Cellular/Molecular

Neurotensin Release from Dopamine Neurons Drives Long-Term Depression of Substantia Nigra Dopamine Signaling

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Midbrain dopamine neurons play central physiological roles in voluntary movement, reward learning, and motivated behavior. Inhibitory signaling at somatodendritic dopamine D2 receptor (D2R) synapses modulates excitability of dopamine neurons. The neuropeptide neurotensin is expressed by many inputs to the midbrain and induces LTD of D2R synaptic currents (LTD_{DA}); however, the source of neurotensin that is responsible for LTD_{DA} is not known. Here we show, in brain slices from male and female mice, that LTD_{DA} is driven by neurotensin released by dopamine neurons themselves. Optogenetic stimulation of dopamine neurons was sufficient to induce LTD_{DA} in the substantia nigra, but not the VTA, and was dependent on neurotensin receptor signaling, postsynaptic calcium, and vacuolar-type H^+-ATPase activity in the postsynaptic cell. These findings reveal a novel form of signaling between dopamine neurons involving release of the peptide neurotensin, which may act as a feedforward mechanism to increase dopamine neuron excitability.

Key words: **dopamine; neurotensin; plasticity; retrograde; substantia nigra; VTA**

Significance Statement

Dopamine neurons in the midbrain play a critical role in reward learning and the initiation of movement. Aberrant dopamine neuron function is implicated in a range of diseases and disorders, including Parkinson's disease, schizophrenia, obesity, and substance use disorders. D2 receptor-mediated PSCs are produced by a rare form of dendrodendritic synaptic transmission between dopamine neurons. These D2 receptor-mediated PSCs undergo LTD following application of the neuropeptide neurotensin. Here we show that release of neurotensin by dopamine neurons themselves is sufficient to induce LTD of dopamine transmission in the substantia nigra. Neurotensin signaling therefore mediates a second form of interdopamine neuron communication and may provide a mechanism by which dopamine neurons maintain excitability when nigral dopamine is elevated.

Introduction

Midbrain dopamine neurons of the SNc and VTA play central roles in the initiation of movement and motivated behavior. A complete understanding of the determinants of dopamine neuron excitability is of critical importance in understanding their role in complex behavior. Dopamine neurons inhibit each other through a relatively slow form of dendritic neurotransmission mediated by postsynaptic dopamine D2 autoreceptors (D2Rs) (Beckstead et al., 2004; Robinson et al., 2017). Dendritic dopamine neurotransmission can be measured in mouse brain slices by electrically evoking dopamine release and electrophysiologically recording the subsequent D2R activation of G-protein-coupled potassium channel-mediated IPSCs (D2-IPSCs) (Beckstead et al., 2004). Previous work from our laboratory shows that D2-IPSCs undergo LTD (LTD_{DA}) subsequent to low-frequency electrical stimulation (Beckstead and Williams, 2007; Piccart et al., 2015) through a mechanism likely involving signaling by the neuropeptide neurotensin (NT) (Piccart et al., 2015).

NT is expressed throughout the CNS; and in both the midbrain and striatum (a major dopamine projection region), it can affect dopamine-mediated behaviors, including food- and psychostimulant-reinforced responding (Kempadoo et al., 2013; Leinninger et al., 2011; Patterson et al., 2015; Dominguez-Lopez et al., 2018; Tschumi and Beckstead, 2018, 2019). Exogenous NT application induces synaptic plasticity of GABAergic, dopaminergic, and

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glutamatergic input to dopamine neurons, as well as activating cell-autonomous endocannabinoid signaling in those cells (Kortleven et al., 2012; Kempadoo et al., 2013; Bose et al., 2015; Stuhrman and Roseberry, 2015; Piccart et al., 2015; Gantz and Bean, 2017; Tschumi and Beckstead, 2018; Tschumi and Beckstead, 2019). NT release from lateral hypothalamic inputs to the VTA drives plasticity at glutamatergic synapses (Kempadoo et al., 2013), but the endogenous source of NT which drives plasticity at dendrodendritic dopamine synapses is not known. Therefore, physiological delineation of NT signaling is central to understanding synaptic plasticity in dopamine neurons and its effect on excitability.

Here we show in mouse brain slices that LTD_{DA} in the SNc is driven by NT release from dopamine neurons. We used transgenic and viral targeting to enable optogenetic stimulation of dopamine neurons or NT terminals projecting from either the lateral hypothalamus (LH) or NAc to the VTA. Targeted stimulation of dopamine neurons drove LTD_{DA} in the SNc that was prevented by NT receptor antagonism, as well as postsynaptic chelation of calcium or inhibition of vacuolar-type H^+-ATPase (V-ATPase). Conversely, neither stimulation of dopaminergic, lateral hypothalamic, or accumbal NT-expressing inputs was sufficient to drive LTD_{DA} in the VTA. These findings provide evidence for a second form of interdopamine neuron signaling specific to the SNc which proceeds through local NT release.

Materials and Methods

Animals. Male and female mice (age range: 6-113 weeks) were used for all experiments. Genetic lines were obtained from Jackson ImmunoResearch Laboratories (C57Bl/6J, *DAT-Cre*, and *Cre-ChR2*), except for *NT-Cre* mice, which were generously provided by Dr. Gina Leinninger (Leinninger et al., 2011). Mice were group-housed when littermates were available in polycarbonate chambers with rodent bedding. The vivarium was on a reverse 12/12 light cycle (lights off at 0900). Animal usage was reviewed and approved by Institutional Animal Care and Use Committees at the University of Texas Health Science Center at San Antonio and the Oklahoma Medical Research Foundation.

Mouse genotyping. DNA was extracted from mouse ear punches using Extract-N-Amp (Sigma) per the manufacturer's instructions and used for PCR amplification. Genotyping was performed either on the University of Texas Health Science Center at San Antonio campus using the protocols listed below or performed by Transnetyx using real-time PCR.

DAT-Cre mice ($DAT^{IREScre}$, $Slc6a3^{tm.1(cre)Bkmn}$) and *Cre-ChR2* mice (B6.Cg-$Gt(ROSA)26Sor^{tm32(CAGCOP4*H134R/EYFP)Hze}$/J) were genotyped using a multiplex PCR reaction as per the outlined protocols (https://www.jax.org/Protocol?stockNumber=006660&protocolID=23107) and (https://www.jax.org/Protocol?stockNumber=017525&protocolID=26086), respectively. *NT-Cre* mice ($NTS^{IRES-Cre}$, B6;129-$Nts^{tm1(cre)Mgmj}$/J) were genotyped by multiplex PCR using primers designed by Dr. Richard Palmiter, University of Washington: 5′CAATGGTGCGCCTGCTGGAAG3′, 5′CATGAATGCAAGAAACATCACATCC3′, and 5′TCCAGGAAGATATCCTTGATAACG3′.

Stereotaxic surgery and vector delivery. Surgeries were conducted using a stereotaxic instrument (Kopf Instruments). Mice were anesthetized with isoflurane kept steady at 1.5%-2% using a Somnosuite low-flow anesthesia system (Kent Scientific). Access holes were drilled bilaterally in the skull, and 33-gauge stainless-steel injectors were lowered to one set of the following coordinates in mm: SNc; ML: 1.25, AP: −3.4, DV: −4.7; LH; ML: 0.9, AP: −1.1, DV: −5.25; NAc; ML: 2.44, AP: 1.65, DV: −4.35 @ 20 degrees. At least 3 weeks before electrophysiological experiments, mice were injected with 400 nl of cre-inducible viral vector AAV5-EF1a-DIO-hChR2(H134R)-EYFP-WPRE-pA (University of North Carolina Vector Core, Chapel Hill, NC) synthesized in the laboratory of Dr. Karl Deisseroth, and mice received injections of 20 mg/ml ticarcillin (antibiotic; Sigma-Aldrich) and 0.8 mg/ml ketoprofen (analgesic; Hospira) after surgery.

Brain slice electrophysiology. On the day of the experiment, mice were anesthetized with isoflurane and immediately decapitated. Brains were extracted and placed in ice-cold carboxygenated (95% O_2, 5% CO_2) aCSF containing the following (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.4 NaH_2PO_4, 25 $NaHCO_3$, and 11 D-glucose. For Ca^{2+}-free experiments, $CaCl_2$ was replaced with $MgCl_2$ (for a total of 3.7 mM) and 0.5 mM EGTA. Kynurenic acid (1 mM) was added to the buffer for the slicing procedure. Horizontal midbrain slices (200 μm) containing the SNc and VTA were obtained using a vibrating microtome (Leica). Slices were incubated for at least 30 min at 34°C-36°C with carboxygenated aCSF that also contained the NMDA receptor antagonist MK-801 (10-20 μM) unless noted otherwise.

Slices were placed in a recording chamber attached to an upright microscope (Nikon Instruments) and maintained at 34°C-36°C with aCSF perfused at a rate of 1.5 ml/min. Dopamine neurons were visually identified using gradient contrast optics based on their location in relation to the midline, medial lemniscus, and the medial terminal nucleus of the accessory optic tract. Neurons were further identified physiologically by the presence of spontaneous pacemaker firing (1-5 Hz) with wide extracellular waveforms (>1.1 ms) and a hyperpolarization-activated current (I_H) of > 100 pA. Recording pipettes (1.5-2.5 MΩ resistance) were constructed from thin-wall capillaries (World Precision Instruments) with either a PC-10 or a PC-100 puller (Narishige International). Whole-cell recordings were obtained using an intracellular solution containing the following (in mM): 115 K-methylsulfate, 20 NaCl, 1.5 $MgCl_2$, 10 HEPES, 2 ATP, 0.4 GTP, and either 10 BAPTA or 0.025 EGTA, pH 7.35-7.40, 269-274 mOsm.

D2-IPSCs were evoked during voltage-clamp recordings (holding voltage, −55 mV) using electrical stimulation (D2-eIPSCs, 5 stimulations of 0.5 ms applied at 50 Hz) delivered by a bipolar stimulating electrode that was inserted into the slice 100-200 μm caudal to the patched cell in the presence of the following receptor antagonists: picrotoxin (100 μM, $GABA_A$), CGP 55845 (100 nM, $GABA_B$), DNQX (10 μM, AMPA), hexamethonium (100 μM, nAChR), MPEP hydrochloride (300 nM, mGluR5), and JNJ 16259685 (500 nM, mGluR1). D2-IPSCs were also evoked via blue light (D2-oIPSCs) with four stimulations of 5 ms applied at 50 Hz generated either by laser (Optoengine, 100 mW max power, 473 nm) and delivered through a fiber optic cable (200 μm core, 0.39 NA, Thorlabs), the tip of which was placed 100-200 μm caudal to the patched cell, or generated by high-power LED (Solis-3C, 20 mW max power, Thorlabs) and delivered through the microscope objective after passing through a filter cube (TLV-TE2000, Thorlabs). Both D2-eIPSCs and D2-oIPSCs were evoked once every 50 s. The stimulation protocol to electrically (0.5 ms pulse) or optogenetically (10 ms pulse) induce LTD_{DA} was 10 Hz stimulation for 2 min. D2-oIPSCs shown in Figure 2*D1*, *D2* were recorded in *DAT-Cre* mice in which ChR2 was expressed by AAV delivered into the SNc (see previous section). For antagonist and inhibition experiments, compounds were either included in the recording pipette and allowed to dialyze for 10 min after breaking into the cell, or bath-perfused for at least 10 min immediately before repeated stimulation and continued throughout the length of the recording.

Fluorescent labeling and imaging. Brain samples were embedded in OCT cryo-matrix (Sakura Finetek) with the coronal fissure oriented to the bottom of the mold. Enough OCT was added to the mold to cover the sample halfway, leaving ~50% of the sample exposed. The brains were sectioned coronally on a Leica CM3050 cryostat at 40 μm. Each sample was collected at three different areas of interest (Paxinos and Franklin, 2019) (NAc [1.98-0.74 mm], LH [−0.58 to −2.54 mm], VTA [−2.54 to −3.80 mm]). Sections were transferred to a well plate containing a 1× PBS. Once completed, the well plates were sealed with Parafilm and stored at 4°C.

Sections were blocked at room temperature for 1 h with 5% normal donkey serum (Abcam ab7475). Sections were washed in PBS + 0.1% Tween-20, then incubated with primary antibodies + 2% normal donkey serum for 22 h at 4°C. Sections were then washed in PBS and incubated with secondary antibodies for 2 h at room temperature. After final washes in PBS, sections were mounted on gelatin-coated slides and

coverslips were applied using Prolong Diamond anti-fade reagent (Thermo Fisher Scientific). Primary antibodies used were goat anti-GFP (1:500; Novus NB100-1770) for eYFP, and chicken anti-TH (1:1000; Abcam ab76442). Secondary antibodies used were donkey anti-goat AlexaFluor-488 (1:750; Jackson ImmunoResearch Laboratories, 705-545-147), and donkey anti-chicken Rhodamine Red-X (1:200; Jackson ImmunoResearch Laboratories, 703-295-155). Primary and secondary antibodies were incubated with PBS + 0.3% Triton X-100.

Sections were imaged using a Zeiss LSM 710 confocal microscope with a 40× objective. ZEN Microscope Software (Carl Zeiss Microscopy) was used for image acquisition. Final images were obtained at single planes or through maximum intensity flattened *z* stacks (1 µm between planes) generated using ImageJ (National Institutes of Health).

Pharmacological agents. Methamphetamine hydrochloride was a generous gift from the National Institute on Drug Abuse drug supply program. Kynurenic acid, MK-801, DNQX, picrotoxin, dopamine, sulpiride, hexamethonium, SKF38393, concanamycin A, TTX, and SR142948 were purchased from Sigma-Aldrich. SR48692, CGP55845, JNJ 16259685, and MPEP hydrochloride were purchased from Tocris Bioscience. The active fragment NT_{8-13} was purchased from American Peptide or Sigma-Aldrich.

Experimental design and statistical analyses. Data were collected on a Dell computer running Microsoft Windows (version 7 or 10) using Axograph (version 1.5.4 or 1.7.6) and LabChart (AD Instruments) software. Statistical analyses were performed using GraphPad Prism (version 6 or 9). In most cases, the effect of repetitive stimulation was analyzed using an average of the 5 sweeps immediately preceding stimulation (sweeps 1-5, pre) and sweeps 31-35 (post). In rare cases where <5 sweeps were available, the sweeps within that range (at least 2) were averaged for analysis. In the NT occlusion experiment, 4-6 stable sweeps were obtained after NT_{8-13} bath perfusion before optostimulation at 10 Hz for 2 min. Tukey's *post hoc* tests were performed subsequent to significant ANOVAs. Data are presented as mean ± SEM. In all cases, α was set *a priori* at 0.05.

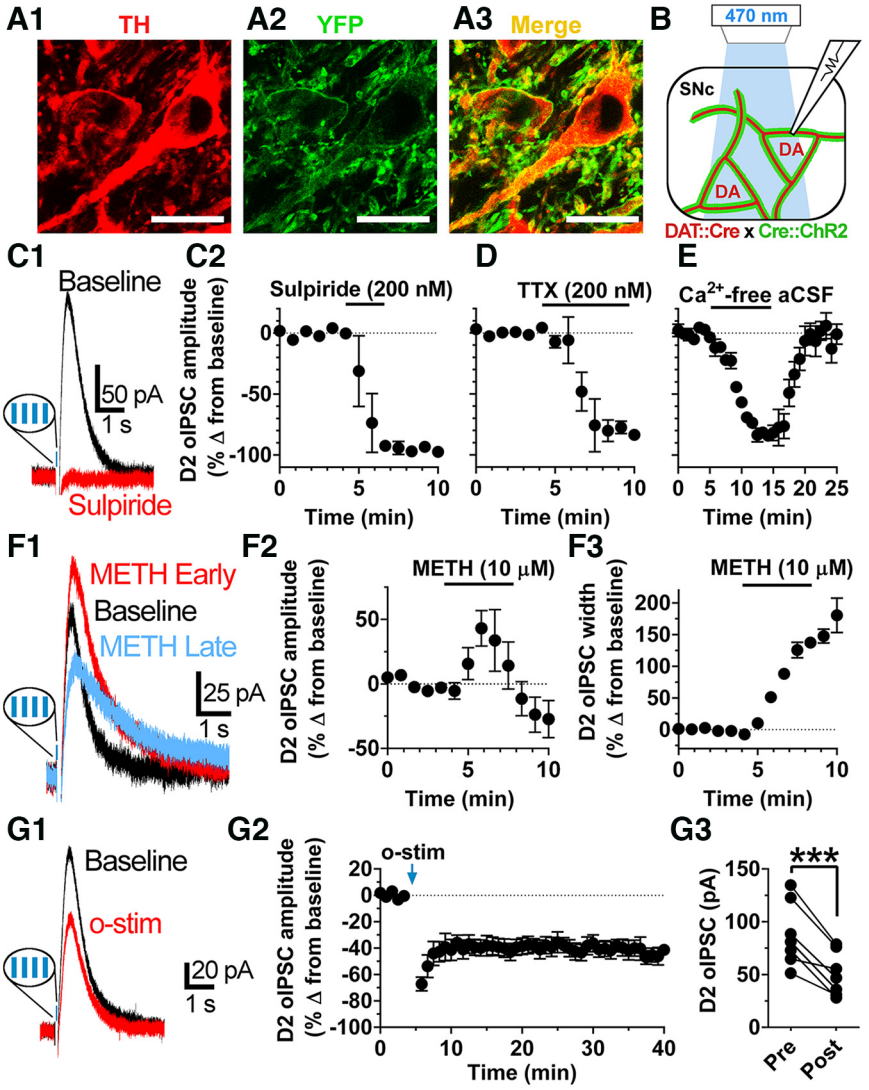

Figure 1. Stimulation of dopamine neurons is sufficient to evoke D2-IPSCs and drive LTD_{DA} in the SNc. Images represent TH (***A1***), eYFP-fused to ChR2 (***A2***), and colocalization (***A3***). Scale bar, 20 µm. Schematic represents dopamine (DA) neurons from *DAT-Cre* mice (*DAT::Cre*) crossed with Ai32 (*Cre::ChR2*) mice, resulting in dopamine neuron-specific expression of eYFP-fused to ChR2 and stimulation by blue light in the SNc (470 nm, ***B***). A blue light elicited D2-oIPSC during baseline and following bath perfusion of sulpiride (***C1***, 4 pulses delivered at 50 Hz, depicted as four blue bars denoting timing of pulses), and time course (***C2***). Time courses represent reduction of D2-oIPSC amplitude during bath perfusion of TTX (***D***) and calcium-free aCSF (***E***). Effects of methamphetamine (METH) on D2-oIPSCs (during the peak-effect of METH on amplitude, and later when the effect of METH on half-width was more pronounced, ***F1***), time course of effect on amplitude (***F2***), and half-width (***F3***). Effect of dopamine-neuron specific stimulation (o-stim, 10 Hz blue light for 2 min) on D2-oIPSCs (***G1***), time course (***G2***), and raw amplitudes (***G3***) during an average of 5 baseline sweeps (Pre) and the 5 sweeps 19-23 min following 10 Hz optostimulation (Post). ***B-F***, Experiments were conducted with BAPTA (10 mM) in the pipette. ***G***, Experiments were conducted with EGTA (0.025 mM). $^{**}p < 0.01$.

Results

Repetitive stimulation of dopamine neurons is sufficient to drive LTD_{DA} in the SNc

We previously found that stimulation-induced LTD_{DA} (Beckstead and Williams, 2007) is driven by endogenous NT signaling (Piccart et al., 2015) and suggested that dopamine neurons might release NT. Thus, to determine the source of NT which drives LTD_{DA}, we used a combination of transgenic and optogenetic tools to delineate the role of dopamine neurons and NT afferents to the midbrain. We first generated a mouse expressing channelrhodopsin (ChR2) specifically in dopamine neurons by cross-breeding dopamine transporter (DAT)-Cre and cre-dependent ChR2 mouse lines (Fig. 1*A1-A3*,*B*). Using whole-cell voltage-clamp (−55 mV) recordings of SNc dopamine neurons with the calcium chelator BAPTA (10 mM) inside the recording pipette, we determined that blue light optical stimulation, like electrical stimulation (Beckstead et al., 2004), generates slow outward synaptic currents that are blocked by the dopamine D2R antagonist sulpiride (D2-oIPSCs; Fig. 1*C1*,*C2*) or by the voltage-gated sodium channel blocker TTX (Fig. 1*D*), and are dependent on the presence of extracellular Ca^{2+} (Fig. 1*E*). This suggests that optogenetic excitation of dopamine neurons and subsequent dopamine release closely resemble previously characterized dopamine neurotransmission (Beckstead et al., 2004). Consistent with our previous studies using electrical stimulation (Branch and Beckstead, 2012), bath perfusion of methamphetamine induced an increase in D2-oIPSC amplitude and width,

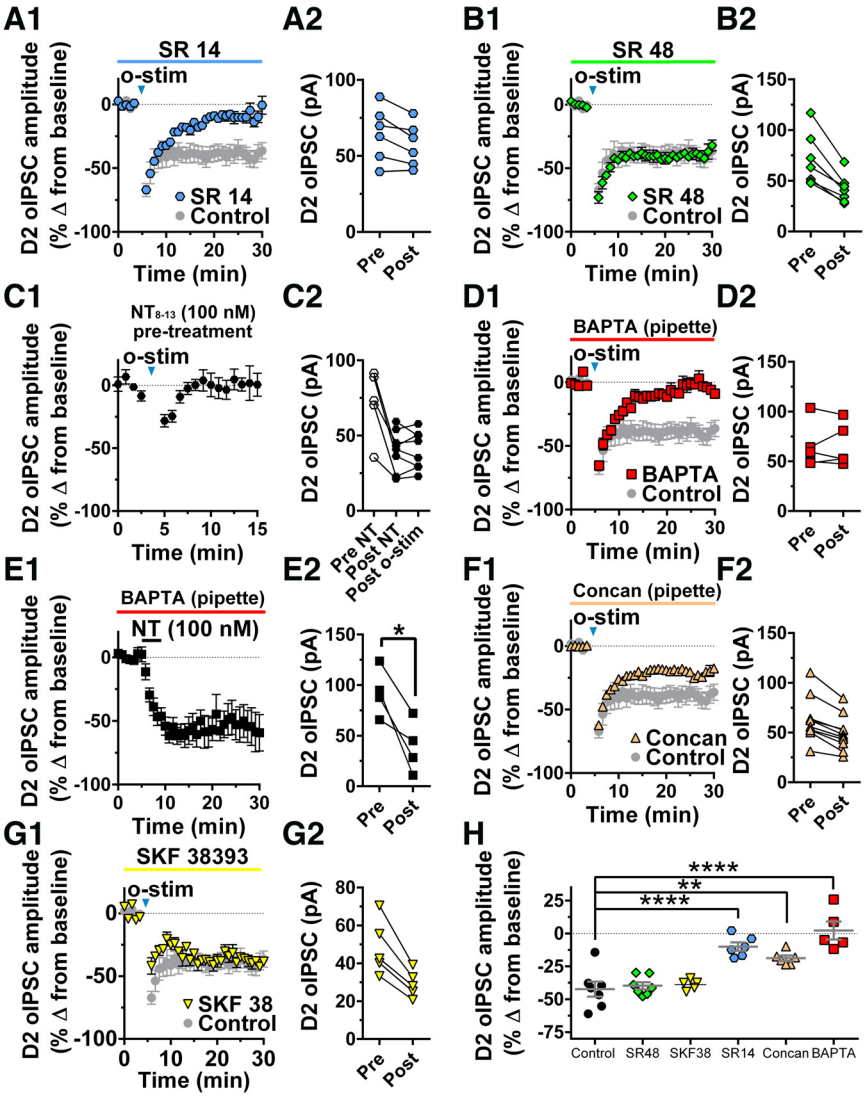

Figure 2. LTD_{DA} requires NT Type 2 receptor signaling, postsynaptic calcium, and V-ATPase. Effect of dopamine neuron-specific stimulation (o-stim, 10 Hz blue light for 2 min) on D2-oIPSCs in aCSF (control, gray circles, data first shown in Fig. 1) or in the presence of the following: the nonselective NT Type 1/2 receptor antagonist SR142948 (1 μM, SR 14, blue hexagons), time course (***A1***) and raw amplitudes (***A2***); the NT Type 1 receptor antagonist SR48692 (green diamonds, ***B1***,***B2***); NT_{8-13} (100 nM) pretreatment (***C1***,***C2***); or with BAPTA (10 mM) added to the recording pipette internal solution (red squares, ***D1***,***D2***). The effect of 5 min bath perfusion of NT_{8-13} on D2-oIPSCs with BAPTA (10 mM) in the recording pipette (***E1***,***E2***). Effect of optostimulation on D2-oIPSCs with concanamycin A (5 μM) in the recording pipette (***F1***,***F2***). Effect of opto-stimulation on D2-oIPSCs recorded from SNc dopamine neurons in the presence of the dopamine D1 receptor antagonist SKF 38393 (10 μM SKF, yellow triangles), time course (***G1***), and raw amplitudes (***G2***). Comparison of percent change from baseline amplitude of D2-oIPSCs following optostimulation between groups (***H***). All raw amplitudes and percent changes are the average of 5 baseline sweeps (Pre) and the 5 sweeps 19-23 min following optostimulation (Post). Arrowhead indicates optostimulation of dopamine neurons (10 Hz, 2 min). *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$. ****$p < 0.0001$.

suggesting that the kinetics of the synaptic current are tightly regulated by the DAT (Fig. 1*F1-F3*). Thus, optogenetic stimulation allows for an assessment of dopamine neuron excitation without stimulation of other cell types and without including blockers of other neurotransmitter receptors.

LTD_{DA} requires NT Type 2 receptor signaling, and postsynaptic calcium and V-ATPase

We previously found that repeated electrical stimulation of midbrain slices induces LTD_{DA} that is dependent on calcium in the postsynaptic cell (Beckstead and Williams, 2007), but the neuronal population that drives electrically induced LTD_{DA} is not known. When calcium buffering was minimized by using a low concentration of EGTA in the recording pipette (0.025 mM), repeated optostimulation of dopamine neurons (10 Hz, 2 min) was sufficient to induce LTD_{DA} ($t_{(5)}$ = 5.04, $p = 0.0040$; Fig. 1*G1-G3*). Because of these findings, further recordings investigating LTD_{DA} were conducted with internal solution that weakly buffers calcium (EGTA, 0.025 mM) unless otherwise noted.

As dopamine neurons express not only Type 1 but also Type 2 NT receptors (Mazella et al., 1988; Sarret et al., 1998), we next investigated whether optostimulation-induced LTD_{DA} is dependent on NT signaling at these receptors. Preincubation with the nonselective NT Type 1/2 receptor antagonist SR 142948 prevented LTD_{DA} (Fig. 2*A1*,*A2*, compare with Fig. 2*H*: one-way ANOVA, $F_{(4,26)}$ = 19.02; $p < 0.0001$, Dunnett's, $p < 0.0001$). Conversely and consistent with our previous findings (Piccart et al., 2015), a selective NT Type 1 receptor antagonist failed to prevent LTD_{DA} (Fig. 2*B1*,*B2*,*H*; $p = 0.99$). Furthermore, LTD_{DA} was occluded by pretreating slices with the active fragment of NT, NT_{8-13} (Fig. 2*C1*,*C2*; $t_{(8)}$ = 0.084, $p = 0.94$). Together with the finding that dopamine neuron activation is sufficient to drive LTD_{DA}, these results suggest that dopamine neurons are themselves releasing NT that induces LTD_{DA} though activation of NT Type 2 receptors.

We next investigated whether NT was being released by presynaptic or postsynaptic dopamine neurons. As NT release is calcium-dependent (Kitabgi et al., 1990), blockade of LTD_{DA} by removing calcium from the postsynaptic cell would suggest postsynaptic release of NT. Indeed, modifying the internal solution in the recording pipette by replacing a low concentration of the relatively weak calcium chelator EGTA with a high concentration of the strong calcium chelator BAPTA (10 mM) prevented optostimulation-induced LTD_{DA} (Fig. 2*D1*, *D2*,*F*; $p < 0.0001$). An alternative possibility is that NT is being released presynaptically but requires calcium signaling in the postsynaptic cell to induce LTD_{DA}. This does not appear to be true, as bath application of NT_{8-13} induces LTD_{DA} in dopamine neurons dialyzed with BAPTA (NT_{8-13}, Fig. 2*E1*,*E2*, $t_{(3)}$ = 4.1, $p = 0.026$). Together, these findings suggest that NT-induced depression of D2-oIPSCs is not dependent on intracellular calcium per se, as NT perfusion can bypass a postsynaptic calcium-dependent step in stimulation-induced LTD_{DA}.

We further investigated whether the postsynaptic dopamine neuron being recorded from releases NT to drive LTD_{DA}. NT is found in dense-core vesicles in dopamine neuron dendrites (Bayer et al., 1991), release of which should be dependent on V-ATPase, which is necessary for both proper trafficking of

neuropeptide-containing dense-core vesicles (Taupenot et al., 2005) and NT release in other brain regions (Krawczyk et al., 2013; Normandeau et al., 2018). Dialyzing the postsynaptic cell with the V-ATPase inhibitor concanamycin A partially blunted LTD_{DA} (Fig. 2*E1*,*E2*,*F*; $p = 0.0012$). One final consideration is that NT-induced synaptic plasticity in the bed nucleus of the stria terminalis is dependent on dopamine D1 receptor (D1R) signaling (Krawczyk et al., 2013), and an alternative explanation here could be that D1R-expressing axon terminals in the midbrain (Levey et al., 1993) could produce NT release subsequent to optostimulation of dopamine neurons. However, the selective D1R antagonist SKF 38393 (10 μM) failed to prevent LTD_{DA} (Fig. 2*G1*,*G2*,*H*; $p = 0.97$). So while these experiments do not definitively exclude the release of other signaling molecules, they do suggest that NT is released in a calcium- and V-ATPase-dependent manner from dopamine neurons located postsynaptic to other dopamine neurons.

Repetitive stimulation of dopamine neurons or other NT inputs is not sufficient to drive LTD_{DA} in the VTA

As NT peptide has been observed in cell bodies and terminals in both the SNc and VTA, we next examined whether dopamine neuron stimulation is sufficient to drive LTD_{DA} in VTA dopamine neurons. Although we have previously shown that bath perfusion of NT induces depression of D2R signaling in VTA dopamine neurons (Piccart et al., 2015), optostimulation surprisingly failed to induce LTD_{DA} in most of the VTA neurons recorded (Fig. 3*A1-A3*; $t_{(4)} = 0.75$, $p = 0.50$), suggesting the presence of brain region-specific interactions between NT and dopamine transmission. However, consistent with our previous findings (Beckstead and Williams, 2007), electrical stimulation did induce LTD_{DA} in the VTA (Fig. 3*B1-B3*). As here we show that optostimulation-induced LTD_{DA} is NT-dependent, and previous findings show that bath-perfused NT depresses D2-IPSCs (Piccart et al., 2015), it is possible that electrical stimulation may elicit NT release from nondopamine cells to induce LTD_{DA} in VTA dopamine neurons.

We next investigated two prominent NT-expressing inputs to the VTA: (1) the LH afferents to the VTA, which have only weak projection to the SNc (Patterson et al., 2015); and (2) the NAc inputs to the VTA, which express increased levels of NT following methamphetamine exposure (Geisler and Zahm, 2006). NT, like many neuropeptides, is coexpressed in neurons that form synaptic connections and generate either GABA- or glutamate-mediated PSCs in VTA dopamine neurons (Kempadoo et al., 2013; Opland et al., 2013; Tschumi and Beckstead, 2019). In order to specifically stimulate these inputs, *NT-Cre* mice were administered bilateral intra-LH or intra-NAc injections of AAV to drive cre-inducible expression of ChR2 (schematic; Figs. 3*C1*, 4*A1*). Both immunohistochemistry (Fig. 3*C2*) and live-slice imaging (observed during recordings, not shown) revealed terminals from NT-expressing LH inputs to the VTA. While synaptic transmission has previously been reported at NT-expressing LH inputs to dopamine neurons in the VTA, the frequency with which these synaptic connections occur, or whether there are also synaptic connections onto nondopamine VTA neurons has not been investigated. Similarly, to our knowledge, a fast synaptic connection from NT NAc to VTA neurons has not been reported. To investigate NT synaptic inputs to midbrain neurons, recordings were performed without blockade of either AMPA or GABA receptors from both putative dopamine neurons (for criteria, see Materials and Methods) and nondopamine neurons, with 10 mM BAPTA in the pipette. This method allowed

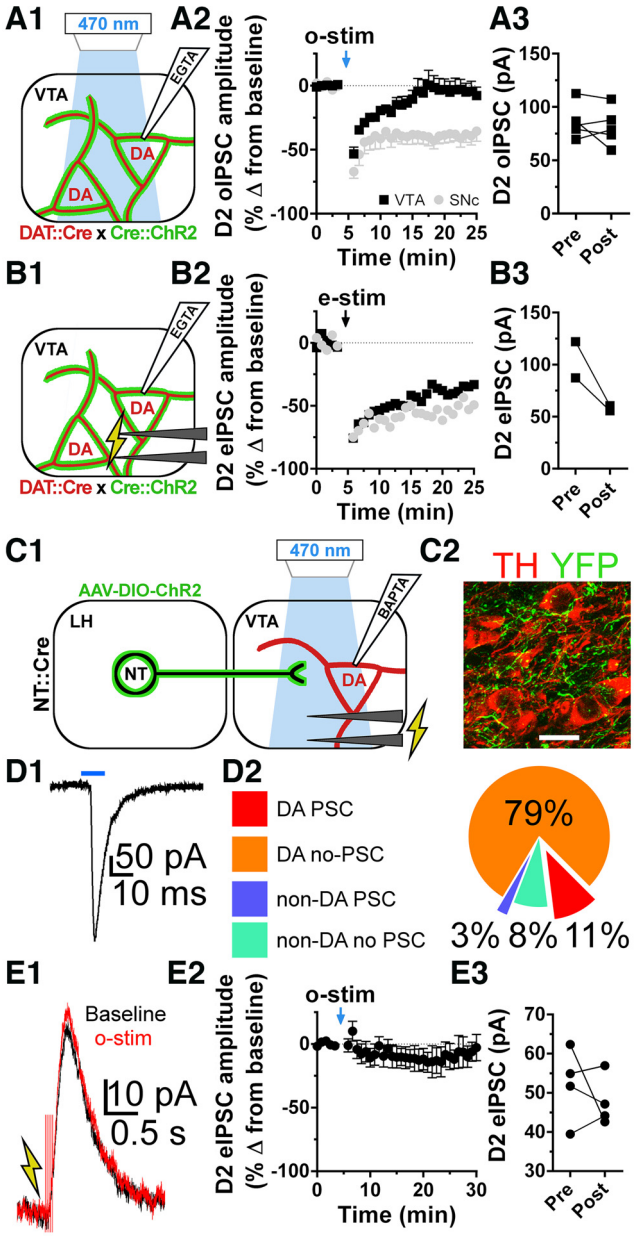

Figure 3. In the VTA, stimulation of dopamine neurons or NT-expressing inputs from the LH does not drive LTD_{DA}. Schematic represents dopamine (DA) neurons from *DAT-Cre* mice (*DAT::Cre*) crossed with Ai32 (*Cre::ChR2*) mice resulting in dopamine neuron-specific expression of eYFP-fused to ChR2 and stimulation by blue light (470 nm, ***A1***) or electrical stimulation (***B1***) in the VTA. Effect of dopamine-neuron specific stimulation (o-stim, 10 Hz blue light for 2 min) on D2-oIPSCs recorded from SNc (gray circles, data first shown in Fig. 1) or VTA dopamine neurons (VTA, black squares), time course (***A2***) and raw amplitudes (***A3***). D2-eIPSCs recorded from VTA dopamine neurons (***B1***), time course (***B2***; cell 1, black squares, cell 2, gray circles), and raw amplitudes (***B3***). Schematic represents NT-expressing neurons in *NT-Cre* mice following intra-LH injection of an AAV that drives cre-inducible expression of eYFP-fused to ChR2 (AAV-DIO-ChR2). These inputs were stimulated either by single pulses of blue light (10 ms, 470 nm) or repeated blue-light stimulation (10 Hz, 2 min, 5 ms pulses) to drive NT release (***C1***). Electrical stimulation (with GABA and glutamate antagonists in the aCSF) was used to elicit D2-eIPSCs. Sample image represents expression of eYFP-fused ChR2 (YFP) near TH-expressing neurons (***C2***, scale bar, 20 μm). Sample trace of a PSC generated by LH-NT input stimulation (***D1***, blue bar represents 10 ms blue-light pulse). Chart represents the percent of dopamine (DA) and nondopamine neurons (non-DA) in which a PSC was or was not elicited by single-pulse stimulation ($N = 38$, ***D2***). Effect of repeated optostimulation of LH NT input to VTA (o-stim, 10 Hz blue light for 2 min) on D2-eIPSCs, sample trace (***E1***), time course (***E2***), and raw amplitudes (***E3***). Raw amplitudes are the average of 5 baseline sweeps (Pre) and 5 sweeps 19-23 min following optostimulation (Post).

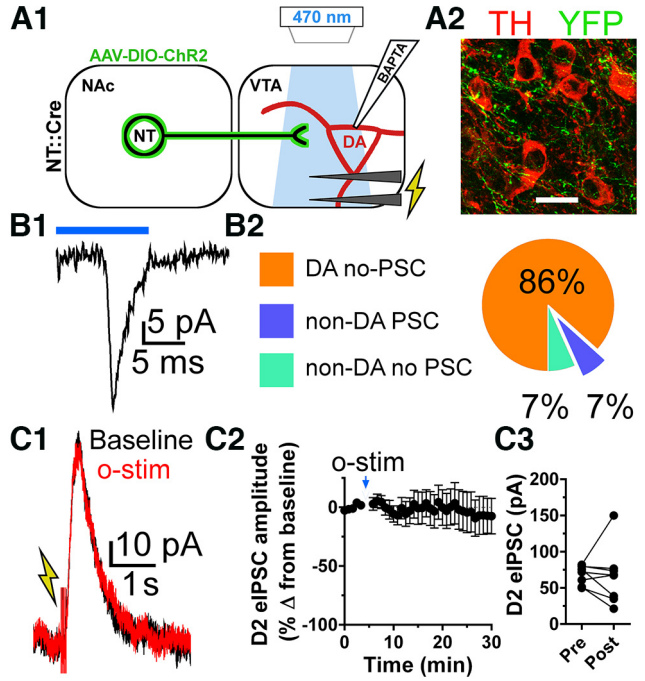

Figure 4. Stimulating NT-expressing inputs from the NAc to the VTA does not drive LTD_{DA}. Schematic represents NT-expressing neurons in *NT-Cre* mice following intra-NAc infusion of an AAV that drives cre-inducible expression of eYFP-fused to ChR2 (AAV-DIO-ChR2). These inputs were stimulated either by single pulses of blue light (10 ms, 470 nm) or repeated blue-light stimulation (10 Hz, 2 min, 5 ms pulses) to drive NT release (***A1***). Electrical stimulation (with GABA and glutamate antagonists in aCSF) was used to elicit D2-eIPSCs. Sample image represents expression of eYFP-fused ChR2 (YFP) near TH-expressing neurons (***A2***, scale bar, 20 µm). Sample trace of a PSC generated by LH-NT input stimulation (***B1***, blue bar represents 10 ms blue-light pulse). Chart represents the percent of dopamine (DA) and nondopamine neurons (non-DA) in which a PSC was or was not elicited by single-pulse stimulation ($N = 15$, ***B2***). Effect of repeated optostimulation of NAc NT input to VTA (o-stim, 10 Hz blue light for 2 min) on D2-eIPSCs, sample trace (***C1***), time course (***C2***), and raw amplitudes (***C3***). Raw amplitudes are the average 5 baseline sweeps (Pre) and 5 sweeps 19-23 min following optostimulation (Post).

for the greatest probability of discovery of synaptic connections. Surprisingly, despite dense labeling of NT terminals in the VTA, optostimulation of NT-expressing LH inputs generated fast PSCs in only a small fraction of both dopamine and nondopamine neurons recorded (Fig. 3*D1*,*D2*). While the kinetics of these currents were universally fast (half-widths $\leq$7 ms), we did not perform pharmacology to definitively determine whether they were because of AMPA or GABA receptor activation. To determine whether NT-expressing LH inputs to the VTA were sufficient to drive LTD_{DA} in the VTA, we stimulated these inputs with the same protocol used to electrically elicit LTD_{DA} (10 Hz, 2 min blue light). However, recordings of D2-IPSCs (isolated with a cocktail of antagonists to block GABA and glutamate signaling) in a separate group of mice revealed no induction of LTD_{DA} by targeted stimulation of NT-expressing LH inputs to the VTA (Fig. 3*E1-E3*; $t_{(3)} = 0.80$, $p = 0.48$). We next investigated NT-expressing NAc inputs to the VTA (Fig. 4*A1*,*A2*) and found these also generated PSCs in only a fraction of cells recorded (Fig. 4*B1*,*B2*) and failed to induce LTD_{DA} (Fig. 4*C1-C3*; $t_{(7)} = 0.223$, $p = 0.87$) with the same stimulation protocol that is sufficient to drive non–input-specific LTD_{DA} in the VTA. Together, these results suggest that, unlike in the SNc, LTD_{DA} in the VTA, at least at some stimulation frequencies, is neither driven by dopamine neuron activity nor by two major NT inputs arising from the NAc and LH.

Discussion

NT release by dopamine neurons

Our data show that selective optostimulation of dopamine neurons is sufficient to evoke D2R-mediated inhibitory synaptic currents (D2-IPSCs), and repeated stimulation in the SNc drives NT-dependent LTD of this form of transmission (LTD_{DA}). NT-induced depression is dependent on NT Type 2 receptors, as well as postsynaptic calcium and V-ATPase (Fig. 5). As both the D2-IPSC and LTD_{DA} can be elicited solely by stimulation of dopamine neurons, it is possible that NT release by dopamine neurons may be acting cell-autonomously, as has recently been postulated for dopamine release (Hikima et al., 2021). However, here we show that LTD_{DA} requires NT Type 2 receptors, which are only sparsely expressed on dopamine neurons and strongly expressed on astrocytes (Woodworth et al., 2018). Furthermore, NT has been shown to regulate intracellular calcium in midbrain astrocytes (Trudeau, 2000), a process that could conceivably drive synaptic plasticity (Guerra-Gomes et al., 2018). The role of astrocytes in synaptic plasticity is well studied in other brain regions, but further work will be needed to elucidate the role of astrocytes on synaptic plasticity in the midbrain.

Alternatively, NT-dependent communication between dopamine neurons meets several criteria of retrograde transmitter release put forth by Regehr et al. (2009): NT is present in the postsynaptic cell (Seroogy et al., 1988), disruption of release from the postsynaptic cell prevents signaling, and NT receptors are located presynaptically (Tanaka et al., 1990; Sarret et al., 1998). Here we show that NT-dependent LTD_{DA} can be inhibited either by (1) blockade of NT receptors or (2) disruption of NT release through either chelation of calcium or inhibition of V-ATPase. We have also previously shown that exposing the presynaptic dopamine neuron to NT by bath application decreases dopamine release (Piccart et al., 2015). Currently available tools do not allow for cell-targeted disruption of NT release from dopamine neurons. NT is not synthesized by dopamine neurons but enters via sequestration through the high-affinity NT receptor (Castel et al., 1990; Laduron, 1995). Therefore, while somewhat limited by peculiarities of NT physiology, the findings presented here provide evidence that NT is released postsynaptically from dopamine neurons and could act as a retrograde messenger to decrease dopamine release presynaptically.

While the findings here suggest that NT is released preferentially from postsynaptic dopamine neurons, it is possible that multiple dopamine neurons in the slice release NT during stimulation. However, it appears that the critical source of NT that drives LTD for a single dopamine neuron is that neuron (as single-cell manipulations in the recorded neuron block LTD). A postsynaptic locus of effect of NT may result from limited diffusion of NT through the extracellular space, possibly because of low concentrations of NT being released combined with rapid degradation of NT by peptidases (McDermott et al., 1982). Alternatively, NT dense-core vesicle may be concentrated at "postsynaptic" varicosities (i.e., near D2Rs but not dopamine vesicles) such that effects of NT release are preferentially observed in postsynaptic cells. Importantly, as with our previous work on LTD_{DA} (Beckstead and Williams, 2007; Piccart et al., 2015), the neuron being recorded is not firing action potentials during the stimulation protocols, because of carefully scaled excitation parameters that do not break the voltage clamp. Additionally, we have previously reported that postsynaptic depolarization alone is insufficient to induce LTD (Beckstead and Williams, 2007). This suggests that voltage-gated calcium channels are an unlikely source of calcium

during calcium-dependent NT release. Alternatively, D2R activation has been shown to increase intracellular calcium in cell lines, dissociated neurons, and striatal brain slice preparations (Iversen et al., 1978; Nishi et al., 1997; Yan et al., 1999; Hernandez-Lopez et al., 2000; Hu et al., 2005). Thus, synchronized synaptic activation of D2Rs with dopamine may drive a D2R-dependent increase in intracellular calcium that permits NT release.

Dopamine neurons also release endocannabinoids, which act as retrograde messengers at both GABA and glutamate inputs, or as autocrine signals (Covey et al., 2017). However, the lack of CB-1 receptors on dopamine neurons suggests that endocannabinoids do not act as retrograde messengers at dendrodendritic dopamine synapses (Julian et al., 2003). Notably, NT signaling via NT Type 1 receptors mobilizes endocannabinoid-induced depression of glutamatergic inputs to dopamine neurons (Kortleven et al., 2012). Thus, NT release by dopamine neurons may also induce endocannabinoid release from neighboring dopamine neurons. Together, our findings suggest that NT release may act as a dopamine neuron-driven feedback mechanism, which results in both depression of local dopamine signaling and endocannabinoid-induced plasticity that alters the balance of excitatory and inhibitory inputs to dopamine neurons.

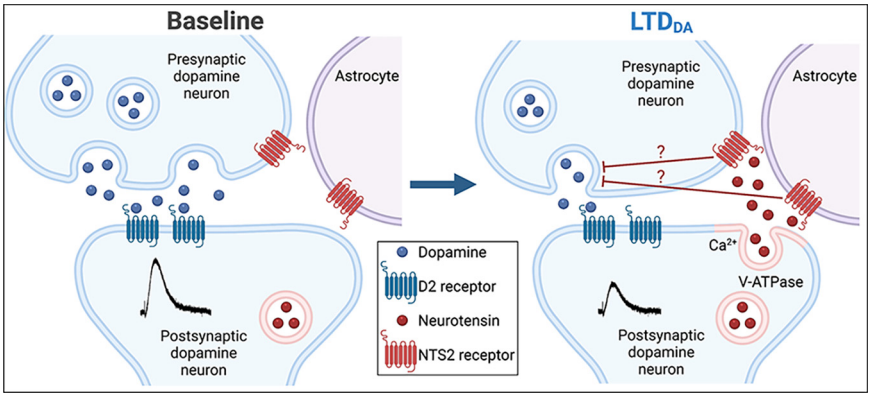

Figure 5. Schematic diagram of LTD_{DA}. Left, Dopaminergic transmission between dopamine neuron dendrites at baseline (blue neurotransmitters and receptors). Right, D2-IPSCs are reduced in amplitude following LTD. NT release from the postsynaptic neuron (red neurotransmitters) is dependent on intracellular calcium and V-ATPase in the postsynaptic dopamine neuron. Activation of Type 2 NT receptors on astrocytes and/or dopaminergic neurons (red receptors) inhibits presynaptic release of dopamine through a mechanism that has yet to be determined. Created with www.BioRender.com and adapted.

NT-induced depression of D2 signaling could have a significant impact on the consequences of dopamine release, including the initiation of voluntary movement and reward-related learning. Burst firing of dopamine neurons, canonically generated by unanticipated rewards (Schultz, 1998), strongly drives inhibitory dendrodendritic dopamine signaling (Beckstead et al., 2004) and is critical for reward-driven behavior (Zweifel et al., 2009). Dendrodendritic D2 signaling occurs at a timescale that is ideal to terminate burst-firing (Beckstead et al., 2004), and could enhance temporal specificity of the bursts. However, prolonged exposure to salient stimuli that increase dopamine neuron firing may drive NT release that depresses dendrodendritic D2 signaling, bypassing normal inhibitory feedback and allowing for continued burst firing to signal the presence of unexpected rewards. For example, transgenic mice lacking functional D2Rs show enhanced measures of sensitivity to the rewarding effects of psychostimulants, including conditioned place preference, responding on a progressive ratio, acquisition of self-administration, and responding to psychostimulant-paired cues (Bello et al., 2011; de Jong et al., 2015; Holroyd et al., 2015; McCall et al., 2017). Furthermore, psychostimulants, such as methamphetamine, upregulate NT mRNA expression (Frankel et al., 2011), and blockade or ablation of NT receptors decreases methamphetamine self-administration (Dominguez-Lopez et al., 2018, 2021), suggesting that, during methamphetamine use, NT may depress D2 signaling, which in turn drives increased drug self-administration.

NT in the VTA

In the VTA, the endogenous source of NT that drives LTD_{DA} remains unclear. Both electrical stimulation and bath application of NT depress D2-IPSCs in the VTA (Piccart et al., 2015; Stuhrman and Roseberry, 2015). One possibility is that this difference is explained by divergent calcium signaling mechanisms between the SNc and VTA. NT release in other brain regions is calcium-dependent (Iversen et al., 1978; Kitabgi et al., 1990), and there are substantial differences in calcium binding proteins and ion channel activation between SNc and VTA dopamine neurons (Rogers, 1992; Liang et al., 1996; Wolfart et al., 2001; Neuhoff et al., 2002). Specifically, a much higher proportion of VTA dopamine neurons express the calcium-binding protein calbindin compared with the SNc (Neuhoff et al., 2002). Furthermore, in SNc dopamine neurons, D2R activation drives dendritic calcium influx through T-type calcium channels, an effect that occurs only in neurons that do not express calbindin (Evans et al., 2017). Notably, calbindin is not necessary for dopamine-induced dendritic calcium influx, but its expression is an indicator of neuronal subpopulations in which this phenomenon occurs (Evans et al., 2017). We did not costain recorded neurons for calbindin in this study. However, in the relatively large population of calbindin-negative SNc dopamine neurons, D2R and subsequent dendritic calcium influx may drive calcium-dependent NT release, whereas this effect does not occur in the relatively large population of calbindin-positive VTA dopamine neurons. Future studies may also determine that electrical stimulation in the VTA releases an as yet unidentified transmitter or modulator that is necessary for induction of LTD in that area, but not in the SNc.

Here we show that NT inputs from both the NAc and the LH to the VTA form synaptic connections with VTA neurons but generate PSCs in only a small fraction of the neurons recorded and fail to drive LTD_{DA}. In agreement with findings reported here of minimal fast-transmitter release from LH and NAc NT inputs, only 10% of NT-expressing terminals form synaptic contacts with dopamine neurons in the VTA (Beaudet and Woulfe 1992). To our knowledge, this is the first report of NT NAc-inputs forming functional synaptic connections with dopamine neurons. Together, it remains to be determined whether NT input originating from a single brain region to the VTA drives NT-induced LTD_{DA}, and the degree to which upregulation of NT leads to NT release which in turn drives LTD_{DA}.

In conclusion, our findings show that stimuli that increase dopamine neuron population activity may induce release of NT that enables firing even in the presence of high concentrations of

dopamine in the SNc, ultimately leading to increased terminal dopamine release in SNc output regions.

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