

Ventral tegmental area interneurons revisited: GABA and glutamate projection neurons make local synapses

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This manuscript provides **convincing** evidence derived from diverse state-of-the-art approaches to suggest that non-dopaminergic projection neurons in the ventral tegmental area (VTA) make local synapses. These **important** findings challenge the prevailing wisdom that VTA interneurons exclusively form local synaptic contacts and instead reveal that VTA neurons expressing interneuron markers also form long-range projections to forebrain targets such as the cortex, ventral pallidum, and nucleus accumbens. Given the importance of VTA interneurons to many models of VTA-linked behavioral functions, these findings have significant implications for our understanding of the neural circuits underlying reward, motivation, and addiction.

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Abstract

The ventral tegmental area (VTA) contains projection neurons that release the neurotransmitters dopamine, GABA, and/or glutamate from distal synapses. VTA also contains GABA neurons that synapse locally on to dopamine neurons, synapses widely credited to a population of so-called VTA interneurons. Interneurons in cortex, striatum, and elsewhere have well-defined morphological features, physiological properties, and molecular markers, but such features have not been clearly described in VTA. Indeed, there is scant evidence that local and distal synapses originate from separate populations of VTA GABA neurons. In this study we tested whether several markers expressed in non-dopamine VTA neurons are selective markers of interneurons, defined as neurons that synapse locally but not distally. Challenging previous assumptions, we found that VTA neurons genetically defined by expression of parvalbumin, somatostatin, neurotensin, or mu-opioid receptor project to known VTA targets including nucleus accumbens, ventral pallidum, lateral habenula, and prefrontal cortex. Moreover, we provide evidence that VTA GABA and glutamate projection neurons make functional inhibitory or excitatory synapses locally

within VTA. These findings suggest that local collaterals of VTA projection neurons could mediate functions prior attributed to VTA interneurons. This study underscores the need for a refined understanding of VTA connectivity to explain how heterogeneous VTA circuits mediate diverse functions related to reward, motivation, or addiction.

Significance statement

GABA neurons in VTA are key regulators of VTA dopamine neurons and considered central to the mechanisms by which opioids and other drugs of abuse can induce addiction. Conventionally, these VTA GABA neurons are considered interneurons, but GABA projection neurons are also abundant in VTA, and it is unclear if these represent separate populations. We found that several markers enriched in non-dopamine neurons of VTA, including Mu-opioid receptor, are also expressed in projection neurons, and thus are not selective interneuron markers. Moreover, we found that VTA GABA and glutamate projection neurons collateralize within VTA where they make local synapses. These data challenge the notion of a VTA interneuron that synapses only within VTA and suggest that inhibitory projection neurons can serve functions previously attributed to VTA interneurons.

Introduction

The ventral tegmental area (VTA) is a central component of the brain's reward circuitry, and a common attribute of addictive drugs is their ability to increase dopamine release from VTA projections (Luscher, 2016; Nestler, 2005). The VTA projects to and receives inputs from many brain structures involved in reward-related behavior, including nucleus accumbens (NAc), ventral pallidum (VP), lateral habenula (LHb), and prefrontal cortex (PFC) (Fields et al., 2007; Morales & Margolis, 2017). The VTA is often simplified as a region containing dopamine (DA) projection neurons and inhibitory GABA 'interneurons' that regulate DA neurons (Johnson & North, 1992; Luscher & Malenka, 2011; Nestler, 2005). However, VTA neurons are highly heterogeneous. The VTA contains distinct populations of DA neurons that can be segregated by gene expression, projection target, and function (Azcorra et al., 2023; Poulin et al., 2020; Roeper, 2013). GABA-releasing VTA neurons make local intra-VTA synapses (Bayer & Pickel, 1991; Omelchenko & Sesack, 2009), but also project widely outside the VTA, including dense projections to LHb, VP, and VP-adjacent areas of basal forebrain (Kaufling et al., 2010; Oades & Halliday, 1987; Taylor et al., 2014). Glutamate neurons are also prevalent in VTA and overlap with other populations such that ~25% of VTA glutamate neurons co-express a DA marker and ~25% express a GABA marker (Conrad et al., 2024; Ma et al., 2023; Phillips et al., 2022). VTA glutamate neurons release glutamate locally within VTA and from distal axons in medial NAc, PFC, VP, LHb, and elsewhere (Dobi et al., 2010; Gorelova et al., 2012; Hnasko et al., 2012; Root et al., 2014; Taylor et al., 2014; Yamaguchi et al., 2011).

It is now understood that DA signals can induce or correlate with distinct behavioral responses depending on their projection targets (Azcorra et al., 2023; Badrinarayan et al., 2012; de Jong et al., 2019; Faget et al., 2024). This is true also for VTA GABA and glutamate neurons. For example, activating VTA GABA neurons either locally within VTA or from distal processes can drive behavioral avoidance, disrupt reward seeking, or modify opioid reinforcement (Corre et al., 2018; Root et al., 2020; Shields et al., 2021; Soden et al., 2020; Tan et al., 2012; van Zessen et al., 2012; Zhou et al., 2022). On the other hand, stimulation of VTA GABA projections to LHb can be rewarding (Lammel et al., 2015; Stamatakis et al., 2013). Likewise, stimulation of VTA glutamate neurons can drive robust positive reinforcement or behavioral avoidance depending on the projection target and behavioral assay (Root et al., 2018; Root et al., 2014; Wang et al., 2015; Yoo et al., 2016). These responses can depend also on the co-release of

distinct transmitters. For example, activation of VTA glutamate projections to NAc drives positive reinforcement through the release of glutamate and avoidance via DA co-release (Warlow et al., 2024 [↗](#); Zell et al., 2020 [↗](#)). Thus, VTA neurons can mediate approach or avoidance behaviors through their specific connectivity and neurotransmitter content, and understanding the circuit mechanisms regulating activity in diverse VTA cell types is crucial to understanding the mechanisms by which mesolimbic circuits control motivated behaviors.

Local intra-VTA GABA modulation of VTA output, particularly DA output, may underlie key aspects of behavioral reinforcement. For example, inhibitory inputs to VTA from lateral hypothalamus, bed nucleus of stria terminalis, or VP can drive positive reinforcement and approach behaviors through inhibition of VTA GABA neurons and disinhibition of VTA DA neurons (Faget et al., 2024 [↗](#); Nieh et al., 2015 [↗](#); Soden et al., 2020 [↗](#); Soden et al., 2023 [↗](#)). VTA GABA circuits also appear to be critical for the generation of DA reward prediction error signals (Eshel et al., 2015 [↗](#); Keiflin & Janak, 2015 [↗](#)). Moreover, drugs of abuse can induce rapid or plastic changes in DA signaling through mechanisms that depend on intra-VTA GABA transmission (Corre et al., 2018 [↗](#); Gomez et al., 2019 [↗](#); Luscher & Malenka, 2011 [↗](#); Ostroumov & Dani, 2018 [↗](#); Ting & van der Kooy, 2012 [↗](#)). Indeed, the observation that Mu-opioid receptor agonists directly inhibit non-DA VTA neurons and produce disinhibitory effects on VTA DA neurons (Johnson & North, 1992 [↗](#)) helped establish the notion of VTA interneurons into current models of VTA architecture.

Yet there is scant evidence for the existence of VTA GABA interneurons, defined as neurons that make synapses locally within VTA but that do not make distal connections. Interneurons as so defined in cortex, striatum and other brain regions have characteristic morphological features, physiological properties, and molecular markers (Markram et al., 2004 [↗](#); Pelkey et al., 2017 [↗](#); Tepper et al., 2010 [↗](#)). However, no molecular or physiological feature has been described that can clearly distinguish VTA GABA interneurons from GABA projection neurons. Identifying a marker that selectively labels VTA interneurons would enable investigations into distinct roles for VTA interneurons and projection neurons (Bouarab et al., 2019 [↗](#); Paul et al., 2019 [↗](#)).

In this study we first sought to test whether several genes that are expressed in a subset of VTA neurons may be selective for interneurons in VTA. We chose markers that are expressed in non-DA neurons, selectively label interneurons in other brain areas, and/or have been widely presumed to label VTA interneurons. We found that these markers labeled neurons that were primarily non-DA neurons, but that made projections to distinct VTA projection targets, and thus did not selectively label VTA interneurons. We thus sought to test the hypothesis that VTA GABA (or glutamate) projection neurons make intra-VTA collaterals. Indeed, we provide both anatomical and physiological evidence that VTA GABA neurons projecting to NAc, VP, or PFC make local synapses within VTA. This work challenges the presumption of GABA interneurons in VTA by providing direct evidence for an alternative model by which GABA projection neurons can regulate the activity of neighboring VTA cells.

Results

PV, SST, MOR, and NTS are not selective interneuron markers in VTA

We selected four genes with well-validated Cre lines to test as putative genetic markers that might selectively label VTA interneurons: Parvalbumin (PV), Somatostatin (SST), Mu-opioid receptor (MOR), or Neurotensin (NTS). We injected adeno-associated virus (AAV) into the VTA for Cre-dependent expression of Channelrhodopsin-2 (ChR2) fused to mCherry that labels distal axons (Figure 1A [↗](#)). To test whether the labeled VTA neurons project distally we assessed expression in known VTA projection sites including nucleus accumbens (NAc), ventral pallidum (VP), prefrontal cortex (PFC), and lateral habenula (LHb).

Parvalbumin (PV) is a marker of interneurons in cortex and striatum (Kawaguchi, 1993 [↗](#); Kawaguchi & Kondo, 2002 [↗](#); Tepper et al., 2018 [↗](#)), but is also expressed in VTA GABA neurons (Olson & Nestler, 2007 [↗](#)). Injections into VTA of PV-Cre mice labeled neurons located in medial VTA. We also detected a dense concentration of axonal fibers in LHb, with scant labeling in other known VTA projection targets (**Figure 1B** [↗](#)). These data suggest that PV labels VTA projection neurons and PV is not a selective marker of VTA interneurons.

Like PV, Somatostatin (SST) is an interneuron marker in cortex (Kawaguchi & Kondo, 2002 [↗](#)). SST is expressed in VTA GABA neurons that can inhibit neighboring VTA DA neurons (Nagaeva et al., 2020 [↗](#)). Injections into SST-Cre mice labeled cell bodies in VTA (**Figure 1C** [↗](#)). We again identified axons in distal targets, here with notably dense labeling in VP.

MOR is expressed in VTA GABA neurons, inhibiting GABA release from synapses on to VTA DA neurons, thereby increasing DA neuron firing, and is often described as a marker of VTA interneurons (Gysling & Wang, 1983; Johnson & North, 1992 [↗](#); Luscher & Malenka, 2011 [↗](#); Nestler, 2005 [↗](#); Phillips et al., 2022 [↗](#)). Injections into MOR-Cre mice led to labeled neurons throughout VTA, but also labeled axons in PFC, NAc, LHb, and especially VP (**Figure 1D** [↗](#)).

NTS is expressed in a subpopulation of VTA GABA neurons (Phillips et al., 2022 [↗](#)) and NTS can stimulate mesolimbic DA cells through activation of NTS receptor 1 (Caceda et al., 2006 [↗](#); Kalivas, 1983 [↗](#)). Injections into NTS-Cre mice labeled neurons in VTA, as well as axons in VP, with weaker labeling in other VTA projection sites (**Figure 1E** [↗](#)).

We also stained VTA sections for Tyrosine hydroxylase (TH) to estimate the proportion of ChR2:mCherry neurons colocalizing with DA neurons. In all cases only a minority of mCherry-labeled neurons expressed TH, ranging from 2% for PV to 12% for NTS (**Figure 1F** [↗](#)). In total, our data suggest that these four markers label primarily non-DA neurons in VTA, but that none are selective for interneurons, and instead are inclusive of VTA projection neurons.

Anatomical evidence that VTA projection neurons make local synapses

Each of the markers tested are also expressed in neurons proximal to VTA and our injections led to variable spread to neighboring regions, including interpeduncular nucleus (IPN) and red nucleus. While these regions are not known to project to PFC, NAc, VP, or LHb, we nonetheless aimed to validate the above findings with a secondary approach involving a combination of retrograde labeling and intersectional genetics to target VTA projection neurons. We injected AAV-fDIO-mGFP-Synaptophysin:mRuby into VTA of each Cre line, plus retroAAV-DIO-Flp into a projection target receiving dense innervation. This approach allowed for Cre-plus Flp-dependent expression of both membrane-localized GFP and the synaptic vesicle marker Syn:Ruby (Beier et al., 2015). The intersectionality of this approach allows for precise targeting of VTA projection neurons, and Syn:Ruby highlights putative release sites, either local to or distal from VTA.

Using this approach to label PV-Cre projectors to LHb (**Figure 2A** [↗](#)), or SST-Cre projectors to VP (**Figure 2G** [↗](#)), revealed GFP-positive soma well-restricted within VTA borders delineated by TH immunolabel (**Figure 2B,C,H,I** [↗](#)). We also observed GFP-positive axons and Syn:Ruby-positive puncta in LHb of PV-Cre, or VP of SST-Cre mice (**Figure 2E,F,K,L** [↗](#)). Using high magnification we observed Syn:Ruby puncta proximal to TH-positive cells in VTA (**Figure 2D,J** [↗](#)), suggesting that these VTA projection neurons collateralize within VTA and synapse on to DA neurons.

The same approach was used to label VTA projectors to VP in MOR-Cre or NTS-Cre mice (**Figure 3A** [↗](#)). VP-projecting GFP-positive cell bodies in MOR-Cre mice were contained within and throughout VTA (**Figure 3B** [↗](#)). We also observed axonal fibers densely filling VP, delineated by Substance P immunolabel (**Figure 3F-H** [↗](#) and **Supplemental Figure 1** [↗](#)). Using high

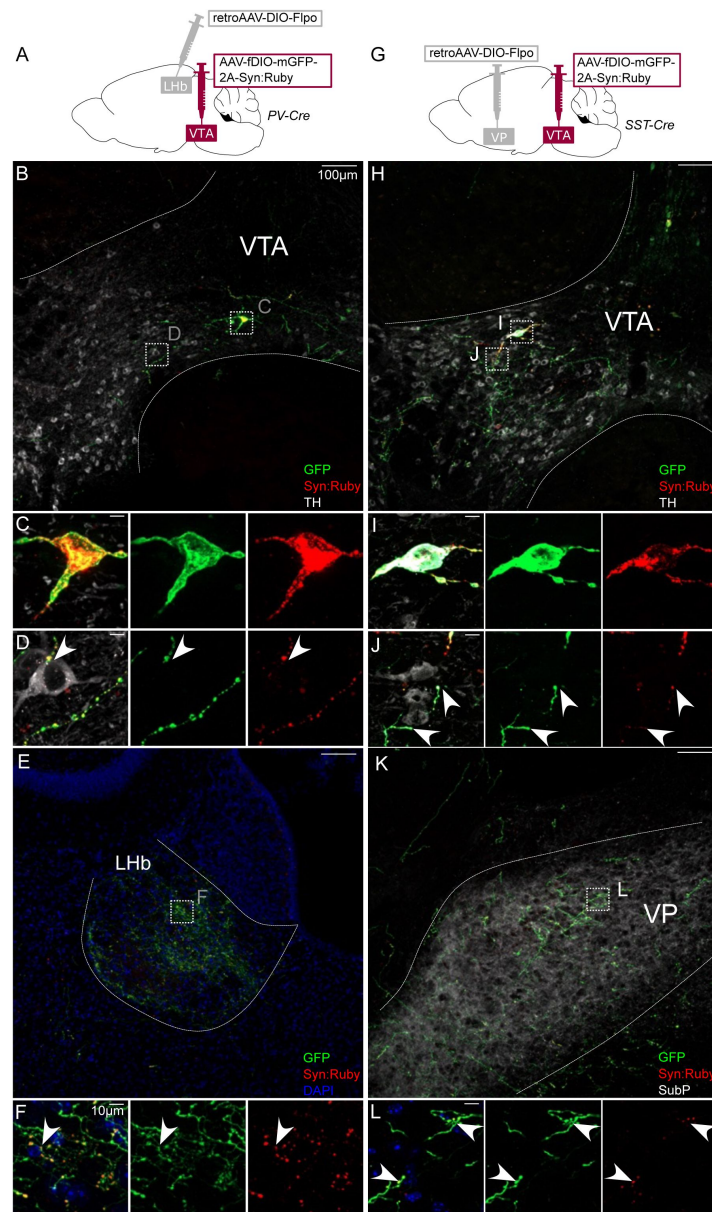


Figure 2

Intersectional approach to label projections of PV- and SST-expressing VTA neurons.

(A) Dual AAV approach for Cre-dependent expression of Flp injected in LHb plus Flp-dependent expression of GFP and Syn:Ruby in VTA of PV-Cre mice. (B) LHb-projecting PV-Cre neurons in VTA with (C,D) high magnification insets showing putative release sites proximal to TH+ DA neurons. (E) VTA axons in LHb with (F) high magnification insets. (G) Dual AAV approach for Cre-dependent expression of Flp injected in VP plus Flp-dependent expression of GFP and Syn:Ruby in VTA of SST-Cre mice. (H) VP-projecting SST-Cre neurons in VTA with (I,J) high magnification insets showing putative release sites proximal to TH+ DA neurons. (K) VTA axons in VP with (L) high magnification insets. Scale bars 100µm, or 10µm for high magnification insets.

magnification, we observed Syn:Ruby puncta proximal to TH-positive VTA neurons, again suggestive of synapses made within VTA by labeled projection neurons (**Figure 3C-E**). Using the same approach to target VP-projecting neurons in NTS-Cre mice we found similar results, with GFP-positive soma in VTA and Syn:Ruby-positive puncta in both VTA (**Figure 3I-J**) and VP (**Figure 3K-L**), though signals were notably less dense. These results corroborate the findings in **Figure 1** and suggest that multiple markers that had been suggested to label putative interneurons instead label VTA projection neurons that may make local synapses through axon collaterals.

There is evidence indicating that PV, SST, MOR and NTS neurons in VTA express GABA markers or release GABA (Nagaeva et al., 2020; Olson & Nestler, 2007; Phillips et al., 2022). However, some neurons positive for those markers may express VGLUT2 and release glutamate (Miranda-Barrientos et al., 2021). We therefore used VGAT-Cre and VGLUT2-Cre mice to selectively express GFP and Syn:Ruby, here targeting NAc-projecting VTA neurons. In VGAT-Cre mice (**Figure 4A**) we identified GFP-positive cell bodies that were restricted to VTA (**Figure 4B**) and GFP-positive fibers in NAc (**Figure 4E-F**). At higher magnification, we observed a pattern of GFP fibers and Syn:Ruby puncta surrounding TH-positive cell bodies (**Figure 4C-D**), suggesting that NAc-projectors make collaterals on to VTA DA neurons. We observed similar results when using VGLUT2-Cre mice, suggesting that NAc-projecting VTA glutamate neurons can also make local collaterals within VTA (**Figure 4G-L**). As expected VTA glutamate cell bodies were concentrated in medial VTA, where they are most dense (Conrad et al., 2024; Kawano et al., 2006; Yamaguchi et al., 2011).

Physiological evidence that VTA projection neurons make local synapses

Our anatomical results suggest that multiple types of VTA projection neurons collateralize locally within VTA. Next, to functionally assess whether VTA projection neurons make local synapses in VTA, we used a combination of optogenetics and electrophysiology. We selectively expressed ChR2 in NAc-projecting VTA neurons by injecting retroAAV-Cre into NAc and AAV-DIO-ChR2:mCherry into VTA of wild-type mice (**Figure 5A**). We then made acute brain slices to record from VTA neurons negative for ChR2:mCherry to test if they received synaptic inputs from NAc-projecting VTA neurons (**Figure 5B**). Using wild-type mice allowed us to express opsin in both GABA and glutamate projection neurons, and assess for optogenetic-evoked postsynaptic currents (oPSC) that were either inhibitory (oIPSC) or excitatory (oEPSC) from the same cell.

As expected, the medial shell of NAc showed dense mCherry-positive fibers (**Figure 5C**), and mCherry-positive cell bodies were restricted to VTA (**Figure 5D**). We patched ChR2:mCherry-negative VTA neurons (**Supplemental Figure 2**), flashed 2-ms blue light pulses, and observed oPSCs in 59% of neurons; 44% displayed short-latency oIPSCs (mean 84 ± 17 pA), 6% had short-latency oEPSCs (mean -28 ± 6 pA), 41% had no response (responses less than 5 pA were considered unconnected), and 11% had oPSCs with long-latency to onset (>5 ms) (**Figure 5E-F**). Note that unconnected and long-latency cells are not included in **Figures 5F, I**. The GABAA receptor antagonist picrotoxin (PTX) blocked oIPSCs while oEPSCs were blocked by the AMPA receptor antagonist DNQX (**Figure 5G,H**), confirming that these responses are mediated by evoked GABA or glutamate release, respectively.

Most responses displayed onset latencies more than 2 ms and less than 5 ms, consistent with monosynaptic connectivity (3.2 ± 0.1 ms and 3.8 ± 0.2 ms for oIPSCs and oEPSCs, respectively) (**Figure 5I**). To confirm connections are monosynaptic we performed additional pharmacology. We found that the amplitude of oPSCs was diminished following the application of the voltage-gated sodium channel blocker tetrodotoxin (TTX, voltage-gated sodium channel are necessary for the propagation of action potentials), and that oPSCs recovered with bath application of the

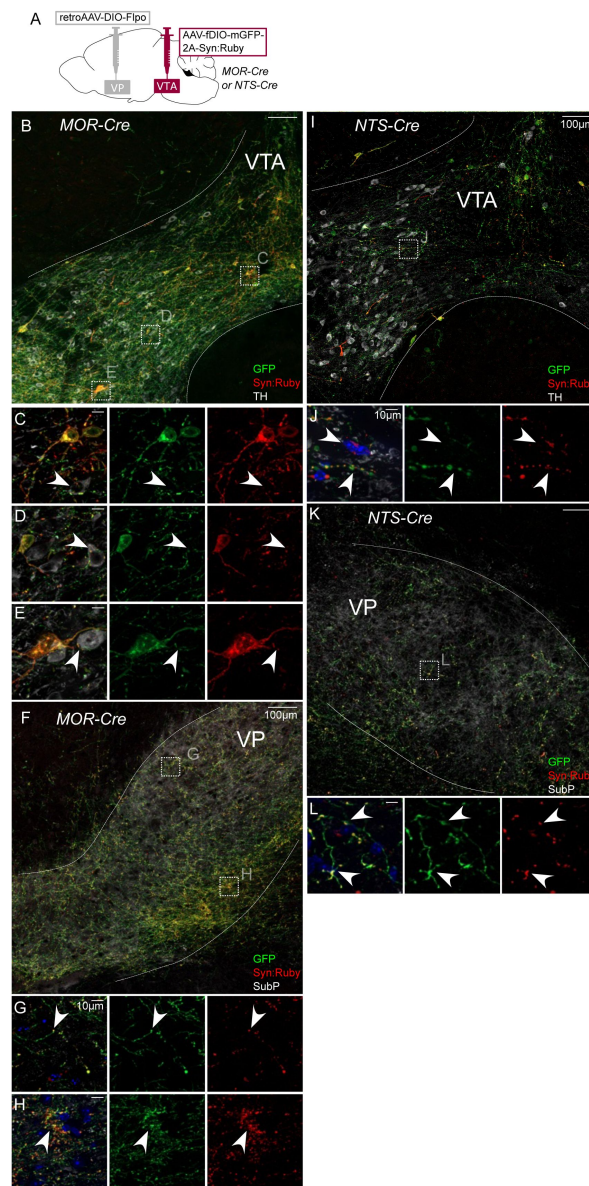


Figure 3

Intersectional approach to label projections of MOR- and NTS-expressing VTA neurons.

(A) Dual AAV approach for Cre-dependent expression of Flp injected in VP plus Flp-dependent expression of GFP and Syn:Ruby in VTA of MOR-Cre and NTS-Cre mice. (B) VP-projecting MOR-Cre neurons in VTA with (C,D,E) high magnification insets showing putative release sites proximal to TH+ DA neurons. (F) VTA axons in VP with (G,H) high magnification insets. (I) VP-projecting NTS-Cre neurons in VTA with (J) high magnification insets showing putative release sites. (K) VTA axons in VP with (L) high magnification insets. Scale bars 100µm, or 10µm for high magnification insets.

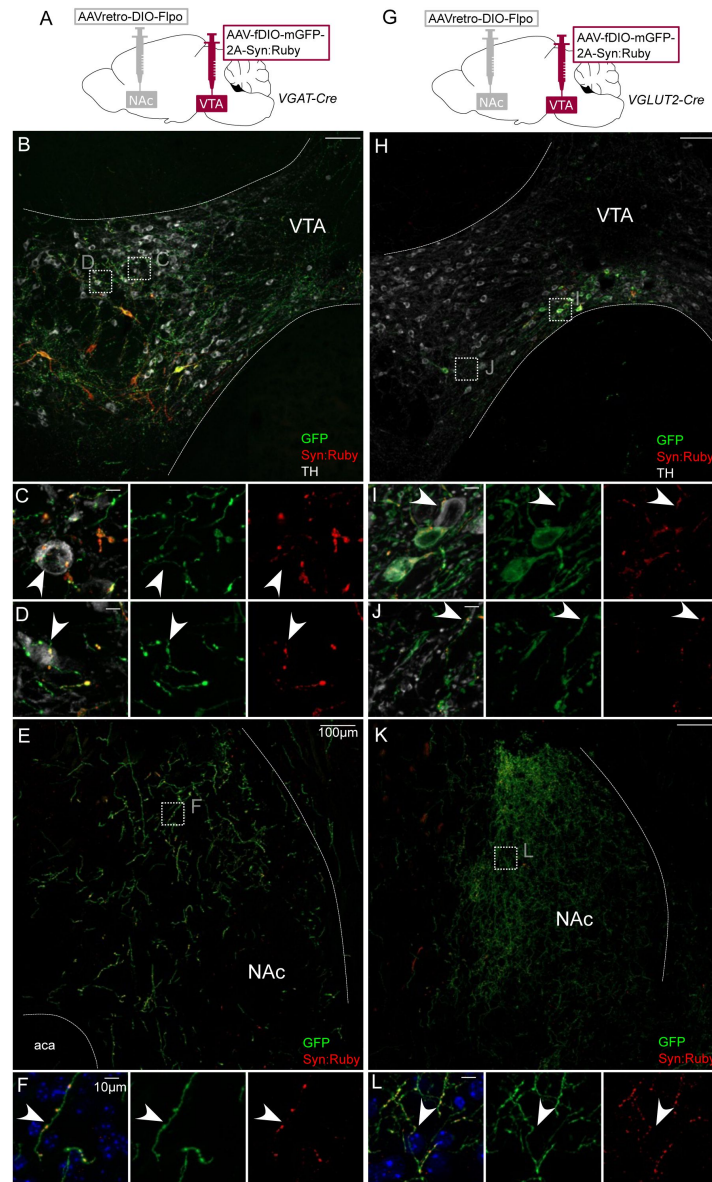


Figure 4

Intersectional labeling of VTA GABA and glutamate projection neurons suggests intra-VTA collaterals.

(A) Dual AAV approach for Cre-dependent expression of Flp injected in NAc plus Flp-dependent expression of GFP and Syn:Ruby in VTA of VGAT-Cre mice. (B) NAc-projecting VGAT-Cre neurons in VTA with (C,D) high magnification insets showing putative release sites proximal to TH+ DA neurons. (E) VTA axons in NAc of VGAT-Cre mice, with (F) high magnification insets. (G) Dual AAV approach for Cre-dependent expression of Flp injected in NAc plus Flp-dependent expression of GFP and Syn:Ruby in VTA of VGLUT2-Cre mice. (H) NAc-projecting VGLUT2-Cre neurons in VTA with (I,J) high magnification insets showing putative release sites proximal to TH+ DA neurons. (K) VTA axons in NAc of VGLUT2-Cre mice with (L) high magnification insets. Scale bars 100µm, or 10µm for high magnification insets.

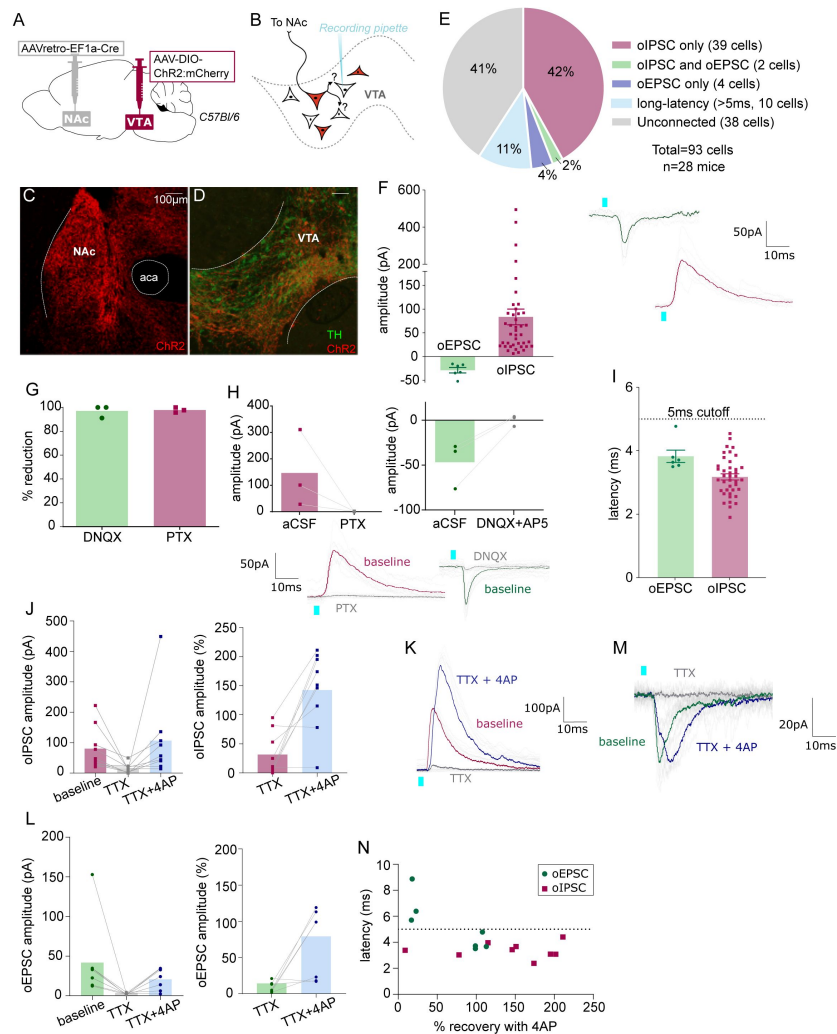


Figure 5

NAc-projecting VTA GABA and glutamate neurons make intra-VTA synapses.

(A) Dual AAV approach to express Chr2:mCherry in NAc-projecting VTA neurons in wild-type mice. (B) Patch-clamp recordings from Chr2:mCherry-negative neurons of VTA to test for collateralizing synapses made by NAc-projectors. (C) Coronal images showing Chr2:mCherry expression in NAc and (D) VTA; scale bars 100 μ m. (E) Chr2:mCherry-negative VTA neuron responses to optogenetic stimulation of NAc-projectors. (F) Peak amplitude of connected cells that displayed an oEPSC and/or oIPSC (excluding long-latency), with example traces. (G) Percent reduction in oEPSC or oIPSC by DNQX or PTX, respectively. (H) Peak amplitude of oIPSCs before and after bath application of PTX, or of oEPSCs before and after bath application of DNQX, with example traces. (I) Latency to oPSC onset (excluding long-latency). (J) Peak oIPSC amplitude before and after bath application of TTX and recovery with 4AP (Friedman's test Chi-square=10.9, $p=0.0029$) and (K) example traces. (L) Peak oEPSC amplitude before and after bath application of TTX and recovery with 4AP (Friedman's test Chi-square=11.6, $p=0.0013$) and (M) example traces (N) Scatter plot showing relationship between initial (pre-treatment) latency to oPSC onset and 4AP recovery. Green dots represent oEPSCs and red squares oIPSCs.

inhibitor of voltage-sensitive potassium channels 4-aminopyridine (4AP). When this strategy was applied to oIPSCs (**Figure 5J** [↗](#)), 8 out of 9 TTX-diminished currents were restored by the application of 4AP (**Figure 5J,K** [↗](#)). Similarly, 4 of 7 oEPSCs were recovered by 4AP (**Figure 5L,M** [↗](#)). We plotted the latency of oPSC onset against the percent oPSC recovery mediated by 4AP and found that 3 of 4 neurons that failed to recover had a latency >5ms, whereas only 1 of 13 neurons that had a latency <5 ms failed to recover (**Figure 5N** [↗](#)). Therefore, we used 5 ms as a ‘short latency’ cutoff to consider an oPSC as monosynaptic. In total we recorded ten cells with oPSC latency >5 ms (identified as long latency on **Figure 5E** [↗](#)). Out of these 10 long-latency oPSCs, 8 were oEPSCs and 2 oIPSCs. This proportion (8:2) of neurons with oEPSCs versus oIPSCs was strikingly greater than that for short-latency responses (6:41), suggesting that in some cells/slices optogenetic stimulation of projection neurons recruited a more extensive intra-VTA excitatory network.

We used a similar approach to assess whether VTA neurons projecting to VP or PFC also made local collaterals in VTA. We used the same combination of viruses but here injected retroAAV-Cre into VP of wild-type mice (**Figure 6A** [↗](#)), again recording from mCherry-negative VTA neurons (**Figure 6B** [↗](#)). As expected, we observed dense mCherry-positive fibers in VP and mCherry-positive cell bodies restricted to VTA (**Figure 6C-D** [↗](#) and **Supplemental Figure 3** [↗](#)). We found that 52% of mCherry-negative neurons were connected (13 of 25), 32% displayed short-latency oIPSCs, 4% had short-latency oEPSCs, and 20% were connected but with long latency (>5 ms) (**Figure 6E-G** [↗](#)). We also patched from postsynaptic neurons in VP and found 89% displayed oPSCs, all with short latency, and as in VTA most currents were inhibitory (**Figure 6H-K** [↗](#)).

Finally, we repeated the same approach but for PFC-projecting VTA neurons (**Figure 6L-M** [↗](#)). We observed mCherry-positive fibers in PFC (**Figure 6N** [↗](#)) arising from sparse cell bodies found within the bounds of VTA (**Figure 6O** [↗](#) and **Supplemental Figure 3** [↗](#)). In VTA, we found that 36% of ChR2:mCherry-negative neurons were connected, all of which displayed short-latency oIPSCs (**Figure 6P-R** [↗](#)). Altogether our data indicate that VTA GABAergic projection neurons, and to a lesser extent glutamatergic projection neurons, make functional synapses within VTA.

The use of WT mice in these experiments allowed us to assay for the presence of inhibitory currents mediated by GABA-releasing neurons and for excitatory currents mediated by glutamate-releasing neurons, from the same postsynaptic cells. However, we also performed similar experiments using VGAT-Cre and MOR-Cre mice. In these experiments we used an intersectional approach similar to **Figures 2** [↗](#)-**4** [↗](#). We targeted NAc-projecting VGAT-Cre neurons by injecting retroAAV for Cre-dependent expression of Flp into NAc, plus AAV for Flp-dependent expression of ChR2:YFP into VTA (**Figure 7A-D** [↗](#)). We found that the majority of YFP-negative VTA neurons that we recorded displayed short-latency oIPSCs (**Figure 7E-G** [↗](#)) (note that we did not assay for oEPSCs in this experiment).

Finally, we did a similar experiment using MOR-Cre mice to target VP-projecting VTA neurons (**Figure 7H-K** [↗](#)). Here we found that half of the recorded YFP-negative cells were connected, showing primarily short-latency oIPSCs along with fewer short- and long-latency oEPSCs (**Figure 7L-N** [↗](#)).

Discussion

The VTA plays consequential roles in the orchestration of motivated behaviors and is composed of heterogeneous populations of DA, GABA, and glutamate neurons that send dense projections to diverse forebrain regions (Fields et al., 2007 [↗](#); Morales & Margolis, 2017 [↗](#)). Yet VTA DA, GABA, and glutamate neurons also release their neurotransmitters locally within VTA. DA is released from somatodendritic compartments, activating DA autoreceptors (Ford, 2014 [↗](#)). Multiple lines of evidence indicate that GABA and glutamate neurons resident to VTA synapse locally on to VTA DA

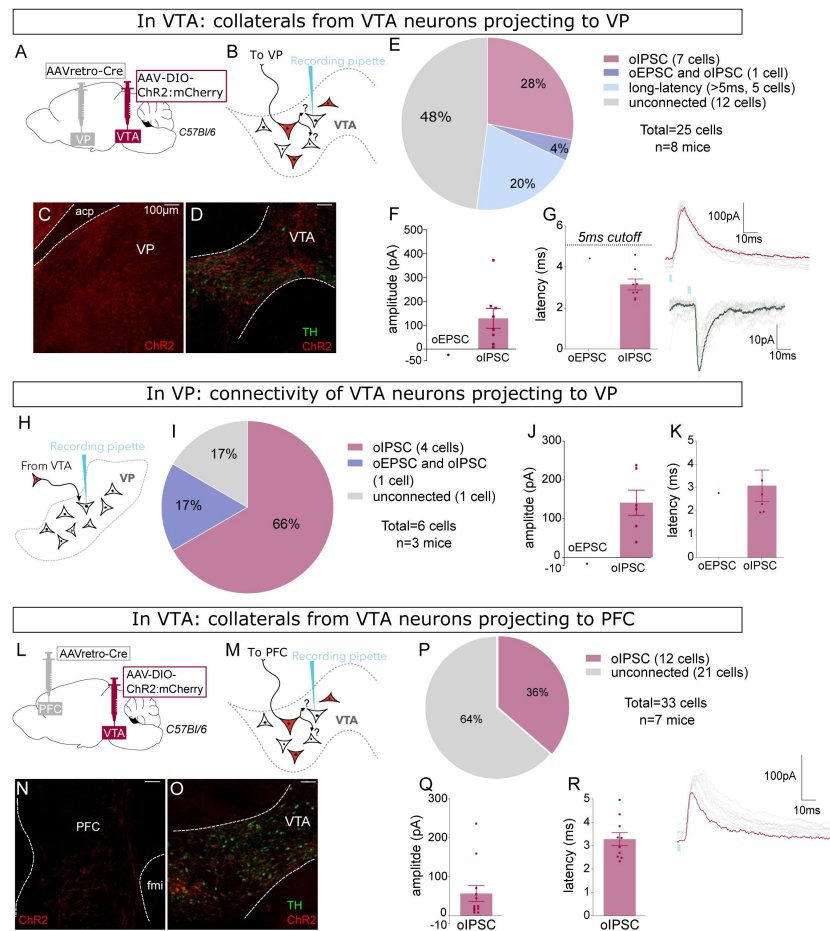


Figure 6

VP-projecting and PFC-projecting VTA GABA and glutamate neurons make intra-VTA synapses.

(A) Dual AAV approach to express ChR2:mCherry in VP-projecting VTA neurons in wild-type mice. (B) Patch-clamp recordings from ChR2:mCherry-negative neurons of VTA to test for collateralizing synapses made by VP-projectors. (C) Coronal images showing ChR2:mCherry expression in VP and (D) VTA; scale bars 100 μ m. (E) ChR2:mCherry-negative VTA neuron responses to optogenetic stimulation of VP-projectors. (F) Peak amplitude and (G) onset latency of connected cells that displayed an oEPSC and/or oIPSC (excluding long-latency), with example traces. (H) Recordings of oPSCs from neurons in VP and (I) VP responses to optogenetic stimulation of VP-projecting VTA neurons from approach described in panel A. (J) Peak amplitude and (K) onset latency of connected VP neurons that displayed an oEPSC and/or oIPSC, with example traces. (L) Dual AAV approach to express ChR2:mCherry in PFC-projecting VTA neurons in wild-type mice. (M) Patch-clamp recordings from ChR2:mCherry-negative neurons of VTA to test for collateralizing synapses made by PFC-projectors. (N) Coronal images showing ChR2:mCherry expression in PFC and (O) VTA; scale bars 100 μ m. (P) ChR2:mCherry-negative VTA neuron responses to optogenetic stimulation of PFC-projectors. (Q) Peak amplitude and (R) onset latency of connected cells that displayed an oEPSC and/or oIPSC, with example trace.

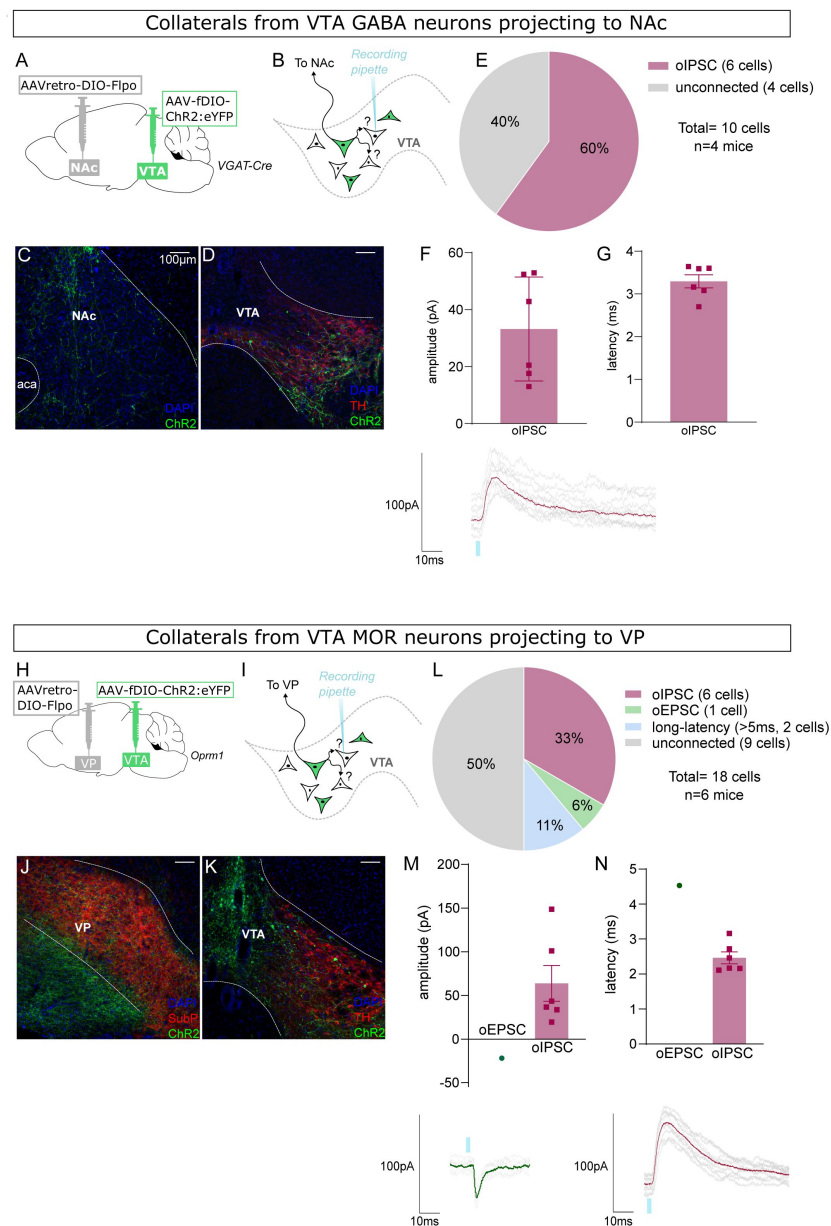


Figure 7

NAc-projecting VTA GABA neurons, and VP-projecting VTA MOR neurons, make intra-VTA synapses.

(A) Dual AAV approach to express ChR2:eYFP in NAc-projecting VTA neurons in VGAT-Cre mice. (B) Patch-clamp recordings from ChR2:eYFP-negative neurons of VTA to test for collateralizing synapses made by NAc-projectors. (C) Coronal images showing ChR2:eYFP expression in NAc and (D) VTA; scale bars 100 μ m. (E) ChR2:eYFP-negative VTA neuron responses to optogenetic stimulation of NAc-projectors. (F) Peak amplitude of connected cells that displayed an oIPSC, with example trace. (G) Latency to oIPSC onset. (H) Dual AAV approach to express ChR2:eYFP in VP-projecting VTA neurons in MOR-Cre mice. (I) Patch-clamp recordings from ChR2:eYFP-negative neurons of VTA to test for collateralizing synapses made by VP-projectors. (J) Coronal images showing ChR2:eYFP expression in VP and (K) VTA; scale bars 100 μ m. (L) ChR2:eYFP-negative VTA neuron responses to optogenetic stimulation of VP-projectors. (M) Peak amplitude of connected cells that displayed an oEPSC and/or oIPSC (excluding long-latency), with example traces. (N) Latency to oPSC onset (excluding long-latency).

and non-DA neurons (Beier, 2022 [↗](#); Dobi et al., 2010 [↗](#); Omelchenko & Sesack, 2009 [↗](#); Soden et al., 2020 [↗](#); Tan et al., 2012 [↗](#)). VTA GABA neurons that make local synapses within VTA have frequently been described as interneurons (Bonci & Williams, 1996 [↗](#); Johnson & North, 1992 [↗](#); Luscher & Malenka, 2011 [↗](#); O'Brien & White, 1987 [↗](#)). But there is scant evidence that VTA GABA interneurons and projection neurons represent distinct cell types. In this study we used retroAAV to target VTA neurons that project to NAc, VP, PFC, or LHb for recombinase-dependent expression of synaptic tags to image putative release sites, or of opsin for optogenetic stimulation of projection neurons while recording synaptic events in neighboring VTA neurons. Both approaches point to the same conclusion, that at least a subset of GABA and glutamate projection neurons collateralize locally and make intra-VTA synapses.

In cortex, hippocampus and other areas dominated by glutamate projection neurons the term interneuron is often used to describe inhibitory GABA neurons that synapse on to neurons within the same structure as their soma reside. However, the limbic basal ganglia circuits in which VTA neurons are embedded include many GABAergic projection neurons, at least subsets of which are understood to make both distal and local synapses. For example, DA D2 receptor-expressing medium spiny neurons are GABA projection neurons that also make extensive local collaterals that laterally inhibit and regulate other striatal neurons (Dobbs et al., 2016 [↗](#); Tunstall et al., 2002 [↗](#)). Thus, a meaningful definition of the term in the context of mesolimbic circuitry, and the definition of interneuron we use in this study, is a neuron that synapses locally but not distally. Indeed, this definition captures many well characterized populations of neurons throughout the cortex, hippocampus, striatum, or cerebellum (Lim et al., 2018 [↗](#); Maccaferri & Lacaille, 2003 [↗](#); Pelkey et al., 2017 [↗](#); Petilla Interneuron Nomenclature et al., 2008). For example, cortical or striatal interneurons that express PV, SST, or cholinergic markers can be readily distinguished at the molecular level, but also by physiological properties that distinguish them from projection neurons or other cell types (Huang & Paul, 2019 [↗](#); Markram et al., 2004 [↗](#); Tepper et al., 2018 [↗](#)).

The VTA has been known to contain non-DA GABA neurons since at least the early 1980's (Gysling & Wang, 1983; Oertel & Mugnaini, 1984 [↗](#); Waszczak & Walters, 1980 [↗](#); Yim & Mogenson, 1980 [↗](#)). Moreover, VTA GABA neurons were demonstrated to make inhibitory synapses on to VTA DA neurons (Bayer & Pickel, 1991 [↗](#); Johnson & North, 1992 [↗](#)). VTA DA neurons may be distinguished from non-DA neurons (at least in lateral VTA) based on firing rate and other physiological and pharmacological features (Bunney et al., 1973 [↗](#); German et al., 1980 [↗](#); Waszczak & Walters, 1980 [↗](#)). These observations serve as the primary basis for the notion of a VTA interneuron. However, those observations could instead be explained by VTA GABA projection neurons that collateralize locally. Indeed, one notable study used in vivo electrophysiology to identify a population of non-DA projection neurons and showed that they were reliably activated by antidromic stimulation of the internal capsule (Steffensen et al., 1998 [↗](#)). This suggests that the population of non-DA VTA neurons they were able to identify through in vivo recordings were projection neurons. Likewise, substantia nigra (SN) compacta DA neurons were inhibited by antidromically identified GABA projection neurons in SN reticulata (Tepper et al., 1995 [↗](#)), suggesting a parallel between our findings in VTA and those in SN.

If GABA interneurons represent one or more bona fide VTA cell types, then it is reasonable to suppose that they would be distinguishable by a molecular marker (or a constellation of markers). Markers that confer a GABAergic identity label VTA GABA projection neurons, and thus could not distinguish putative interneurons from projection neurons. However, several other markers have been shown to co-localize with a subset of VTA GABA but not DA neurons and thus represent potential interneuron markers (Bouarab et al., 2019 [↗](#); Nagaeva et al., 2020 [↗](#); Olson & Nestler, 2007 [↗](#); Phillips et al., 2022 [↗](#)). We selected four of these markers that had well-validated Cre lines: PV, SST, MOR, and NTS. Using two different tracing strategies we confirmed that these markers are expressed in a subset of VTA neurons that are primarily non-dopaminergic. We found that PV⁺ VTA neurons project densely to LHb (and weakly to other projection targets), while SST⁺, NTS⁺, and especially MOR⁺ VTA neurons project densely to VP (and other projection targets).

Thus, while it is possible that there exists VTA interneurons that express one or more of these markers, none of these markers can be used on its own to discriminate between VTA projection neurons and VTA interneurons.

While the retroAAV approach resulted in strong labeling of projection neurons, including GABA- and glutamate-releasing neurons, it is likely that the intrinsic tropism of retroAAV influenced the population of projection cells that we labeled. Indeed, prior work showed that midbrain DA neurons are not efficiently targeted by the retroAAV vector we used (Tervo et al., 2016). Thus, populations of neurons that release DA and co-release GABA or glutamate may not contribute to the signals we measured.

In addition to GABA projection neurons, our experiments revealed that glutamate projection neurons in VTA also make local synapses. Therefore, the local excitatory synaptic events observed in prior studies (Dobi et al., 2010; McGovern et al., 2023; Yoo et al., 2016) may be driven by collaterals made by VTA glutamate projection neurons rather than a population of glutamate interneurons. Interestingly, we found that optogenetic activation of unspecified VTA projection neurons induced intra-VTA oEPSCs more rarely than intra-VTA oIPSCs. However, we also observed long-latency oPSCs, that were likely the result of activating VTA glutamate projection neurons that make intra-VTA excitatory collaterals and drive feed-forward recruitment of other VTA cells that also make local synapses.

The VTA integrates a large number of inhibitory inputs from a multitude of brain regions. However, recent studies indicate that neurons local to VTA preferentially inhibit DA neurons compared to GABAergic afferents from distal sources (Beier, 2022; Soden et al., 2020). Yet these studies cannot determine whether the local neurons synapsing on to VTA DA neurons are interneurons versus collaterals made by projection neurons. Moreover, VTA has been shown to receive an important GABAergic input from the rostral medial tegmental nucleus (RMTg) (Jhou, 2005; Perrotti et al., 2005). While VTA and RMTg are considered separate structures, the boundary between caudal VTA and rostral RMTg is ambiguous, and this area is dominated by GABA neurons (Smith et al., 2019). Interestingly, RMTg inhibitory synapses on to VTA DA neurons are strongly inhibited by MOR activation, and thus some of the functions classically attributed to VTA interneurons may be mediated by these short-range projection neurons (Jhou, 2021; Jhou et al., 2009; Kaufling & Aston-Jones, 2015; Matsui et al., 2014; St Laurent et al., 2020).

Within VTA, we found that MOR and several other potential interneuron markers were instead expressed in projection neurons. Other interneuron marker candidates have been suggested but, to our knowledge, no other VTA marker has been shown to be expressed selectively within a VTA interneuron population (Bouarab et al., 2019; Paul et al., 2019). One promising candidate is neuronal nitric oxide synthase (nNOS) which labels a subset of VTA GABA neurons in the parabrachial pigmented area of VTA that may not project distally, but also labels DA and glutamate neurons in adjacent areas of VTA and substantia nigra (Paul et al., 2018). Future work, for example using intersectional labeling, may resolve whether nNOS selectively labels bona fide GABA interneurons in VTA. Another candidate marker of interest is prepronociceptin (PNOC). A recent report showed that PNOC labels a population of paranigral non-DA neurons that make dense intra-VTA synapses without projecting to NAc (Parker et al., 2019). However, VTA PNOC neurons express both GABA and glutamate markers, and it is not clear whether they project to other VTA projection targets, such as VP or LHB.

In sum, we provide multiple lines of evidence that VTA GABA (and glutamate) neurons that project to distal targets also collateralize locally and make intra-VTA synapses. We also demonstrate that several candidate markers, including MOR, are expressed in VTA projection neurons. Future efforts may reveal positive evidence for the existence of VTA interneurons, for example through the identification of a marker, or a combinatorial set of markers, that labels VTA neurons that

make local but not distal connections. At present, however, there is little evidence to support the notion of a VTA interneuron. We suggest that some functions prior attributed to VTA interneurons, such as MOR-mediated disinhibition of DA neurons, may instead be mediated by VTA projection neurons that make synaptic collaterals on to DA neurons. In this way the actions of opioids on VTA neurons would not only disinhibit DA neurons, but simultaneously inhibit GABA (or glutamate) release from distal VTA projections to VP and elsewhere. Indeed, in light of our increasing understanding for the roles of VTA GABA and glutamate projections in processes underlying behavioral reinforcement, their direct effects on distal targets may contribute to opioid-induced behaviors or adaptations relevant to drug addiction distinct from their effects on VTA DA neurons.

Methods

Animals

Mice were group-housed (up to 5 mice/cage), bred at the University of California, San Diego (UCSD), kept on a 12h light-dark cycle, and had access to food and water ad libitum. Initial breeders were acquired from The Jackson Laboratory (**Table 1** [↗](#)), except for the MOR-Cre (Bailly et al., 2020 [↗](#)) obtained from the lab of Brigitte Kieffer (University of Strasbourg). All mice were bred with a C57Bl/6 background and used as a mix of heterozygotes and homozygotes. Male and female mice were used in all experiments. All experiments were performed in accordance with protocols approved by the UCSD Institutional Animal Care and Use Committee.

Stereotaxic surgery

Mice >5 weeks (and up to 6months old) were deeply anesthetized with Isoflurane (502017, Primal Critical Care) and placed on a stereotaxic frame (Kopf 1900) for microinjection into discrete brain areas (**Table 2** [↗](#)). After ensuring the skull is flat small holes were drilled (1911-C Kopf) and AAVs (**Table 3** [↗](#)) infused with Nanoject (3-000-207, Drummond) using glass injectors (3-000-203-G/X, Drummond) pulled on a horizontal pipette puller (P-1000 Sutter Instrument). After infusion the injector was left for 3 to 5 min then withdrawn. Analgesia was provided via injections with 5mg/kg S.C. Carprofen (510510 Vet One). Electrophysiology was performed >3 weeks after surgery, histology >5 weeks.

Histology

Mice were deeply anesthetized with pentobarbital (200 mg.kg-1, i.p., 200-071, Virbac) and transcardially perfused with 30mL of PBS (BP399, Fisher bioreagents) followed by 50mL of 4% PFA (18210, Electron Microscopy Sciences) in PBS. Brains were removed, post-fixed in 4% PFA overnight, and dehydrated in 30% sucrose (S0389, Sigma-Aldrich) in PBS for 48h then flash-frozen in isopentane. Brains were cut in 30µm coronal sections on a cryostat (CM3050S, Leica). Sections were selected to encompass the VTA and efferents to PFC, NAc, VP, and LHb. Sections were blocked in 5% normal donkey serum/0.4% Triton X-100 in PBS for 1h at room temperature and incubated with primary antibodies (**Table 4** [↗](#)) overnight at 4°C in the blocking buffer. Next day, slides were washed three times in 0.4% Triton X-100 in PBS for 5 min and incubated with secondary antibodies for two hours at room temperature shielded from the light. Finally, sections were washed three times in 0.4% Triton X-100 in PBS for 5 min and coverslipped with Fluoromount-G (Southern Biotech) containing 0.5µg/mL of DAPI (Roche). Images were taken using a Zeiss Axio Observer Epifluorescence microscope. For **Supplemental Figure 1** [↗](#), the same procedure was used but the brains were cut sagittally at a 15° angle.

Abbreviation	Mouse line	Jackson Labs #
VGAT-Cre	B6J.129S6(FVB)-Slc32a1 ^{tm2(cre)Low} /MwarJ	028862
VGLUT2-Cre	STOCK Slc17a6 ^{tm2(cre)Low} /J	016963
PV-Cre	B6.129P2-Pvalb ^{tm1(cre)Arbr} /J	017320
NTS-Cre	B6;129-Nts ^{tm1(cre)Mgm} /J	017525
SST-Cre	B6N.Cg-Sst ^{tm2.1(cre)Zjh} /J	018973
R26-ZsGreen	B6.Cg-Gt(ROSA)26Sor ^{tm6(CAG-ZsGreen1)Hze} /J	007906

Table 1.
Mouse lines

Table 2.**Stereotaxic coordinates**

Injection site	ML	AP	DV
VTA	-0.35	-3.35	-4.3
NAc	-0.8	1.34	-4.5
PFC	-0.4	+1.9	-1.7
VP	-1.45	+0.55	-5.35
VTA (MOR-Cre)	-0.6	-.3.4	-4.4

Table 3.**AAV vectors**

AAV	Titer	Packaged by	Volume	Addgene #
AAVretro-EF1a-Cre	3*10 ¹³	Salk GT3	150nL	55636
AAV5-EF1α-DIO-hChr2(H134R)-mCherry	2*10 ¹³	Addgene	150nL for ephys 100nL for histology	20297
AAVDJ-hSyn1-FLEXFRT mGFP-2A-Synaptophysin:mRuby	2*10 ¹³	Addgene	150nL	71761
AAVretro-hSyn1-DIO-Flpo	2*10 ¹²	Salk GT3	150nL	NA

Primary antibody	Species	Catalog #	Company	Dilution
TH	Sheep	P60101	Pel-Freez	1:2000
DsRed	Rabbit	632496	Clontech	1:2000
GFP	Chicken	A10262	Invitrogen	1:2000
Substance P	Rat	MAB356	Millipore	1:200
Chat	Goat	AB144P	Millipore	1:400
Donkey secondary antibody	Alexa Fluor conjugate	Catalog #	Company	Concentration
anti-Sheep	488	713-545-003	Jackson Immuno Research	3 µg/mL
anti-Sheep	594	713-585-147		
anti-Sheep	647	713-605-147		
anti-Rabbit	594	711-585-152		
anti-Chicken	488	703-546-155		
anti-Rat	647	712-605-153		
anti-Goat	647	705-605-147		

Table 4.

Antibodies

Colocalization with TH and counting

For each genetic marker, 3–4 mice and 4 sections through VTA per mouse were stained with antibodies against TH and DsRed. All sections were imaged at 10X with the same exposure parameters, using a Zeiss AxioObserver equipped with Apotome2 for structured illumination. The same display settings were applied to all images within condition. TH signal was used to define the boundaries of VTA and align to Bregma point. Cells expressing mCherry were identified first, then scored for presence or absence of TH expression. The counts were done independently by two experimenters and a high correlation was observed between the experimenters ($R^2=0.77$, $p<0.001$, 60 total sections). Each cell that was only identified by one observer was reassessed for inclusion in final dataset.

Single injection tracing

For evaluation of projection targets following a single AAV injection into VTA, we excluded subjects that had <30% of labeled cell bodies outside the VTA (**Table 5**). We also excluded subjects that had mCherry-labeled cell bodies in supramammillary nucleus. But we did not exclude mice with spread to red nucleus or IPN because these regions are not known to project to NAc, PFC, VP, or LHB (Liang et al., 2011; McLaughlin et al., 2017).

Electrophysiology

Mice were deeply anesthetized using pentobarbital (200 mg/kg, i.p., Virbac) and transcardially perfused with 30 ml cold N-methyl D-glucamine (NMDG)-artificial Cerebro-Spinal Fluid (aCSF, containing in mM: 92 NMDG, 2.5 KCl, 1.25 NaH_2PO_4 , 30 NaHCO_3 , 20 HEPES, 25 D-glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, 10 MgSO_4 , 0.5 CaCl_2 , pH 7.3) saturated with carbogen (95% O_2 –5% CO_2). Sections (coronal, 200 μm) were cut through VTA while immersed in cold NMDG-aCSF using a vibratome (VT1200S, Leica). Slices were incubated at 33°C for 25–30 min in a holding chamber containing NMDG-aCSF saturated with carbogen. During the incubation NaCl concentration was slowly increased in 5-min increments by spiking the holding-aCSF with a 2M NaCl solution diluted with the NMDG-aCSF (Ting et al., 2018). Slices were incubated at 25°C for 30–45 min in a holding chamber containing holding-aCSF (containing in mM: 115 NaCl, 2.5 KCl, 1.23 NaH_2PO_4 , 26 NaHCO_3 , 10 D-glucose, 5 sodium ascorbate, 2 Thiourea, 3 sodium pyruvate, 2 MgSO_4 , 2 CaCl_2 , pH7.3) saturated with carbogen. While recording, slices were superfused with 31°C recording-aCSF (containing in mM: 125 NaCl, 2.5 KCl, 1.20 NaH_2PO_4 , 26 NaHCO_3 , 12.5 D-glucose, 2 MgSO_4 , 2 CaCl_2) using an in-line heater (TC-324B, Warner) at 1.5 ml/min. Whole-cell patch-clamp recordings from mCherry-negative VTA neurons were performed under visual guidance with infrared illumination and differential interference contrast using a Zeiss AxioCam MRm, Examiner.A1 equipped with a 40X objective. 6–7 M Ω patch pipettes were pulled from borosilicate glass (Sutter Instruments) and filled with internal solution (containing in mM: 133.4 cesium-methanesulfonate, 22.7 HEPES, 0.45 EGTA, 3.2 NaCl, 5.7 tetraethylammonium-chloride, 0.48 NA-GTP, 4.5 $\text{Na}_2\text{-ATP}$, pH to 7.3 with Cesium-OH). Postsynaptic currents were recorded in whole-cell voltage clamp (Multiclamp 700B amplifier, Axon Instruments), filtered at 2 kHz, digitized at 20 kHz (Axon Digidata 1550, Axon Instruments), and collected using pClamp 10 software (Molecular Device). Neurons were first held at -65mV to record excitatory currents and then at 0mV to record inhibitory currents. Optogenetic-evoked postsynaptic currents (oPSCs) were induced by flashing blue light (two 10Hz 2ms pulses, every 15s) through the light path of the microscope using a light-emitting diode (UHP-LED460, Prizmatix, 50mW) under computer control. We discarded likely ChR2^+ cells, displaying photocurrent (**Supplemental Figure 5**), identified as starting within 1 ms of the light pulse, as well as cells where the series resistance varied by more than 20%. After breaking-in we waited 2–3 minutes before beginning optogenetic stimulation. For each cell we first recorded a baseline period (4–6 min) and for some cells baseline was followed by 4–6 min bath

Figure 1	Surgeries (n)	Tracing cases (M/F)	Excluded: spread	Excluded: technical failure	TH counting cases (M/F)
PV	9	2/1	4	2	3/1
SST	5	0/3	2	0	0/3
MOR	13	3/0	8	2	3/1
NTS	5	0/4	1	3	0/4

Table 5.

Cases included/excluded for Figure 1.

application of drug: 1 μ M TTX, 50-100 μ M 4AP, 10 μ M DNQX, 100 μ M PTX ([Table 6](#)). For each condition we averaged the last 10 sweeps; amplitude represented the peak current, and latency calculated as the duration from light onset to current onset.

Statistics

Data values are presented as means $\pm$ SEM. Effects of drug application were subjected to Friedman's test (nonparametric ANOVA) followed by a Dunn's posthoc test. Statistical significance was set at $p < 0.05$.

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Additional information

Author Contributions

Lucie Oriol: [Contribution](#), methodology, investigation (stereotaxic surgeries, histology, electrophysiology, microscopy, cell counting), analysis and validation, visualization, project coordination and writing. [Competing interests](#), no competing interests declared

Melody Chao: [Contribution](#), investigation (histology) and visualization. [Competing interests](#), no competing interests declared.

Grace J. Kollman: [Contribution](#), investigation (cell counting) and resources (breeding). [Competing interests](#), no competing interests declared.

Dina S. Dowlat: [Contribution](#), investigation (LHb stereotactic injections) and resources (reagents provision). [Competing interests](#), no competing interests declared.

Sarthak M. Singhal: [Contribution](#), investigation (Electrophysiology in VP). [Competing interests](#), no competing interests declared.

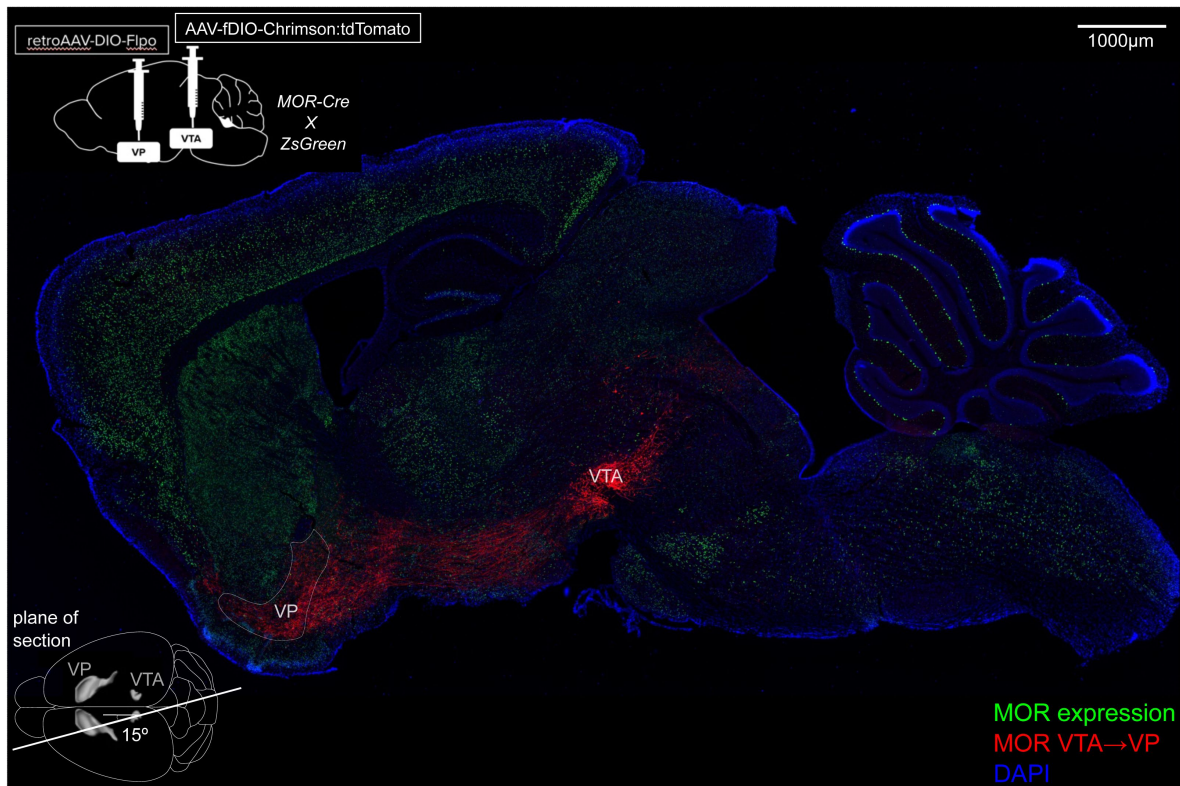
Thomas Steinkellner: [Contribution](#), investigation (cloning). [Competing interests](#), no competing interests declared.

Thomas S. Hnasko: [Contribution](#), conceptualization, methodology, supervision, funding acquisition, validation, resources, and writing. [Competing interests](#), no competing interests declared.

Table 6.

Drugs and physiology reagents

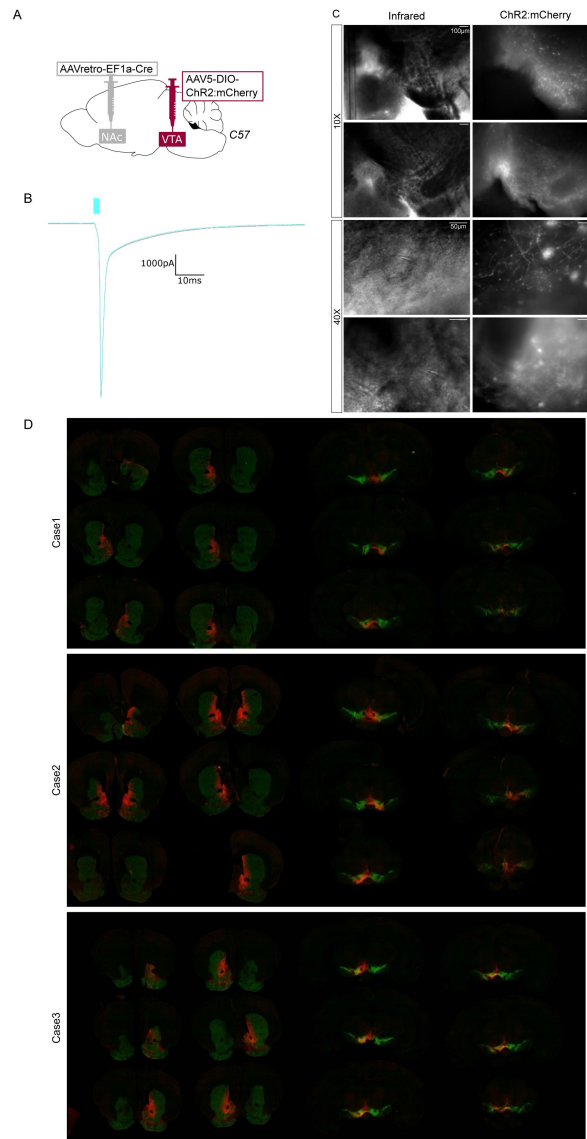
Reagent	Catalog #	Company
4AP	0940	Tocris
CaCl ₂ .2H ₂ O	BP510	Fisher bioreagents
Ces met	2550-61-0	Sigma-Aldrich
D-Glucose	G8270	Sigma
DNQX	D0540	Sigma
EGTA	E3889	Sigma
HEPES	H3375	Sigma
KCl	BP366	Fisher bioreagents
MgSO ₄ .7H ₂ O	M80	Fisher bioreagents
Na-GTP	G8877	Sigma
Na ₂ -ATP	A2383	Sigma
NaCl	BP358	Fisher bioreagents
NaH ₂ PO ₄	BP329	Fisher bioreagents
NaHCO ₃	BP328	Fisher bioreagents
NMDG	M2004	Sigma-Aldrich
PTX	P1675	Sigma
Sodium ascorbate	A7631	Sigma
Sodium pyruvate	P2256	Sigma-Aldrich
TEA chloride	86616	Fluka
Thiourea	T8656	Sigma-Aldrich
TTX	1069	Tocris



Supplemental Figure 1

Projection of MOR-Cre-expressing VTA neurons to VP (related to Figure 3).

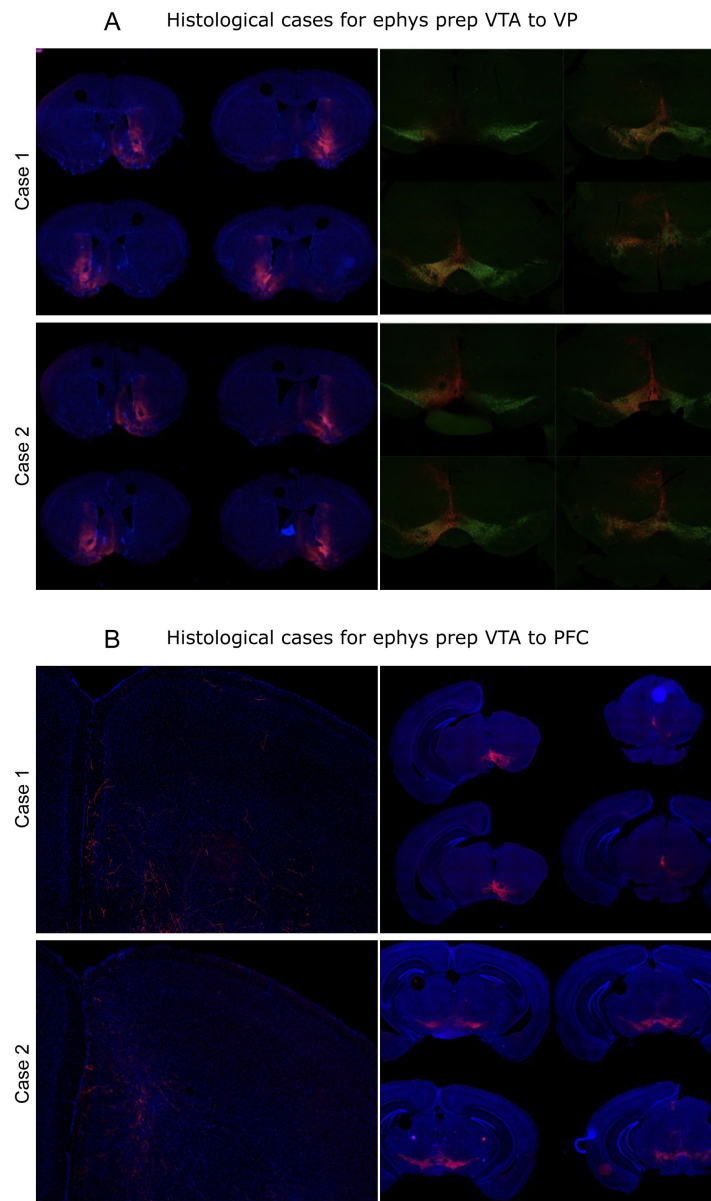
Sagittal image, genotypes, and schematics of dual AAV approach and approximate location and sectioning angle of the cut. ZsGreen (green) labels all cells that have expressed MOR-Cre, Chrimson:tdTomato (red) labels cells/fibers from MOR-Cre VTA neurons projecting to VP, DAPI (blue) labels nuclei.



Supplemental Figure 2

Photocurrent and histological validation of approach used in Figure 5 (related to Figure 5).

(A) Dual AAV approach to express ChR2:mCherry in NAc-projecting VTA neurons in wild-type mice. (B) Example opsin-mediated photocurrent from ChR2:mCherry-positive neuron of VTA. (C) Example images under DIC IR light and mCherry expression around patch-clamp pipettes. (D) Additional cases of histology.



Supplemental Figure 3

Histological validation of approach used in Figure 6 (related to Figure 6).

(A) Additional cases of histology with expression of ChR2:mCherry in VP-projecting VTA neurons in wild-type mice. (B) Additional cases of histology with expression of ChR2:mCherry in PFC-projecting VTA neurons in wild-type mice.

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Reviewer #1 (Public review):

The manuscript by Lucie Oriol et al. revisits the understanding of interneurons in the ventral tegmental area (VTA). The study challenges the traditional notion that VTA interneurons exclusively form local synapses within the VTA. Key findings of the study indicate that VTA GABA and glutamate projection neurons also make local synapses within the VTA. This evidence suggests that functions previously attributed to VTA interneurons could be mediated by these projection neurons.

The study tested four genetic markers-Parvalbumin (PV), Somatostatin (SST), Mu-opioid receptor (MOR), and Neurotensin (NTS)-to determine if they selectively label VTA interneurons. The findings indicate that these markers label VTA projection neurons rather than selectively identifying interneurons. Using a combination of anatomical tracing and brain slice physiological recordings, the study demonstrates that VTA projection neurons make functional inhibitory or excitatory synapses locally within the VTA. These data challenge the conventional view that VTA GABA neurons are purely interneurons and suggests that inhibitory projection neurons can serve functions previously attributed to VTA interneurons. Thus, some functions traditionally ascribed to interneurons may be carried out by projection neurons with local synapses. This has significant implications for understanding the neural circuits underlying reward, motivation, and addiction.

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Reviewer #2 (Public review):

Summary:

In this manuscript, authors use a combination of transgenic animals, intersectional viruses, retrograde tracing, and ex-vivo slice electrophysiology to show that VTA projections neurons synapse locally. First, the authors injected a cre-dependent channelrhodopsin into the VTA of PV, SST, MOR, and NTS-Cre mice. Importantly, PV, SST, MOR, and NTS are molecular markers previously used to describe VTA interneurons. Imaging of known VTA target regions identified that these neurons are not localized to the VTA and instead project to the PFC, NAc, VP, and LHb. Next, the authors used an intersectional viral strategy to label projections neurons with both GFP (membrane localized) and Syn:Ruby (release sites). These experiments identified that VTA projection neurons also make intra-VTA synapses. Finally, the authors use a combination of optogenetics and ex-vivo slice electrophysiology to show that neurons projecting from the VTA to the NAc/VP/PFC also synapse locally. Overall, the conclusions are well supported by the data.

Strengths:

Previous literature has described Pvalb, Sst, Oprm1, and Nts as selective markers of VTA interneurons. Here, the authors make use of cre driver lines to show that neurons defined by these genes are not classically-defined interneurons and project to known VTA target regions. Additionally, the authors convincingly use intersectional viral approaches and slice electrophysiology to show that projection neurons synapse onto neighboring cells within the VTA

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Reviewer #3 (Public review):

Summary:

This study from Oriol et al. first uses transgenic animals to examine projection targets of specific subtypes of VTA GABA neurons (expressing PV, SST, MOR, or NTS). They follow this with a set of optogenetic experiments showing that VTA projection neurons (regardless of genetic subtype) make local functional connections within the VTA itself. Both of these findings are important advances in the field. Notably, both GABAergic and glutamatergic neurons in the VTA likely exhibit these combined long/short-range projections.

Strengths:

The main strength of this study is the series of optogenetic/electrophysiological experiments that provide detailed circuit connectivity of VTA neurons. The long-range projections to the VP (but not other targets) are also verified to have functional excitatory and inhibitory components. Overall, the experiments are well executed and the results are very relevant in light of the rapidly growing knowledge about the complexity and heterogeneity of VTA circuitry.

Another strength of this study is the well-written and thoughtful discussion regarding the current findings in the context of the long-standing question of whether the VTA does or does not have true interneurons.

Comments on revisions:

The authors have addressed all of my questions admirably, and the final result is considerably improved and remains a valuable contribution to the field.

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Author response:

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

Reviewer #1:

Regarding the manuscript's clarity, the sentence on page 5, "We also stained VTA sections for Tyrosine hydroxylase (TH) to estimate the rate of ChR2 colocalization with DA neurons," reads awkwardly. Removing the word "rate" could improve clarity.

We have made the recommended clarifying edit (page 5, lines 30-31).

Additionally, the anatomical data and findings are largely non-quantitative in nature. However, solid microscopy images are presented to support each claim. Additional quantification would strengthen the paper, specifically the quantification of projection density for each population and the proportion of each subpopulation that projects to their regions of interest.

To rigorously quantify the projection density of each subpopulation would require a level of exhaustivity our study was not designed for. This is because during microscopy we focused efforts on imaging regions containing dense signals but did not exhaustively image regions receiving apparently weak or no input. While we considered including a semi-quantitative table of projection density, based on the data available we could not discriminate with confidence between, e.g., regions recipient of minimal input versus no input from VTA populations. Thus, while we stand by our descriptive statements we do not expand on those further.

The authors should consider discussing the possibility that subpopulations of these cells could still be true interneurons especially if cells were looked at the single neuron level of resolution.

We agree that some of the VTA populations we studied could include subpopulations that are bona fide interneurons. The identification of alternate markers or combinations of markers, or use of single-cell imaging approaches may indeed support this possibility in future. This is discussed in the context of currently available evidence on page 5 lines 32-34, page 11 lines 2-4, page 12 lines 2-11, and page 12 lines 15-16.

Overall, the paper is well-written and important for the field and beyond.

Thank you!

Reviewer #2:

Weaknesses:

While the authors use several Cre driver lines to identify GABAergic projection neurons, they then use wild-type mice to show that projection neurons synapse onto neighboring cells within the VTA. This does not seem to lend evidence to the idea that previously described "interneurons" are projection neurons that collateralize within the VTA.

We think the use of WT mice is a strength because it allows us to measure both GABA and non-GABA synapses made by VTA projections on to the same cells within VTA. However, we have also done this experiment targeting NAc-projecting VTA VGAT-Cre neurons, and VP-projecting VTA MOR-Cre neurons. Consistent with the WT dataset, we find that these defined projection neurons also make intra-VTA synapses. These data are now included as Figure 7.

More broadly. Our review of the literature finds very little evidence to support the notion of a VTA interneuron as we define it: VTA neurons that makes only local connections. But the absence of evidence need not imply evidence of absence, thus we do not claim that all VTA neurons previously presumed to be interneurons must be projection neurons. We do express confidence in our findings that VTA projection neurons (that include GABA-releasing neurons) make local synapses in VTA. We argue that in the absence of compelling positive evidence for the existence of VTA interneurons, such as a selective marker, "we", "the field", should not presume their existence.

Other suggestions:

(1) While the authors present evidence that some projection neurons also synapse locally, there is no quantification as to the proportion of each neuronal subtype that collateralizes within the VTA. This would be a useful analysis.

We agree this would be useful information. But our experiments were not designed to answer this question. Indeed, we have not conceived of a feasible method to discriminate between collateralizing and non-collateralizing VTA projection neurons at the single-cell level, thus we do not know how we would calculate such proportions.

(2) There is significant interest in the molecular heterogeneity and spatial topography of the VTA. Additional analyses of the spatial topography of labeled projectors would be useful. For example, knowing if Pvalb+ projection neurons are distributed throughout the VTA or located along the midline would be a useful analysis.

Prior studies and public databases (e.g., Allen brain atlas, GENSAT) allow one to visualize the location of VTA neurons positive for Pvalb and the other markers we investigated (Olson & Nestler, 2007). However, these label the entire population of neurons and thereby include those that project to any of the various projection targets. There are also studies that have used retrograde labeling approaches to map the distribution of labeled VTA cells projecting to one or another target (Beier et al., 2015; Lammel et al., 2008; Margolis et al., 2006). For example, finding that LHb-projecting neurons (a major target of Pvalb+ VTA neurons) are enriched in medial VTA (Root et al., 2014). From this evidence we might infer that Pvalb+ VTA neurons that project to LHb are likely to be medially biased. Future studies may more carefully map the intersection of specific projection targets for each VTA subpopulation.

Reviewer #3 (Recommendations For The Authors):

Weaknesses:

This study has a few modest shortcomings, of which the first is likely addressable with the authors' existing data, while the latter items will likely need to be deferred to future studies:

(1) Some key anatomical details are difficult to discern from the images shown. In Figure 1, the low-magnification images of the VTA in the first column, while essential for seeing what overall section is being shown, are not of sufficient resolution to distinguish soma from processes. A supplemental figure with higher-resolution images could be helpful.

We uploaded a higher resolution file for figure 1.

Also, where are the insets shown in the second column obtained from? There is not a corresponding marked region on the low-magnification images. Is this an oversight, or are these insets obtained from other sections that are not shown?

This was an oversight, we added the corresponding marked region to the low-magnification images.

Lastly, there is a supplemental figure showing the NAc injection sites corresponding to Figure 5, but not one showing VP or PFC injection sites in Figure 6. Why not?

We added a figure with histology examples for the VP and the PFC injection sites as done for Figure 5, included as Supplemental Figure 3.

(2) Because multiple Chr2 neurons are activated in the optogenetic experiments, it is not clear how common is it for any specific projection neuron to make local connections. Are the observed synaptic effects driven by just a few neurons making extensive local collateralizations (while other projection neurons do not), or do most VTA projection neurons have local collaterals? I realize this is a complex question, that may not have an easy answer.

This is a great question but, indeed, we don't know the answer. As mentioned in response to Reviewer #2, we are not convinced there is a currently feasible way to discriminate between collateralizing and non-collateralizing cells at the single cell level.

(3) There is something of a conceptual disconnect between the early and later portions of this paper. Whereas Figures 1-4 examine forebrain projections of genetic subtypes of VTA neurons, the optogenetic studies do not address genetic subtypes at all. I do realize that is outside of the scope of the author's intent, but it does give the impression of somewhat

different (but related) studies being stitched together. For example, the MOR-expressing neurons seem to project strongly to the VP, but it is not addressed whether these are also the ones making local projections. Also, after showing that PV neurons project to the LHb, the opto experiments do not examine the LHb projection target at all.

This too was raised by Reviewer #2. While addressing this question for all the populations we investigated feels redundant, we now include optogenetic data showing that NAc-projecting VTA VGAT-Cre and VP-projecting VTA MOR-Cre neurons also make local collaterals (Figure 7). We think this allows us to connect the two approaches to a greater degree. Based on our findings using a dual virus approach to express Syn:Ruby in each population of VTA projection neuron, we think it very likely that we'd continue to find similar results using optogenetics-assisted slice electrophysiology for each population.

Other suggestions:

(1) I appreciated the extensive and high-quality anatomical figures shown in Figures 2-4. However, the layout was sometimes left-to-right, and sometimes right-to-left, which felt distracting. At some point, the text refers to "Fig. 3KJ", i.e. with the letters being in backward alphabetical order, and Figures 3I and 3L do not appear mentioned anywhere in the main text, leading me to wonder if that text was intended to read "Fig. 3I-L".

Thank you for noting this. We have harmonized the layout of Figures 2-4 and adjusted the in-text Figure call-outs.

Also, the inset in Figure 3J appears to show local collaterals of NTS neurons in the VTA, since there is no soma in that inset. This is interesting, and worth reporting, but is not explained in either the main text or Figure legend.

We added a more complete description in the result section (page 6 line 25-30).

(2) Perhaps I missed it, but I could not find any mention of the intensity of the LED light delivered during the optogenetic experiments. While acknowledging that this can be variable, do the authors have at least a rough range?

We have added this information to the methods, page 17 line 8.

Editor's Note:

Should you choose to revise your manuscript, please double check that you have fully reported all statistics including exact p-values wherever possible alongside the summary statistics (test statistic and df) and 95% confidence intervals.

We confirm that we have fully reported all statistics including exact p-values wherever possible alongside the summary statistics (test statistic and df) and 95% confidence intervals.

Note to Editor and Readers

While reanalyzing our data for resubmission, we discovered that some of the short-latency optogenetic evoked postsynaptic currents (oPSCs) we detected were erroneously categorized. Specifically, some VTA cells that showed large outward currents (oIPSCs) when held at 0 mV, also had small inward currents when held at -60 mV. These small inward currents were initially categorized as oEPSCs, suggesting these VTA cells received input from populations of VTA projection neurons that released GABA and/or glutamate. However, the kinetics of these small inward currents were slow and aligned with the within-cell kinetics of the oIPSCs, indicating that these were very likely mediated by GABA_A receptors. In one case the opposite

was apparent, with a small PSC initially miscategorized as an oIPSC. These miscategorized oEPSCs and oIPSC were presumably detected because our holding potentials were not precisely identical to the reversal potentials for GABA_A and AMPA receptors, respectively. For this reason, we removed these 14 oEPSCs and 1 oIPSCs from our analyses in the revised version. The revised dataset suggests that VTA glutamate projection neurons may be less likely to collateralize widely within VTA compared to GABA projection neurons. But, importantly, this correction does not affect any of our conclusions.

Citations:

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