurons results in place aversion mediated by glutamate receptors and GABA receptors. (a) Diagram of virus injection in VTA, intra-nAcc microinjections of receptor antagonists and nAcc photostimulation of VTA VGluT2 inputs. (b) Experimental timeline. VGluT2-Chr2-eYFP mice received intra-nAcc microinjections of antagonists of glutamate receptors (MK-801 + CNQX), GABA receptors (bicuculline + saclofen), D1 receptor (SCH23390), D2 receptor (eticlopride), or both D1 and D2 receptors 10 min before photostimulation. (c) Effects of intra-nAcc microinjections of antagonists for glutamate receptors (n = 6), GABA receptors (n = 7), D1 receptor (n = 7), D2 receptor (n = 7) or both D1 and D2 receptors (n = 6) on place aversion induced by nAcc mShell photostimulation. Mice that received intra-nAcc ACSF injections (n = 11) avoided the photostimulation-paired chamber on training day (*P < 0.05). Microinjection of glutamate receptor or GABA-receptor antagonists blocked photostimulation-induced place aversion, whereas injection of dopamine-receptor antagonists did not (main effect group, F_{1,38} = 1.35, P = 0.266; group × chamber interaction, F_{10,38} = 1.7, P = 0.096; two-way ANOVA, *P < 0.05, Newman–Keuls post hoc test). In box plots, center lines denote medians, edges represent upper and lower quartiles, and whiskers indicate minimum to maximum values.
Photoactivation of these VGAT mesoaccumbens fibers did not induce place aversion, place preference (Supplementary Fig. 6f,g) or change in locomotion (Supplementary Fig. 6h). Thus findings from these studies together with those obtained from 6-OHDA lesions suggest that GABA release from mesoaccumbens fibers does not mediate the signaling of GABA receptors in nAcc.

**VGLUT2-expressing mesoaccumbens fibers activate PV interneurons**

Considering that the VGLUT2-expressing mesoaccumbens fibers did not seem to corelease glutamate and GABA, we hypothesized that VGLUT2-expressing mesoaccumbens fibers synapsing on nAcc GABAergic interneurons induce the release of GABA, accounting for activation of nAcc GABA receptors evoked by nAcc photostimulation of VGLUT2-expressing mesoaccumbens fibers. To test this hypothesis, we determined whether photoactivation of VGLUT2-expressing mesoaccumbens fibers induced expression of c-Fos (a marker of neuronal activation) in nAcc GABAergic interneurons (PV or nitric oxide synthase (NOS) interneurons) and found that most of them (81.56%) expressed c-Fos. Considering that the VGLUT2-expressing mesoaccumbens fibers did not seem to corelease glutamate and GABA, we hypothesized that VGLUT2-expressing mesoaccumbens fibers activate PV interneurons (Supplementary Fig. 7 and Supplementary Table 1). In contrast to observations for c-Fos induction in PV GABAergic interneurons in nAcc of VGLUT2-ChR2-eYFP mice, the number of MSNs coexpressing c-Fos was lower in these mice than in the control (VGLUT2-eYFP) mice (Supplementary Table 2), suggesting that activation of PV GABAergic interneurons affects c-Fos expression in MSNs. Because c-Fos induction was preferentially observed in PV GABAergic interneurons, we next determined whether VGLUT2-expressing mesoaccumbens fibers synapse on PV GABAergic interneurons.

Using confocal microscopy, we detected mCherry-VGLUT2 immunofluorescence varicosities in nAcc mShell. After three-dimensional reconstruction of mCherry-VGLUT2 immunofluorescence varicosities, we determined that they made contacts along the proximal and distal aspects of individual PV dendrites or around the PV cell body (Fig. 6). We analyzed the distribution of 447 of these dual mCherry-VGLUT2 immunofluorescence varicosities contacting PV interneurons and found that most of them (81.56% ± 1.03%) made contacts on PV dendrites, with the remainder (18.44% ± 1.03%) making contact on PV cell bodies ($t_{1}=30.72$, $P<0.01$). Using immunoelectron microscopy and serial sectioning, we confirmed that dual mCherry-VGLUT2 varicosities corresponded to axon terminals. These mCherry-VGLUT2 axon terminals formed multiple asymmetric (excitatory-type) synapses with a single PV dendrite (Fig. 6d–g) or with a single PV cell body (Fig. 6i–k). We analyzed 138 of these mCherry-VGLUT2 terminals making asymmetric synapses on PV interneurons and found that most of them (79.98% ± 6.50%) synapsed on PV dendrites and that the remainder (20.02% ± 6.50%) synapsed on PV cell bodies ($t_{1}=4.613$, $P=0.0192$). In a separate study, we found that synapses between VGLUT2 axon terminals and PV interneurons were maintained after the development of nAcc 6-OHDA lesions (Supplementary Fig. 6h).

**Figure 5** The majority of VTA VGluT2 neurons projecting to nAcc mShell lack Gad65 and Gad67 mRNA. (a) The retrograde tracer FluoroGold (FG) was delivered into nAcc mShell. (b) FG immunoreactivity was confined at nAcc mShell. (c–f) VTA detection of FG neurons (red; indicated by a single arrow, a double arrow and an arrowhead in c). Vglut2 mRNA expression (white aggregated grains; d) and TH (green; e). Scale bar in e applies to c and d. (f) Four phenotypes of FG neurons coexpressing Vglut2 mRNA are seen in VTA: FG-VGluT2 neurons that do not express either Gad65 and Gad67 mRNA or TH (single arrows); FG-VGluT2 neurons coexpressing TH (double arrows); and FG-VGluT2 neurons coexpressing Gad65 and Gad67 mRNA (arrowheads). Scale bar applies to all images in panel. (g) Frequency of the four phenotypes of VTA VGluT2 neurons innervating the nAcc mShell (mean ± s.e.m.). Among VTA VGluT2 neurons innervating nAcc mShell, 26.3% ± 0.8% did not express either Gad65 and Gad67 mRNA or TH, 63.6% ± 0.3% coexpressed TH, 7.8% ± 2.5% coexpressed Gad65 and Gad67 mRNA, and 2.24% ± 1.6% coexpressed both Gad65 and Gad67 mRNA and TH. Neurons were counted from bregma −3.08 mm to −3.88 mm ($n=3$ mice, 12–13 sections per mouse). Each symbol represents a single mouse; horizontal bars and error bars indicate mean and s.e.m., respectively. GAD, glutamate acid decarboxylase. Scale bars, (b) 250 µm, (e) 100 µm or (f) 10 µm.
Figure 6 A single nAcc mShell PV interneuron forms asymmetric synapses with several axon terminals from VTA vGlut2 neurons. (a) Diagram of fibers from VTA neurons expressing mCherry (under vglut2-promoter regulation) innervating the nAcc mShell (mesoaccumbens axons). (b,c) Varicosities from a mesoaccumbens axon coexpressing mCherry (red) and VGlut2 (green; arrowheads) are in contact with a dendrite (arrows) containing PV (blue) (b). Contacts are better seen after 3D reconstruction (c). Scale bar in b applies to all images in the panel. (d,e) Electron micrograph (d) and corresponding diagram (e) showing detection of PV (gold particles; blue arrowheads) in a dendrite (outlined in blue) that establishes synaptic contacts with four mesoaccumbens axon terminals (AT1–AT4) coexpressing mCherry (scattered dark material) and VGlut2 (gold particles). (f,g) Asymmetric synapses (green arrows) between a PV dendrite and both AT2 (f) and AT3 (g) in serial sections. Blue arrowheads, PV; green arrowheads, VGlut2. (h) Varicosities from a mesoaccumbens axon coexpressing mCherry (red) and VGlut2 (green) are in contact (arrowheads) with the cell body of a PV interneuron (blue). Scale bar applies to all images in the panel. (i–k) A cell body expressing PV (gold particles; blue arrowheads) forming an asymmetric synapse (green arrows) with two mesoaccumbens terminals (AT1 and AT3) coexpressing mCherry (scattered dark material) and VGlut2 (gold particles; green arrowheads). Scale bars, (b,h) 2 μm, (d) 500 nm or (f,g,k) 200 nm.

Fig. 5d,e), suggesting a major input to PV neurons from VGlut2-only neurons. The detection of multiple terminals from VGlut2-expressing mesoaccumbens fibers containing VGlut2 and making asymmetric synapses on individual PV neurons suggested that VGlut2-expressing mesoaccumbens fibers provide strong monosynaptic excitatory regulation of PV GABAergic interneurons. On the basis of these anatomical observations, we hypothesized that nAcc activation of mesoaccumbens fibers evokes firing of PV interneurons. To test this hypothesis, we recorded the response of nAcc PV interneurons to optical activation of mesoaccumbens fibers (Fig. 7).

Because the number of nAcc PV interneurons is very small, we obtained cellular recordings in nAcc slices from PV interneurons expressing eYFP under the regulation of the pv promoter (Fig. 7a–c). Photoactivation of mesoaccumbens fibers evoked EPSCs in fluorescent PV neurons (Fig. 7a–c), which were characterized as fast-spiking interneurons (Fig. 7d and Supplementary Fig. 8). A single pulse of photoactivation of mesoaccumbens fibers elicited potential firing in nAcc PV interneurons (Fig. 7e), the amplitude of which was not significantly affected by bicuculline but was blocked by CNQX (Fig. 7f). Given that photoactivation of mesoaccumbens fibers elicited the firing of PV interneurons, and because it is well established that PV interneurons provide feed-forward inhibition to MSNs, we determined whether photoactivation of mesoaccumbens fibers induces polysynaptic GABA release onto MSNs. To discriminate direct excitatory inputs from mesoaccumbens fibers and indirect local inhibitory inputs onto MSNs, we recorded nAcc shell MSNs at a holding potential of ~70 mV or ~45 mV (Supplementary Fig. 9). Photoactivation of mesoaccumbens fibers evoked inward currents (EPSCs) in MSNs when recorded at ~70 mV (Supplementary Fig. 9b) and fast inward currents followed by delayed outward currents (inhibitory postsynaptic currents (IPSCs)) when recorded at ~45mV (Supplementary Fig. 9c). Bath application of bicuculline blocked the IPSCs without affecting the EPSCs (Supplementary Fig. 9c). In contrast, CNQX application alone or in combination with bicuculline blocked both EPSCs and IPSCs, indicating that the IPSCs depended on the activation of glutamate receptors (Supplementary Fig. 9c,d) and suggesting that photoactivation of mesoaccumbens fibers evokes polysynaptic release of GABA from PV interneurons onto MSNs (Supplementary Fig. 9e).

Activation of nAcc PV interneurons promotes place aversion
To directly examine the participation of nAcc PV GABAergic interneurons in aversion, we delivered the viral vector AAV-DIO-Chr2-eYFP into the nAcc of PV::Cre mice (PV-Chr2-eYFP mice). PV-Chr2-eYFP and control (PV-eYFP) mice were tested under the place-conditioning paradigm (Supplementary Fig. 10). Whereas photostimulation of nAcc had no effects on place preference in control mice, the PV-Chr2-eYFP mice not only avoided the place where the stimulation was given when the stimulation was present, but also avoided the chamber where they had previously received the photostimulation (Supplementary Fig. 10f) when no stimulation was given. Thus, PV-Chr2-eYFP mice avoided the photostimulation and acquired a conditioned aversion to the stimulation-paired chamber.

DISCUSSION
The nAcc consists of a major population of projection MSNs (95% of nAcc neurons) and a smaller population of GABAergic and cholinergic...
interneurons, of which PV GABAergic interneurons constitute around 3% (ref. 33). Here we provide converging evidence showing that PV GABAergic interneurons are activated in the nAcc shell by glutamatergic inputs from VTA neurons. We found that activation of these glutamatergic mesoaccumbens inputs evoked conditioned aversion in mice that was mediated by activation of both glutamate and GABA receptors but was independent of the activation of DA receptors. Because we found that the vast majority of glutamatergic mesoaccumbens neurons lack GABAergic markers, we propose that VTA glutamatergic inputs drive aversion by a mechanism in which MSN activity is inhibited by the release of GABA from PV interneurons, which are excited by VTA glutamatergic inputs. We provide evidence that activation of VTA glutamatergic inputs to the nAcc is aversive rather than rewarding.

**VTA VGluT2-only neurons control nAcc PV interneurons**

Converging evidence has shown that although all VTA glutamatergic neurons selectively express Vglut2 mRNA, they are diverse in their biochemical composition and connectivity18,27,29,34. The diversity among VTA VGluT2 neurons has led to the suggestion that these neurons participate in multiplexed neurochemical signaling35. A major class of VTA VGluT2 neurons lacks both TH and GABAergic markers (VGluT2-only neurons)18. A minor class of VTA VGluT2 neurons coexpresses TH (VGluT2-TH neurons), and some of these coexpress molecules necessary for DA synthesis and DA vesicular transport34. In another minor class of VTA VGluT2 neurons coexpressing GABAergic markers, a single axon terminal coreleases glutamate and GABA, and these constitute the major mesohabenular input to lateral habenula glutamatergic neurons29. We found here that the VGluT2 GABAergic neurons rarely innervate nAcc mShell, and that VGluT2 neurons (more than half of them coexpressing TH) constitute nearly one-quarter of the mesoaccumbens neurons targeting nAcc mShell. Thus there is a possibility that excitatory inputs from both VTA VGluT2-only neurons and VTA VGluT2-TH neurons may contribute to the aversion mediated by excitation of glutamatergic mesoaccumbens fibers synapsing on nAcc PV GABAergic interneurons. However, we found that nAcc degeneration of DA axons did not eliminate synapsing of glutamatergic mesoaccumbens fibers on PV GABAergic interneurons. Moreover, nAcc degeneration of DA axons did not eliminate conditioned aversion induced by activation of glutamatergic mesoaccumbens fibers. Thus we propose that the glutamatergic mesoaccumbens fibers synapsing on nAcc PV GABAergic interneurons are mostly from VTA VGluT2-only neurons, rather than from VTA VGluT2-TH neurons.

The glutamatergic mesoaccumbens pathway shown here to form asymmetric synapses on PV GABAergic interneurons is to our knowledge the first anatomically identified glutamatergic pathway to GABAergic interneurons in the nAcc. In contrast, it is well established that cortical glutamatergic inputs from motor and somatosensory cortices synapse on dorsal striatum PV GABAergic interneurons and regulate the activity of those interneurons35,36. On the basis of the ultrastructural and electrophysiological findings reported here, we propose that VTA VGluT2 neurons serve as a major source of glutamatergic control over nAcc PV GABAergic interneurons. At the ultrastructural level, we found that a single dendrite from a PV GABAergic interneuron could establish asymmetric synapses with multiple glutamatergic mesoaccumbens terminals, which contain VGluT2 for the vesicular accumulation and release of glutamate. In addition to dendrites, the cell bodies of nAcc PV GABAergic interneurons established asymmetric synapses with glutamatergic mesoaccumbens terminals. These findings indicate that the glutamatergic mesoaccumbens pathway provides to a single nAcc PV GABAergic interneuron multiple synaptic sites for glutamate release along its dendrites and cell body. In addition, we have demonstrated that nAcc photoactivation of mesoaccumbens inputs drives glutamate-receptor-mediated firing of nAcc PV GABAergic interneurons and the in vivo expression of c-Fos in nAcc PV GABAergic interneurons. Prior studies established that the synaptic potential generated from a single PV GABAergic interneuron is able to delay or block the generation of action potentials in a large number of postsynaptic MSNs37.
This powerful regulation is due to the multiple synapses that a single PV interneuron establishes with MSNs, and by the electrotonic synapses among PV interneurons. GABAergic interneurons provide feed-forward inhibition that regulates spike timing in MSNs, thereby regulating striatal output. Here we show that activation of glutamatergic inputs from the VTA innervating nAcc PV GABAergic interneurons releases GABA onto MSNs and induces aversion. This demonstration that glutamatergic mesoaccumbens fibers synapsing on PV interneurons drive aversion is, to our knowledge, the first evidence suggesting a role for nAcc GABAergic interneurons in aversion. Although there are collaterals between nAcc MSN neurons, the major inhibitory control on MSNs is from the PV GABAergic interneurons, which are the predominant target of the VTA-nAcc glutamate projection.

**VGluT2-TH neurons control MSNs and cholinergic interneurons**

Recent neurochemical and ultrastructural studies have shown that VGlut2-TH mesoaccumbens fibers release DA and glutamate from different pools of vesicles. Within these dual VGlut2-TH axons, the VGlut2 vesicles are concentrated in axon terminals that form asymmetric synapses on dendritic spines of MSNs. Although a small number of VGlut2-TH neurons innervate the nAcc, nAcc activation of fibers from these VGlut2-TH neurons reportedly evokes small EPSCs in each tested nAcc MSN without resulting in the firing of these neurons. These electrophysiological findings suggest that VGlut2-TH axon terminals establish infrequent synapses on individual MSNs. Although it is likely that nAcc photoactivation of mesoaccumbens fibers expressing ChR2 under the vglut2 promoter resulted in activation of mesoaccumbens fibers from both VTA VGlut2-only and VTA VGlut2-TH neurons in the current study, this activation did not elicit reward. The lack of reward elicited by activation of glutamatergic mesoaccumbens inputs may be explained in part by the fact that this activation alone does not result in the firing of MSNs. However, it is also conceivable that GABA release from concomitant mesoaccumbens activation of PV interneurons may diminish the glutamatergic mesoaccumbens effects on MSNs (shown in the present study), resulting in induction of a general inhibition of MSN outputs and the mediation of aversion.

*In vitro* electrophysiological studies have shown that, in addition to MSNs, nAcc activation of VGlut2-TH fibers evokes EPSCs on cholinergic interneurons in nAcc mShell. However, in *in vivo* optical stimulation, we did not detect c-Fos induction in nAcc cholinergic interneurons. It is unclear whether the lack of c-Fos induction was due to neuronal network conditions or the postburst hyperpolarization (mediated by SK channels and increased by DA and muscarinic receptors) that cholinergic interneurons experience after activation of VGlut2-TH fibers. The activation of DA receptors, which augments postburst hyperpolarization, may be mediated by the corelease of DA with glutamate from glutamate-DA fibers or by DA release known to be evoked by the firing of cholinergic interneurons. We cannot exclude the possibility that the lack of c-Fos detected in cholinergic interneurons was due to the *in vivo* optical-evoked GABA release from PV GABAergic interneurons, this seems unlikely, as striatal PV GABAergic interneurons do not seem to establish synaptic contacts with cholinergic interneurons.

**A role for nAcc PV GABAergic interneurons in aversion**

Although it is well established that nAcc PV interneurons have a crucial role in the regulation of nAcc neuronal activity, until now there has been a lack of information on the inputs that regulate the activity of these interneurons and their possible roles in behavior. Here we demonstrate that aversion is elicited by direct photoactivation of nAcc PV interneurons (expressing ChR2, under the regulation of the pv promoter). We have shown that these interneurons are a major target of VTA glutamatergic efferents and that the activation of these efferents synapsing on PV GABAergic interneurons releases GABA onto MSNs.

Accumulating evidence has shown biochemical heterogeneity among VTA glutamatergic neurons, leading to the suggestion that VTA glutamatergic neurons participate in multiplexed neurochemical signaling by releasing different neurotransmitters. Although the extent to which the different identified types of VTA glutamatergic neurons participate in diverse behaviors is unclear, emerging evidence shows that subsets of VTA glutamatergic neurons participate in either aversion or reward. Whereas aversion is driven by activation of VTA glutamatergic fibers in either lateral habenula or nAcc (present study), reward is driven by activation of VTA glutamatergic neurons. The aversion driven by mesohabenular glutamatergic fibers in lateral habenula involves activation of glutamate receptors located in glutamatergic neurons, whereas the aversion driven by glutamatergic mesoaccumbens fibers in nAcc involves the activation of glutamate receptors located in PV GABAergic interneurons. In contrast, the reward driven by activation of glutamatergic neurons in the VTA involves the activation of glutamate receptors located in neighboring DA neurons that innervate the nAcc. Thus it seems that the participation of VTA glutamatergic neurons in aversion or reward might depend not only on the phenotype of the neurons, but also on the cellular phenotype of their postsynaptic targets.

**METHODS**

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

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**AUTHOR CONTRIBUTIONS**

M.M. conceptualized the project. J.Q. performed behavioral and pharmacological studies. J.Q. and D.J.B. analyzed data from behavioral experiments. J.Q., S.Z., and H.-L.W. performed neuroanatomy and immunolabeling studies. H.-L.W. performed *in situ* hybridization. J.Q. and S.Z. performed confocal microscopy studies. S.Z. performed electron microscopy studies. J.M.-B. performed electrophysiological studies. All authors participated in conception, experimental design and data interpretation. M.M. wrote the manuscript with the participation of all other authors.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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1. Carlezon, W.A. Jr. & Thomas, M.I. Biological substrates of reward and aversion: a nucleus accumbens activity hypothesis. *Neuropsychopharmacology* 56, 122–132 (2009).
2. Todtenkopf, M.S. *et al.* Brain reward regulated by AMPA receptor subunits in nucleus accumbens shell. *J. Neurosci.* 26, 11665–11669 (2006).
3. Britt, J.P. *et al.* Synaptic and behavioral profile of multiple glutamatergic inputs to the nucleus accumbens. *Neuron* 76, 790–803 (2012).
4. Friedman, D.P., Aggleton, J.P. & Saunders, R.C. Comparison of hippocampal, amygdala, and perirhinal projections to the nucleus accumbens: combined anterograde and retrograde tracing study in the Macaque brain. *J. Comp. Neurol.* 450, 345–365 (2002).
5. Grace, A.A., Floresco, S.B., Goto, Y. & Lodge, D.J. Regulation of firing of dopamine neurons and control of goal-directed behaviors. Trends Neurosci. 30, 220–227 (2007).

6. Kelley, A.E. Memory and addiction: shared neural circuitry and molecular mechanisms. Neuron 44, 161–179 (2004).

7. O’Dell, S. & Grace, A.A. Synaptic interactions among excitatory afferents to nucleus accumbens neurons: hippocampal gating of prefrontal cortical input. J. Neurosci. 15, 3622–3639 (1995).

8. Phillips, P.E., Stuber, G.D., Heien, M.L., Wightman, R.M. & Carelli, R.M. Subsecond dopamine release promotes cocaine seeking. Nature 422, 614–618 (2003).

9. Stuber, G.D. et al. Reward-predictive cues enhance excitatory synaptic strength onto midbrain dopamine neurons. Science 321, 1690–1692 (2008).

10. Sesa, S.R., Carr, D.B., Omelchenko, N. & Pinto, A. Anatomical substrates for glutamate-dopamine interactions: evidence for specificity of connections and extrasynaptic actions. Ann. NY Acad. Sci. 1003, 36–52 (2003).

11. Papp, E. et al. Glutamatergic input from specific sources influences the nucleus accumbens–ventral pallidum information flow. Brain Struct. Funct. 217, 37–48 (2012).

12. Stuber, G.D. et al. Excitatory transmission from the amygdala to nucleus accumbens facilitates reward seeking. Nature 475, 377–380 (2011).

13. Morales, M. & Root, D.H. Glutamate neurons within the midbrain dopamine regions. Neuroscience 282C, 60–68 (2014).

14. Stuber, G.D., Hinasko, T.S., Britt, J.P., Edwards, R.H. & Bonci, A. Dopaminergic terminals in the nucleus accumbens but not the dorsal striatum corelease glutamate. J. Neurosci. 30, 8229–8233 (2010).

15. Tecuapetla, F. et al. Glutamatergic signaling by mesolimbic dopamine neurons in the nucleus accumbens. J. Neurosci. 30, 7105–7110 (2010).

16. Van Bockstaele, E.J. & Pickel, V.M. GABA-containing neurons in the ventral tegmental area project to the nucleus accumbens in rat brain. Brain Res. 682, 215–221 (1995).

17. van Zeijen, P., Phillips, J.L., Budgijn, E.A. & Stuber, G.D. Activation of VTA GABA neurons disrupts reward consumption. Neuron 73, 1184–1194 (2012).

18. Yamaguchi, T., Sheen, W. & Morales, M. Glutamatergic neurons are present in the rat ventral tegmental area. Neuron 35, 581–592 (2002).

19. Zhang, S. et al. Multiplexed neurochemical signaling by mesolimbic dopamine neurons in the ventral midbrain. Eur. J. Neurosci. 28, 60–68 (2008).

20. Papp, E. et al. Glutamatergic signaling by mesolimbic dopamine neurons in the rat dorsal striatum. Eur. J. Neurosci. 28, 1529–1540 (2013).

21. Brown, M.T. et al. Ventral tegmental area GABA projections pause accumbal cholinergic interneurons to enhance associative learning. Nature 492, 452–456 (2012).

22. Kawano, M. et al. Particular subpopulations of midbrain and hypothalamic dopamine neurons express vesicular glutamate transporter 2 in the rat brain. J. Comp. Neurol. 498, 581–592 (2006).

23. Trude, U.E. et al. The multilingual nature of dopamine neurons. Prog. Brain Res. 211, 141–164 (2014).

24. Yamaguchi, T., Sheen, W. & Morales, M. Glutamatergic neurons are present in the rat ventral tegmental area. J. Neurosci. 25, 105–116 (2005).

25. Chelum, N., Minagawa, S., Moore, H. & Rayport, S. Dopamine neurons control striatal cholinergic neurons via regionally heterogeneous dopamine and glutamate signaling. Neuron 81, 901–912 (2014).

26. Zhang, S. et al. Dopaminergic and glutamatergic microdomains in a subset of rodent mesoaccumbens axons. Nat. Neurosci. 18, 386–392 (2015).

27. Root, D.H., Meijas-Aponte, C.A., Qi, J. & Morales, M. Role of glutamatergic projections from ventral tegmental area with lateral habenula in aversive conditioning. J. Neurosci. 34, 13906–13910 (2014).

28. Yamaguchi, T., Qi, J., Wang, H.L., Zhang, S. & Morales, M. Glutamatergic and dopaminergic neurons in the mouse ventral tegmental area. Eur. J. Neurosci. 41, 1776–1787 (2015).

29. Root, D.H. et al. Single rodent mesohabenular axons release glutamate and GABA. Nat. Neurosci. 17, 1543–1551 (2014).

30. Tisch, N.X., Ding, J.B. & Sabatini, B.L. Dopaminergic neurons inhibit striatal output through non-canonical release of GABA. Nature 490, 262–266 (2012).

31. Taylor, S.R. et al. GABAergic and glutamatergic efferents of the mouse ventral tegmental area. J. Comp. Neurol. 552, 3308–3334 (2014).

32. Kawaguchi, Y., Wilson, C.J., Augood, S.J. & Emson, P.C. Striatal interneurons: chemical, physiological and morphological characterization. Trends Neurosci. 18, 527–539 (1995).

33. Kita, H., Kosaka, T. & Heizmann, C.W. Parvalbumin-immunoreactive neurons in the rat neostriatum: a light and electron microscopic study. Brain Res. 536, 1–15 (1990).

34. Li, X., Qi, J., Yamaguchi, T., Wang, H.L. & Morales, M. Heterogeneous composition of dopamine neurons of the rat A10 region: molecular evidence for diverse signaling properties. Brain Struct. Funct. 218, 1159–1176 (2013).

35. Parthsarathy, H.B. & Graybiel, A.M. Cortically driven immediate-early gene expression reflects modular influence of sensorimotor cortex on identified striatal neurons in the squirrel monkey. J. Neurosci. 20, 2477–2491 (1997).

36. Ramanathan, S., Hanley, J.J., Deniau, J.M. & Bolam, J.P. Synaptic convergence of motor and somatosensory cortical afferents onto GABAergic interneurons in the rat striatum. J. Neurosci. 22, 8156–8168 (2002).

37. Tepper, J.M., Wilson, C.J. & Koss, T. Feedback and feedforward inhibition in neostriatal GABAergic spiny neurons. Brain Res. Rev. 58, 272–281 (2008).

38. Koss, T. & Tepper, J.M. Inhibitory control of neostriatal projection neurons by GABAergic interneurons. Nat. Neurosci. 2, 467–472 (1999).

39. Gittis, A.H., Nelson, A.B., Thwin, M.T., Palop, J.J. & Kreitzer, A.C. Distinct roles of GABAergic interneurons in the regulation of striatal output pathways. J. Neurosci. 30, 2223–2234 (2010).

40. Planert, H., Szydrowski, S.N., Hjorth, J.J., Griller, S. & Silberberg, G. Dynamics of synaptic transmission between fast-spiking interneurons and striatal projection neurons of the direct and indirect pathways. J. Neurosci. 30, 3499–3507 (2010).

41. Taverna, S., Canciani, B. & Pernazz, C.M. Membrane properties and synaptic connectivity of fast-spiking interneurons in rat ventral striatum. Brain Res. 1152, 49–56 (2007).

42. Koos, T., Tecuapetla, F. & Tepper, J.M. Glutamatergic signaling by midbrain dopaminergic neurons: recent insights from optogenetic, molecular and behavioral studies. Curr. Opin. Neurobiol. 21, 393–401 (2011).

43. Cachope, R. et al. Selective activation of cholinergic interneurons enhances accumbal phasic dopamine release: setting the tone for reward processing. Cell Rep. 2, 33–41 (2012).

44. McNeil, S. et al. Striatal dopamine release is triggered by synchronized activity in cholinergic interneurons. Neuron 75, 58–64 (2012).

45. Chang, H.T. & Kita, H. Interneurons in the rat striatum: relationships between parvalbumin neurons and cholinergic neurons. Brain Res. 574, 307–311 (1992).

46. Bernal, K., Root, D.H., Zhang, S. & Morales, M. Multiplexed neurochemical signaling by neurons of the ventral tegmental area. J. Chem. Neuroanat. http://dx.doi.org/10.1016/j.jchemneu.2012.05.010 (4 January 2012).

47. Wang, H.L., Qi, J., Zhang, S., Wang, H. & Morales, M. Rewarding effects of optical stimulation of ventral tegmental area glutamatergic neurons. J. Neurosci. 35, 15948–15954 (2015).
Methods were described previously. Briefly, vibratome tissue sections were rinsed and incubated with 1% sodium borohydride to inactivate free aldehyde groups, rinsed and then incubated with blocking solution. Sections were then incubated with primary antibodies (mouse anti-mCherry (1:1,000, Clontech Laboratories, 625243), guinea pig anti-VGluT2 (1:500, Frontier Institute, VGluT2-GP-AF400-1) and rabbit anti-PV (1:500, Frontier Institute, PV-Rb-AF750)). All primary antibodies were diluted with 1% normal goat serum, 4% BSA in phosphate buffer (PB) supplemented with 0.02% saponin. Incubations were for 24 h at 4 °C. Sections were rinsed and incubated overnight at 4 °C in the corresponding secondary antibodies. Sections were rinsed in PB and then in double-distilled water, and rinsing was followed by silver enhancement of the gold particles with the Nanoprobe silver kit (2012, Nanoprobes) for 7 min at room temperature. Next, sections were incubated in avidin-biotinylated horseradish peroxidase complex in PB (1:1,000, Vector Laboratories, PK-6100) for 30 min, washed with PB, incubated with 0.003% DAB and 0.01% H2O2 in PB, washed, and then incubated with blocking solution. Finally, sections were incubated with gold-conjugated secondary antibodies (1:500, Alexa Fluor 594–anti-mCherry, Alexa Flour 488–anti–guinea pig + Alexa Fluor 594–anti-mouse + Alexa Flour 647–anti-Rb) for 2 h at room temperature. After rinsing, sections were mounted on slides. Fluorescent images were collected with an Olympus BX51 Confocal System (Olympus). Images were taken sequentially with different lasers with 100× oil-immersion objectives, and z-axes stacks were collected at 0.2 µm. Imaris microscopy software (Bitplane Inc., South Windsor, CT) was used to analyze z-stacks of confocal images from four VGluT2-ChR2-mCherry mice (64 × 64 × 10 µm for each image; eight images of nAcc from each mouse) to obtain 3D quantification of axon terminals expressing mCherry. VGluT2 and cell bodies or dendrites expressing PV. Amira microscopy software (Visualization Sciences Group) was used to analyze z-stacks of confocal images from four VGluT2-ChR2-mCherry mice (64 × 64 × 10 µm for each image; four images from each animal) to obtain 3D reconstructions of putative synapses. This experiment was successfully repeated three times.

Electron microscopy. Methods were described previously. Briefly, vibratome tissue sections were rinsed and incubated with 1% sodium borohydride to inactivate free aldehyde groups, rinsed and then incubated with blocking solution. Sections were then incubated with primary antibodies (mouse anti-mCherry (1:1,000, Clontech Laboratories, 625243), guinea pig anti-VGluT2 (1:500, Frontier Institute, VGluT2-GP-AF400-1) and rabbit anti-PV (1:500, Frontier Institute, PV-Rb-AF750)). All primary antibodies were diluted with 1% normal goat serum, 4% BSA in phosphate buffer (PB) supplemented with 0.02% saponin. Incubations were for 24 h at 4 °C. Sections were rinsed and incubated overnight at 4 °C in the corresponding secondary antibodies. Sections were rinsed in PB and then in double-distilled water, and rinsing was followed by silver enhancement of the gold particles with the Nanoprobe silver kit (2012, Nanoprobes) for 7 min at room temperature. Next, sections were incubated in avidin-biotinylated horseradish peroxidase complex in PB for 2 h at room temperature and washed. Peroxidase activity was detected with 0.025% 3,3′-diaminobenzidine (DAB) and 0.003% H2O2 in PB for 5–10 min. Sections were rinsed with PB, fixed with 0.5% osmium tetroxide in PB for 25 min, washed in PB followed by double-distilled water, and then contrasted in freshly prepared 1% uranyl acetate for 35 min. Sections were dehydrated by a series of graded alcohols and with propylene oxide. Afterward they were flat-embedded in Durcupan ACM epoxy resin (14040, Electron Microscopy Sciences). Resin-embedded sections were polymerized at 60 °C for 2 d. Sections of 65 nm were cut from the outer surface of the tissue with an ultramicrotome (UC7, Leica Microsystems) using a diamond knife (Diatome). The sections were collected on formvar-coated single-slot grids and counterstained with Reynolds lead citrate. Sections were examined and photographed using a Tecnai G2 12 transmission electron microscope (FEI Company) equipped with a digital micrograph 3.4 camera (Gatan).

Ultrastructural analysis of brain tissue. Serial ultrathin sections of the nAcc (bregma, 2.76 mm to 0.96 mm) were obtained from four male VGluT2-ChR2-mCherry mice. Synaptic contacts were classified according to their morphology and immunolabel and photographed at a magnification of 6,800–13,000×. The morphological criteria used for identification and classification of cellular components or type of synapse observed in these thin sections were as previously described. In the serial sections, a terminal containing more than five immunogold particles was considered an immunopositive terminal. Pictures were adjusted to match contrast and brightness using Adobe Photoshop (Adobe Systems Incorporated, Seattle, WA). This experiment was successfully repeated three times. Electron microscopy and confocal analysis quantification were done in a blinded fashion.

Fluorescence microscopy and 3D analysis. Methods were described previously. Briefly, free-floating coronal sections (40 µm) from VGluT2-ChR2-mCherry mice (n = 4) were incubated for 1 h in PB supplemented with 4% BSA and 0.3% Triton X-100. Sections were then incubated with cocktails of primary antibodies (mouse anti-mCherry (1:500) + guinea pig anti-VGluT2 (1:500) + rabbit anti-PV (1:500)) overnight at 4 °C. After three rinses (10 min each) in PB, sections were incubated in a cocktail of the corresponding fluorescently labeled secondary antibodies (Alexa Fluor 488–anti–guinea pig + Alexa Fluor 594–anti-mouse + Alexa Fluor 647–anti-Rb) for 2 h at room temperature. After rinsing, sections were mounted on slides. Fluorescent images were collected with an Olympus BX51 Confocal System (Olympus). Images were taken sequentially with different lasers with 100× oil-immersion objectives, and z-axes stacks were collected at 0.2 µm. Imaris microscopy software (Bitplane Inc., South Windsor, CT) was used to analyze z-stacks of confocal images from four VGluT2-ChR2-mCherry mice (64 × 64 × 10 µm for each image; eight images of nAcc from each mouse) to obtain 3D quantification of axon terminals expressing mCherry, VGluT2 and cell bodies or dendrites expressing PV. Amira microscopy software (Visualization Sciences Group) was used to analyze z-stacks of confocal images from four VGluT2-ChR2-mCherry mice (64 × 64 × 10 µm for each image; four images from each animal) to obtain 3D reconstructions of putative synapses. This experiment was successfully repeated three times.

Retrograde tract tracing and in situ hybridization. Methods were described previously. Briefly, midbrain dorsal coronal sections (16 µm) were incubated with rabbit anti-FG (1:1,000, Millipore, AB153) and mouse anti-TH (1:500, Millipore, MAB318) supplemented with RNasin. Sections were rinsed and incubated in biotinylated goat anti-rabbit, fluorescein-conjugated donkey anti-rabbit (1:100, Jackson ImmunoResearch) and fluorescein-conjugated donkey anti-mouse supplemented with RNasin. Sections were rinsed and transferred to 4% PFA to be visualized by fluorescence with an Olympus BX51 microscope to identify FG and TH-labeled neurons. Sections were rinsed, treated with 0.2 N HCl, rinsed and then acetylated in 0.25% acetic anhydride in 0.1 M triethylammonium. Sections were rinsed and postfixed with 4% PFA. Hybridization was performed with buffer containing [35S]- and [33P]-labeled single-stranded antisense rat Vglut2 probes at 107 cpm/µL together with the single-stranded rat digoxigenin (DIG)-labeled antisense probe for the two isofoms of rat glutamic acid decarboxylase (Gad65 and Gad67). Sections were treated with 4 µg/ml RNase A at 37 °C for 1 h and washed with 1× saline–sodium citrate, 50% formamide at 55 °C for 1 h and with 0.1× saline–sodium citrate at 68 °C for 1 h. After the last wash, sections were rinsed with TBS buffer. Afterward, sections were incubated with an alkaline phosphatase–conjugated antibody to DIG for 1–3 h; alkaline phosphatase reaction was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, yielding a purple reaction product. Sections were then photographed under bright-field illumination with an Olympus BX51. Sections were mounted on coated slides. Slides were dipped in 0.05 M nuclear tract emulsion and exposed in the dark at 4 °C for 4 weeks before development.

Sections were viewed, analyzed, and photographed with bright-field or epiluminescence microscopy using an Olympus BX51 microscope. Neurons were observed within each traced region at high power (20× objective lenses) and marked electronically. FG-VGlut2, FG-VGlt2-TG, FG-VGlut2-TD, FG-TH, FG-TH-GAD, FG-GAD, FG-VGlut2-TH-GAD, and labeled material were.
analyzed with epiluminescence to increase the contrast of silver grains, identical to previous studies by our laboratory. For VGluT2 in situ hybridization procedures, FG fluorescent cells containing or lacking TH fluorescent signal were photographed before processing for in situ hybridization. For radioactive in situ hybridization methods (Vglut2 mRNA), a cell was considered to express radioactive transcripts when its soma contained concentric aggregates of silver grains in amounts above the background level. FG-labeled cells (detected by fluorescence and brown DAB label) expressing VGluT2 or Gad65/Gad67 mRNA (detected by nonradioactive in situ hybridization) and TH (detected by immunofluorescence) were counted separately. FG (brown DAB product), nonradioactive Gad65/Gad67 in situ hybridization (DIG probe labeled in purple), and radioactive VGluT2 in situ hybridization (silver grains) triple-labeled material were analyzed by the following procedure: (1) silver grains corresponding to Vglut2 mRNA expression were focused under an epiluminescence microscope, (2) the path of epiluminescence light was blocked without a change in focus, and (3) bright-field light was used to determine whether a brown neuron, expressing FG in focus, contained the aggregates of silver grains seen under epiluminescence. A cell was considered to express DIG-labeled transcripts (Gad65/Gad67 mRNA) when its soma was the same shape as the soma of DAB-labeled FG cells. Neurons were counted when the stained cell was at least 5 µm in diameter. Pictures were adjusted to match contrast and brightness using Adobe Photoshop (Adobe Systems). Cell counting was completed with blinding for injection site. Any FG-labeled neuron localized external to the VTA was excluded from analysis.

Combination of in situ hybridization and c-Fos immunolabeling. Methods were described previously. Coronal free-floating sections (16 µm) were incubated for 10 min in PB containing 0.5% Triton X-100, rinsed twice (5 min each time) with PB, treated with 0.2 N HCl for 10 min, rinsed twice (5 min each time) with PB, and then acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 10 min. Sections were rinsed twice (5 min each time) with PB and post-fixed with 4% PFA for 10 min. Before hybridization and after each rinse with PB, the sections were incubated in hybridization buffer (50% with PB and post-fixed with 4% PFA for 10 min). After hybridization and after a final rinse with PB, the sections were incubated in hybridization buffer (50% formamide, 10% dextran sulfate, 5× Denhardt's solution, 0.62 M NaCl, 50 mM sodium phosphate, pH 7.4, 0.2% SDS, 200 µg/ml salmon sperm DNA, 250 µg/ml tRNA) for 2 h at 55 °C. Sections were hybridized for 16 h at 55 °C in hybridization buffer containing [35S]- and [33P]-labeled single-stranded antisense of rat dopamine D1 receptor (nucleotides 228–1,024, GenBank accession NM-012546.2) or D2 receptor (nucleotides 1,101–1,681, GenBank accession M36831) probes at 10 6 cpm/ml. Sections were treated with 4 µg/ml RNase A at 37 °C for 1 h, washed with 1× SSC, 50% formamide at 55 °C for 1 h, and then washed with 0.1× SSC at 68 °C for 1 h. After the last SSC wash, sections were rinsed with PB and incubated for 1 h in PB supplemented with 4% BSA and 0.3% Triton X-100. This was followed by overnight incubation at 4 °C with a rabbit anti-c-Fos (1:1,000, Santa Cruz Biotechnology, SC-52). After being rinsed three times for 10 min each time in PB, sections were processed with an ABC kit (Vector Laboratories). The material was incubated for 2 h at room temperature in a 1:200 dilution of the biotinylated secondary antibody, rinsed with PB, and incubated with avidin-biotinylated horseradish peroxidase for 1 h. Sections were rinsed, and the peroxidase reaction was then developed with 0.05% 3,3-diaminobenzidine-4 HCl and 0.03% hydrogen peroxide (H2O2). The sections were mounted on coated slides. Slides were dipped in Ilford K.5 nuclear track emulsion (Polyscience; 1:1 dilution in double-distilled water) and exposed in the dark at 4 °C for 4 weeks before development. Sections were viewed, analyzed, and photographed with bright-field or epiluminescence microscopy using an Olympus BX51 microscope. Neurons were observed within each nAcc shell region at high power (20× objective lenses) and marked electronically.

Behavioral test apparatus. For the test we used a three-chamber place-conditioning apparatus consisting of two chambers (20 x 18 x 35 cm) with distinct wall patterns and floors and a connecting chamber (20 x 10 x 35 cm). All animal movements were recorded by a camera with video tracking software. For both negative-reinforcement tasks and positive-reinforcement tasks, sound-attenuated operant chambers were used. Each operant chamber contained a metal grid floor, a house light, and two metal wheels (for negative-reinforcement tasks) or two retractable levers and a food port (for positive-reinforcement tasks) installed on a sidewall. An optical encoder recorded every 90° of rotation for response wheels (Med Associates, St. Albans, VT). Chambers were connected to a computer interface with the software (MED-PC for Windows, Med Associates) that controlled all experimental contingencies.

Real-time place-conditioning test. Methods were described previously. VGluT2::Cre, VGAT-ires-Cre and PV::Cre mice were used in the studies. During the photostimulation pilot study with a Latin square design, a group of VGluT2-ChR2-eYFP mice (n = 6) were tested in the conditioning chamber at different light-stimulation frequencies (5, 10, 20 or 40 Hz) for 10 min. During the real-time place-conditioning training and testing, mice were connected to an optical fiber and allowed to access three chambers freely. On habituation and pretest days (15-min sessions), mice were connected to an optical fiber, but the laser was off. Mice showing a side preference (i.e., staying less than 150 s in either main chamber) before training were excluded. On conditioning days (30-min sessions), one chamber was assigned as the stimulation chamber (counterbalanced across mice). The animals were given bilateral light stimulation (473 nm, ~5 mW, 10-ms duration at 20 Hz, or 532 nm, ~15 mW, constantly on for photoinhibition conditioning) in nAcc mShell whenever they entered the photostimulation-paired chamber. The stimulation continued until the animal left the photostimulation-paired chamber. On test day (15-min sessions), mice were allowed to freely explore all chambers without photostimulation. To determine the duration of the acquired aversion, we tested VGluT2-ChR2-eYFP mice for a second time (test 2 in Supplementary Fig. 2e) 24 h after the first test day (test 1).

To test for the involvement of glutamate receptors, GABA receptors, and DA receptors in the real-time place-conditioning of VGluT2-ChR2-eYFP mice, we used intra-nAcc microinjections of antagonists of glutamate receptors (MK-801 + CNQX), GABA receptors (bicuculline + sqlafen), D1 receptor (SCH23390) or D2 receptor (eticlopride), or a mixture of D1 and D2 receptor antagonists. The infusion lasted 1 min, and the conditioning test was performed 10 min after the infusion. The mice were given optical stimulation on light-conditioning day.

Operant negative-reinforcement task. VGluT2-ChR2-eYFP and VGluT2-eYFP mice with chronic optical fibers were trained in an operant negative-reinforcement task. Mice were placed in operant chambers featuring two wheels. Light stimulation (473 nm, ~5 mW, 10-ms duration at 20 Hz, 0.5 s on/off) was delivered during each session (30 min) daily. For the first 6 d, the right wheel was designated as the ‘active’ wheel, with each 90° rotation of the wheel producing a 2-s time out from optogenetic activation of the VTA to nAcc-mShell pathway. Responses on the left wheel were recorded but had no programmed outcome. During the subsequent four reversal-training sessions, the left wheel was designated as the active wheel.

Operant positive-reinforcement task. VGluT2-ChR2-eYFP and VGluT2-eYFP mice were food restricted to 85% of their free-feeding bodyweight. Mice were weighed in operant chambers containing two retractable levers with a food port positioned between them. Subjects were trained for 1 h per day on an incrementing fixed-ratio (FR) schedule (FR1, FR3, and FR5). Responses on the active lever resulted in the delivery of one 20-mg food pellet and the activation of a 1-s tone and cue light. Sessions concluded at 1 h or after the delivery of 50 pellets, whichever occurred first. Once animals had completed the FR5 task, both levers remained present until mice reached stable responding (defined as continuous days with <20% variability and >250 lever responses). Subjects were then trained for 1 h per day on an incrementing fixed-ratio (FR) schedule (FR1, FR3, and FR5). Responses on the active lever resulted in the delivery of one 20-mg food pellet and the activation of a 1-s tone and cue light. Sessions concluded at 1 h or after the delivery of 50 pellets, whichever occurred first. Once animals had completed the FR5 task, both levers remained present until mice reached stable responding (defined as continuous days with <20% variability and >250 lever responses). Subsequently, a concurrent variable-interval 30-s schedule of optical stimulation (1 s of 20-Hz stimulation at 473 nm, ~5 mW, and 10-ms duration) was imposed on the photostimulation-paired lever. Photostimulation was paired with the left lever in sessions 4 and 5, followed by a no-light test in session 6, a subsequent reversal of the light pairing (to the right lever) in sessions 7 and 8, and a final no-light test in session 9.

Learned helplessness. Methods for the learned-helplessness paradigm were optimized previously. VGluT2-eNpHR-eYFP and VGluT2-eYFP mice with a bilateral optical probe in nAcc mShell were tested in a forced swim protocol to establish a baseline measure for immobility time. Mice were then subjected to 30 inescapable, unsignaled electric foot shocks (5 s, 0.35 mA; 55-s intershock interval) over a period of 30 min for five daily sessions. Exposure to shock in this manner has been shown to produce learned helplessness in mice. Photoinhibition was delivered to VGluT2-eYFP and VGluT2-eNpHR-eYFP mice for a period beginning concomitant with the shock delivery and continuing until 20 s after
the termination of each shock. After the learned-helplessness protocol, mice were again tested in the forced swim procedure. The immobile time each animal spent during the test was manually counted offline, with the evaluator blinded to the treatment of the animals. Immobility time was analyzed using a two-way ANOVA for the forced swim test (pretest versus test) and experimental group (eYFP versus eNpHR).

Slice preparation and optogenetic electrophysiology. Five weeks after virus injection, male PV::Cre mice carrying AAV5-DIO-eYFP in nAcc and AAV8-

hSyn-ChR2-tdTomato in VTA (n = 15) were anesthetized by intraperitoneal injection of chloral hydrate and perfused with ice-cold ACSF saturated with 95% O2 and 5% CO2.

Brains were then removed quickly and modified to contain (in mM) 92 NMDG, 20 HEPES, 25 glucose, 30 NaHCO3, 1.2 NaH2PO4, 2.5 KCl, 5 sodium pyruvate, 2 thiourea, 10 MgSO4, 0.5 CaCl2. Brains were then removed quickly from the mice, placed in this same solution on a VT-1200 vibratome (Leica, Nussloch, Germany), and sectioned through the nAcc in coronal slices (200-µm thickness). The slices were placed in a holding chamber filled with the same solution, but held at 32 °C. After 15 min, slices were transferred to a holding chamber containing room-temperature ACSF modified to contain (in mM) 92 NaCl, 25 glucose, 30 NaHCO3, 1.2 NaH2PO4, 2.5 KCl, 5 sodium ascorbate, 3 sodium pyruvate, 2 thiourea, 1 MgSO4, 2 CaCl2. For recordings, slices were transferred to a chamber superfused with 32 °C ACSF modified to contain (in mM) 125 NaCl, 2.5 KCl, 1.25 NaH2PO4, 1 MgCl2, 2.4 CaCl2, 26 NaHCO3 and 11 glucose. Electrodes (4–6 MΩ) were backfilled with an internal solution containing (in mM) 140 potassium gluconate, 2 NaCl, 1.5 MgCl2, 10 HEPES, 4 Mg-ATP, 0.3 Na2-glycerophosphate, 0.1 EGTA and 0.2% biocytin (pH 7.2; 280–290 mOsm). Cells were visualized on an upright microscope using infrared differential interference contrast video microscopy. Whole-cell voltage-clamp recordings were made using a MultiClamp 700B amplifier (2-kHz low-pass Bessel filter and 10-kHz digitization) with pClamp 10.3 software (Molecular Devices, Sunnyvale, CA). PV interneurons and MSNs in the nAcc shell were identified by membrane resistance, resting membrane potential and green fluorescence in the case of PV interneurons. Both neuron populations were voltage clamped at −70 mV, unless otherwise noted. Series resistance (10–30 MΩ) was monitored by membrane current. In the case of PV interneurons, the peak amplitude of EPSCs was measured with the average of 30 consecutive traces. EPSCs were blocked by bath application of AMPAR antagonist CNQX (10 µM) in some recordings and the peak amplitude of EPSCs was measured with the average of 30 consecutive traces. EPSCs were blocked by bath application of AMPAR antagonist CNQX (10 µM). In some recordings we clamped the membrane potential at −45 mV to see GABAergic outward currents.

Fluorescent immunohistochemistry for animals subjected to behavioral testing. VGluT2-ChR2-eYFP mice (n = 9) and VGluT2-eYFP mice (n = 8) implanted with nAcc mShell bilateral optical fibers were habituated to the conditioned-place-preference apparatus for 2 h with light delivery on three consecutive days. After habituation, mice were placed in the apparatus and photon stimulation was delivered (473 nm, ~70 mW, 20 Hz, 10-ms duration, 5 s on/off for 15 min). Two hours after the onset of light delivery, mice were perfused and nAcc mShell sections (30 µm) were prepared for fluorescent immunostaining with rabbit anti-Phospho-c-Fos (1:400, 5348, Cell Signaling) and goat anti-PI-1 (1:500, Frontier institute, PV-Go-Af610), mouse anti-NOS (1:500, Sigma, N2280) or goat anti-ChAT (1:100, Millipore, AB1144). The sections were washed and incubated with fluorescent secondary antibody. PV-immunoreactive (IR), NOS-IR or ChAT-IR neurons coexpressing c-Fos-IR neurons were counted on the basis of the confocal images. The neuron quantitation areas were within the nAcc mShell (500 µm × 500 µm). All cell counting was done blinded to the performance of the behavioral animals.

Histological verification. After behavioral testing, all mice were deeply anesthetized and coronal sections of nAcc (30 µm) from VGluT2::Cre and VGAT-ires-Cre mice were directly mounted on slides. Free-floating coronal VTA sections from VGluT2::Cre and VGAT-ires-Cre mice were washed before being incubated in blocking solution. Sections were then incubated with primary antibody cocktail (mouse anti-eYFP + rabbit anti-TH) overnight at 4 °C. Sections were rinsed before being incubated at room temperature for 2 h in secondary antibody cocktail after the slice recording, coronal sections of nAcc from PV::Cre mice were incubated with mouse anti-eYFP + goat anti-PV and the appropriate secondary antibody, and the biocytin was stained with Cy5. Sections were then rinsed, mounted on slides, and covered with antifade fluorescent mounting medium. Fluorescence images were taken with an Olympus VS120 microscope. The nAcc sections from the mice with 6-OHDA lesions were incubated with TH antibody and later stained with DAB. After histological verification, mice with incorrect virus injection, lesion injection, cannula or probe implantation were excluded from data analysis.

Statistics. We performed either a two-sided t-test or a one-, two- or three-way ANOVA to analyze all data when applicable. Significant omnibus effects were followed by Newman–Keuls post hoc tests (behavioral experiments) or Dunnett’s post hoc tests (electrophysiology). Statistical analyses were performed with STATISTICA 12 (behavioral experiments) or Graphpad Prism (electrophysiology). Alpha was always set at P < 0.05.

Sample size. The target number of samples in each group for behavioral, anatomical, and electrophysiological experiments was determined on the basis of numbers reported in published studies. The experimenter performing surgeries, including virus vector injection and probe or cannula implantation, was known to hit the targets used (VTA or nAcc) with a probability of 0.9. Our target number of animals in each group was ten. We therefore performed about 12 surgeries in each group.

Replication. All sample sizes indicated in figures for behavioral and electrophysiological experiments represent biological replicates. The anatomical and ultrastructural experiments were successfully repeated three times.

Randomization. All randomization was performed by an experimenter. The same stereotaxic apparatus was used for all surgeries. All surgical and behavioral manipulations performed on each animal were determined randomly. For animals used in behavioral experiments, the virus used in each animal and injection site were determined randomly and counterbalanced across groups.

Exclusion criteria. Data were excluded on the basis of predetermined histological and performance criteria established during pilot experiments. Histological criteria included injection sites and optical fiber or guide cannula placement. Only animals with injection sites in the region of interest were included.

A Supplementary Methods Checklist is available.

48. Borgius, L., Restrepo, C.E., Leao, R.N., Saleh, N. & Kiehn, O. A transgenic mouse line for molecular genetic analysis of excitatory glutamatergic neurons. Mol. Cell. Neurosci. 45, 245–257 (2010).
49. Qi, J. et al. A glutamatergic reward input from the dorsal raphe to ventral tegmental area dopamine neurons. Nat. Commun. 5, 5390 (2014).
50. Li, B. et al. Synaptic potentiation onto habenula neurons in the learned helplessness model of depression. Nature 470, 535–539 (2011).