Cocaine Augments Dopamine-Mediated Inhibition of Neuronal Activity in the Dorsal Bed Nucleus of the Stria Terminalis

James R. Melchior,^{1,2,3} Rafael E. Perez,^{1,2,4} Gregory J. Salimando,^{1,2,3} Joseph R. Luchsinger,^{1,2,6} Aakash Basu,^{1,3} and Danny G. Winder^{1,2,3,4,5,6}

¹Vanderbilt Center for Addiction Research, ²Vanderbilt Brain Institute, ³Department of Molecular Physiology and Biophysics, ⁴Department of Pharmacology, ⁵Department of Psychiatry & Behavioral Sciences, and ⁶Vanderbilt J. F. Kennedy Center, Vanderbilt University School of Medicine, Nashville, Tennessee 37212

The dorsal region of the bed nucleus of the stria terminalis (dBNST) receives substantial dopaminergic input which overlaps with norepinephrine input implicated in stress responses. Using *ex vivo* fast scan cyclic voltammetry in male C57BL/6 mouse brain slices, we demonstrate that electrically stimulated dBNST catecholamine signals are of substantially lower magnitude and have slower uptake rates compared with caudate signals. Dopamine terminal autoreceptor activation inhibited roughly half of the catecholamine transient, and noradrenergic autoreceptor activation produced an $\sim 30\%$ inhibition. Dopamine transporter blockade with either cocaine or GBR12909 significantly augmented catecholamine signal duration. We optogenetically targeted dopamine terminals in the dBNST of transgenic (*TH:Cre*) mice of either sex and, using *ex vivo* whole-cell electrophysiology, we demonstrate that optically stimulated dopamine release induces slow outward membrane currents and an associated hyperpolarization response in a subset of dBNST neurons. These cellular responses had a similar temporal profile to dopamine release, were significantly reduced by the D2/D3 receptor antagonist raclopride, and were potentiated by cocaine. Using *in vivo* fiber photometry in male C57BL/6 mice during training sessions for cocaine conditioned place preference, we show that acute cocaine administration results in a significant inhibition of calcium transient activity in dBNST neurons compared with saline administration. These data provide evidence for a mechanism of dopamine-mediated cellular inhibition in the dBNST and demonstrate that cocaine augments this inhibition while also decreasing net activity in the dBNST in a drug reinforcement paradigm.

Key words: BNST; cocaine; dopamine; electrophysiology; fiber photometry; voltammetry

Significance Statement

The dorsal bed nucleus of the stria terminalis (dBNST) is a region highly implicated in mediating stress responses; however, the dBNST also receives dopaminergic inputs from classically defined drug reward pathways. Here we used various techniques to demonstrate that dopamine signaling within the dBNST region has inhibitory effects on population activity. We show that cocaine, an abused psychostimulant, augments both catecholamine release and dopamine-mediated cellular inhibition in this region. We also demonstrate that cocaine administration reduces population activity in the dBNST, *in vivo*. Together, these data support a mechanism of dopamine-mediated inhibition within the dBNST, providing a means by which drug-induced elevations in dopamine signaling may inhibit dBNST activity to promote drug reward.

Received Feb. 4, 2021; revised May 5, 2021; accepted May 12, 2021.

Author contributions: J.R.M., R.E.P., G.J.S., J.R.L., A.B., and D.G.W. designed research; J.R.M., R.E.P., G.J.S., J.R.L., and A.B. performed research; J.R.M., R.E.P., G.J.S., J.R.L., and A.B. analyzed data; J.R.M. wrote the first draft of the paper; J.R.M., R.E.P., G.J.S., J.R.L., A.B., and D.G.W. edited the paper; J.R.M. and D.G.W. wrote the paper.

This work was supported by National Institutes of Health Grants R37 AA019455 to D.G.W., R01 DA042475 to D.G.W., T32 NS007491 to J.R.M., F32 AA027409 to J.R.M., T32 GM007628 to R.E.P., F30 AA027126 to J.R.L., and T32 GM007347 to J.R.L. We thank Dr. Bob Matthews and the Vanderbilt Cell Imaging Shared Resource Core for help with imaging and analysis.

The authors declare no competing financial interests.

Correspondence should be addressed to Danny G. Winder at danny.winder@vanderbilt.edu.

<https://doi.org/10.1523/JNEUROSCI.0284-21.2021>

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Introduction

The propensity for drugs of misuse to target catecholamine systems has long since implicated catecholamine signaling in the neural processes underlying drug reinforcement and addiction (Wise, 1978, 2008). For example, all drugs of misuse increase dopamine signaling in the NAC, and dopamine transmission in this region is necessary for drug reinforcement learning (Volkow et al., 2019). Conversely, during withdrawal and abstinence, norepinephrine signaling within the extended amygdala is implicated in mediating stress-induced relapse to drug use as a means of negative reinforcement (Koob and Volkow, 2016; Mantsch et al.,

2016). The bed nucleus of the stria terminalis (BNST) is a limbic forebrain component of the extended amygdala and translates information about environmental stressors into appropriate stress responses (Avery et al., 2016; Lebow and Chen, 2016). While norepinephrine innervation heavily targets the ventral BNST (Robertson et al., 2013), in the dorsal BNST (dBNST), norepinephrine innervation is more diffuse and overlaps with dopamine innervation (Freedman and Cassell, 1994), providing the potential for drug reward-related dopamine signaling to modulate stress circuitry. Acute exposure to drugs of misuse robustly increases dopamine signaling in the dBNST, similar to the effects in the NAc (Di Chiara and Imperato, 1988; Carboni et al., 2000). Further, the dBNST projects to the VTA (Silberman et al., 2013), as well as the lateral hypothalamus, a region that governs systemic stress responses and appetitive behaviors (Stuber and Wise, 2016; Giardino et al., 2018); thus, the dBNST is well integrated within classic drug reward pathways to mediate responses to pharmacological reinforcers. Despite the heterogeneity of dBNST neurons (Giardino et al., 2018; Beyeler and Dabrowska, 2020) and highly intersectional connectivity of this region with reward and stress circuitry, nonspecific activation of dBNST neurons is anxiogenic whereas inhibition is anxiolytic (Lee and Davis, 1997; Walker et al., 2003; Sullivan et al., 2004; Resstel et al., 2008; Sajdyk et al., 2008; Kim et al., 2013). Thus, dopamine and norepinephrine signaling, which both occur via volume transmission, can modulate dBNST neuronal populations, potentially in opposition to one another, to favor reward versus stress circuitry, respectively (Park et al., 2012, 2013).

Previous efforts to understand dopaminergic modulation within the dBNST have largely consisted of bath-applying dopamine, or dopamine receptor agonists, to measure changes in presynaptic activity onto dBNST neurons or dBNST field responses (Kash et al., 2008; Krawczyk et al., 2013). However, the use of bath-applied dopamine circumvents endogenous signaling, in which spatiotemporal aspects of transmitter release may encode cell-specific features of neuronal signaling. Nonetheless, these studies have demonstrated examples of dopamine-mediated increases in excitatory activity (Kash et al., 2008) as well as bidirectional changes in inhibitory activity that are dependent on dopamine receptor subtype and drug self-administration history (Krawczyk et al., 2011a,b, 2013). While these studies support the potential for dopaminergic modulation of dBNST activity, their emphasis was on spontaneous presynaptic activity and not direct cellular responses to dopamine, although instances of a dopamine-mediated membrane depolarization were noted in a subset of cells (Kash et al., 2008; Silberman et al., 2013).

Here we wanted to develop a better understanding of the endogenous dopamine signaling profile in the dBNST. We used fast scan cyclic voltammetry (FSCV) in dBNST slices to examine the factors that regulate catecholamine release and to determine the relative contributions of dopamine and norepinephrine to the total evoked catecholamine signal in the dBNST. We show that cocaine, a catecholamine uptake inhibitor, augments dBNST catecholamine signal duration. Using optogenetics to target dBNST dopamine terminal fields combined with whole-cell electrophysiology, we reveal that endogenous dopamine signaling can result in a real-time cellular inhibition of subsets of dBNST neurons, and this effect is also potentiated by cocaine. Further, using fiber photometry to measure calcium fluctuations, we show that cocaine administration, *in vivo*, acutely decreases dBNST population activity in a drug reward paradigm. Together, these data highlight a mechanism for dopamine-mediated inhibition of dBNST neuronal activity.

Materials and Methods

Animals. Adult WT and transgenic mice were maintained in the laboratory mouse colony before experiments. All mice were maintained according to the National Institutes of Health guidelines, and all experimental protocols were approved by the Animal Care and Use Committee at Vanderbilt University Medical Center. WT male C57Bl/6J mice were obtained from The Jackson Laboratory (#00064) at 8 weeks of age and were group-housed in the laboratory mouse colony. For optogenetic studies, the transgenic mouse strain was a BAC-derived (GENSAT) *TH-cre recombinase (Cre)* mouse line (*TH:Cre*) on a C57Bl/6J background (Gerfen et al., 2013; Juarez et al., 2017). Male and female *TH:Cre* mice were bred, genotyped for *Cre recombinase* transgene expression, and maintained in-house. At 8–12 weeks of age, mice underwent intracranial viral injection surgeries. Mice were anesthetized (1.5% isoflurane inhalation) and surgeries performed using an Angle Two stereotaxic frame (Leica Microsystems). A custom-made glass micropipette (Melchior et al., 2015) was inserted bilaterally above the VTA (coordinates from bregma in mm: -3.5 AP, ± 0.3 ML, -3.7 DV), and unilaterally above the caudal linear nucleus (CLi; -4.3 AP, 0.1 ML, -3.5 DV), and ventral periaqueductal gray (vPAG)/dorsal raphe (DR): -4.3 AP, 0.1 ML, -2.75 DV. Microinjections were administered using an air pressure injection system and consisted of applying small pulses of pressure (30 psi, 50–100 ms duration) to the infusion pipette. Individual injections consisted of ~ 0.4 μ l of AAV5-EF1 α -hChR2(H134R)-eYFP (4×10^{12} virus molecules/ml; Virus Vector Core, University of North Carolina). Following surgery, optogenetic mice were returned to the mouse colony, group-housed, and maintained for 6 weeks to allow viral transfection and expression of Channelrhodopsin-2 (ChR2).

Slice preparation for physiological experiments. Animals were anesthetized with isoflurane, transcardially perfused with slice buffer, decapitated, and the brain rapidly removed and placed in pre-oxygenated (95% O₂/5% CO₂) NMDG-based slice buffer (Ting et al., 2014) consisting of the following (in mM): 93 NMDG, 30 NaHCO₃, 25 glucose, 20 HEPES, 2.5 KCl, 1.2 NaH₂PO₄, 10 MgCl₂, 0.5 CaCl₂, 5 Na-ascorbate, 3 Na-pyruvate, 5 N-acetylcysteine, adjusted to pH 7.3–7.4 and 300–310 mOsm. Coronal sections (300 μ m thick) containing the BNST were prepared from each animal with a vibratome (Leica VT1200S; Leica Instruments). Generally, a single experimental slice containing bilateral dBNST, ranging from 0.26 to 0.02 mm from bregma (Franklin and Paxinos, 2007) was obtained from each animal. Slices were transferred to a warm bath (32°C–34°C) of slice buffer and allowed to recover for 12 min. Slices were then transferred to an oxygenated holding buffer as follows: 92 NaCl, 30 NaHCO₃, 25 glucose, 20 HEPES, 2.5 KCl, 1.2 NaH₂PO₄, 2 MgCl₂, 2 CaCl₂, 5 Na-ascorbate, 3 Na-pyruvate, 5 N-acetylcysteine, adjusted to pH 7.3–7.4 and 300–310 mOsm, and maintained at room temperature for > 1 h before physiology experiments. For cyclic voltammetry and whole-cell physiology recordings, slices were transferred to a submersion recording chamber perfused at a rate of 1 ml/min with 32°C oxygenated aCSF containing the following: 126 NaCl, 25 NaHCO₃, 11 glucose, 2.5 KCl, 1.2 NaH₂PO₄, 1.2 MgCl₂, 2.4 CaCl₂, 0.4 L-ascorbic acid, adjusted to pH 7.4 and 300–310 mOsm.

FSCV. WT male C57Bl/6J mice (The Jackson Laboratory) were used for experiments using electrical stimulation, and optogenetic *TH:Cre* male and female mice were used for experiments using optical stimulation. FSCV was performed as described previously (Melchior and Jones, 2017; Harris et al., 2018). Carbon fiber microelectrodes (100–150 μ m length, ~ 7 μ m diameter) were assembled with an epoxied seal and used across experiments. The carbon fiber electrode was placed into the slice ~ 100 μ m below the surface. For electrical stimulation, a stimulating electrode was placed on the surface of the slice in close proximity (~ 150 μ m) to the carbon fiber electrode. For dBNST recordings, the stimulating electrode was placed on the medial side of the dBNST, and the recording electrode was placed on the lateral side of the dBNST; this was done to limit excitation of the neighboring caudate regions lateral to the dBNST. Catecholamine release was evoked by an electrical pulse (350 μ A, 4 ms duration) applied as a multipulse train (5, 10, or 20 pulses) at a frequency of 20 Hz every 10 min. Optical stimulation was delivered by a T-Cube LED Driver (LEDD1B, Thor Labs) and passed through an EN-GFP filter cube (Olympus) to produce blue wavelength light, which

Figure 1. Electrically stimulated catecholamine release in the dBNST *ex vivo*. **A**, Schematic representation of coronal sections containing the BNST (green). Electrically stimulated catecholamine release was measured in the dBNST (1, purple) as well as in the caudate (2, gray) for planned comparison. **B**, Representative voltammetry trace of catecholamine release in dBNST showing current versus time (top), corresponding color plot (bottom), and voltammogram (inset). Scale bar: current (nA) converted to catecholamine (250 nM) versus time (2 sec). **C**, Examples of stimulated catecholamine release measured in dBNST (top, 10 pulse stimulation train) and caudate (bottom, 1 pulse stimulation). Grouped data showing catecholamine release (**D**) and uptake rates (**E**) in the BNST are lower compared with the neighboring caudate. Error bars indicate $\pm$ SEM. $*p < 0.05$. **F**, Correlation analysis, fitted with linear regression line (blue), showing positive relationship between catecholamine release and uptake rate in the dBNST. **G**, Correlation analysis, fitted with linear regression line (blue), showing positive relationship between catecholamine release and uptake rate in the caudate.

was transmitted through the $40\times$ objective (Olympus) on the microscope, positioned directly above the recording region of the slice. Dopamine was evoked by an optical pulse (~ 5 mW, 4 ms duration) applied as a single pulse or a multipulse train (5, 10, or 20 pulses) at a frequency of 20 Hz every 5 or 10 min, dependent on the experiment. During recordings, extracellular catecholamine was monitored at the carbon fiber electrode every 100 ms (10 Hz) using fast-scan cyclic voltammetry (Wightman et al., 1988) by applying a triangular waveform (-0.4 to 1.2 to -0.4 V vs Ag/AgCl, 400 V/s) to the electrode. Following experiments, recording electrodes were calibrated by measuring responses (in electrical current; nA) to a known concentration of dopamine ($3 \mu\text{M}$), using a flow-injection system. The resulting calibration factor was used to convert the measured electrical current (nA) to catecholamine concentration (μM).

Voltammetry experiments began with single-pulse electrical stimulations and recordings in the caudate region of slices containing dBNST, which were repeated every 5 min until the catecholamine signal reached stability. Stability was defined by $<10\%$ variation in magnitude and not trending up or down across three successive recordings; stability was generally achieved ~ 90 min after the slice has been placed in the recording chamber. Once stable, single-pulse evoked catecholamine signals in the caudate were obtained, the recording electrode was repositioned in the dBNST for experiments. Electrical or optical multipulse stimulations (20 Hz) were applied every 10 min until dBNST catecholamine release was stable. All pharmacology experiments began after the establishment of a stable baseline of multipulse stimulated catecholamine release. Once stable baselines were established, drug was added to the perfusion buffer; quinpirole, UK 14304 and raclopride were purchased from Tocris Biosciences. For electrically stimulated catecholamine experiments, Quinpirole ($10 \mu\text{M}$) and UK 14304 ($10 \mu\text{M}$) were applied, in series, with each drug application allowed to perfuse the recording chamber and incubate the slice for 40 min before the next drug was added; catecholamine signals were recorded continuously, at 10 min intervals, throughout the experiment to ensure stability of drug effects before proceeding to the next drug. Similarly, in cocaine experiments, cocaine doses (0.3 – 30

μM) were applied to the perfusion buffer, in a cumulative manner, after the establishment of baseline 10 pulse-stimulated catecholamine signals. Each concentration incubated the slice for 40 min and stable stimulated catecholamine release was obtained with each dose before the next dose was added. For optically stimulated dopamine release experiments, optical stimulations (and dopamine release measurements) occurred with 20 pulse stimulation trains applied every 10 min for 1 h; then 10 pulse stimulation trains were applied every 5 min for 15 min, then 5 pulse stimulation trains were applied every 5 min for 15 min and then a single-pulse stimulation applied once. For drug experiments, this protocol was used to measure baseline optically stimulated dopamine release across stimulation parameters (20, 10, 5, and 1 pulse stimulations) and subsequently either quinpirole ($10 \mu\text{M}$) or raclopride ($10 \mu\text{M}$) was applied to the perfusion buffer. The slice allowed to equilibrate to drug for 30 min, and the same protocol, across stimulation parameters, was applied in the presence of drug.

All voltammetry data were collected and modeled using Demon Voltammetry and Analysis Software (Yorgason et al., 2011) following standard voltammetric modeling procedures (Ferris et al., 2012). The evoked levels of catecholamine and signal half-life measurements are first order kinetic analyses of response curves based on signal magnitude (nA) and the recording electrode calibration factor for converting current (nA) to catecholamine concentration (μM). In experiments comparing the uptake rate in the dBNST versus the caudate, signals were modeled using Michaelis-Menten kinetics (Wightman et al., 1988), which balances the opposing processes of release and uptake. The rate constant (K_m) was set at 160 nM for all analyses of uptake rates (Garris and Wightman, 1994; Wu et al., 2001; Robinson et al., 2008). This is based on the known affinity of dopamine for the dopamine transporter (DAT) (Wu et al., 2001) and that the norepinephrine transporter (NET) has higher affinity for dopamine than for norepinephrine (Gu et al., 1994). Thus, the observed uptake rates for evoked catecholamine signals are standardized to dopamine-DAT affinity and do not provide a kinetic analysis of norepinephrine uptake rates specifically, or the contribution of noncanonical transporter species to catecholamine removal (Holleran

et al., 2020). This K_m value was applied consistently across recordings to provide a qualitative comparison of basal catecholamine signal uptake rates in the dBNST versus the caudate regions, where uptake rate is dominated by DAT function (Holleran et al., 2020).

Whole-cell patch-clamp electrophysiology. Male and female mice were used for whole-cell patch-clamp electrophysiological experiments; no grouped sex differences were detected in *post hoc* analysis of the cellular experimental datasets. All electrophysiology recordings were made using Clampex 10.7 and analyzed using Clampfit 11.1 (Molecular Devices). Recordings were made using a 10 kHz sampling rate and a 2 kHz low pass filter. Recording electrodes for all experiments were filled with a K-glucuronate-based internal solution (in mM) as follows: 125 K-glucuronate, 10 Na-phosphocreatine, 10 HEPES, 4 NaCl, 4 MG-ATP, 0.3 Na-GTP, adjusted to pH 7.3, 280–290 mOsm. The presence of Chr2 in each slice was verified via eYFP fluorescence in fibers proximal to recorded cells. Multiple unidentified cells were patched per slice, and each cell was allowed to equilibrate for 5 min after successful patch access was obtained, before applying optical stimulations. For voltage-clamp experiments, cells were held at -70 mV and membrane currents were recorded; for current-clamp experiments, cells were maintained at resting membrane potential (0 pA current) and membrane voltage responses recorded. In some cells, voltage- and current-clamp experiments were performed in the same cell, whereas other cells were recorded in either voltage- or current-clamp mode. Pipette access resistance was monitored throughout experiments by applying a voltage step between sweeps, and cells in which the access resistance changed by $> 20\%$ were not included in data analysis. Optically stimulated dopamine release occurred via blue light pulses (4 ms) transmitted through the rig microscope objective (see above). Optical stimulation trains (10 pulses at 20 Hz) were applied no more often than once every 5 min.

For pharmacology experiments, raclopride (2 μ M) or cocaine (1 μ M) was applied to the slice perfusion buffer and allowed to incubate the slice for 15 min. Optical stimulations were applied at 5 min intervals throughout, and multiple (2–4) consecutive sweeps, at baseline and following drug incubation, were averaged for data analysis such that each cell had one averaged BL sweep and one averaged drug sweep. Following pharmacological treatments, no further cells were recorded, and the slice was removed. Signal amplitude was determined as the difference in average current (or voltage) measured across a 500 ms sample of the trace immediately before optical stimulation and a 500 ms sample of the trace at the peak of the optically stimulated responses (~ 0.9 s after stimulation onset). In order to calculate the signal half-life of optically stimulated dopamine-mediated slow outward currents or hyperpolarization responses, averaged traces were reduced to a 10 Hz sampling rate, transferred to GraphPad Prism 8, and the descending portion of the curve (14 s following the signal peak) was fit with a nonlinear regression line (exponential, one-phase decay) from which the half-life and r^2 values were determined. Input resistance was determined by applying a brief voltage step (20 mV, 1 s) at baseline and immediately following optical stimulation in the same sweep. The average resistance across the 1 s voltage step was determined and the resistance following optical stimulation was compared with resistance at (prestimulation) baseline.

Histology. Immunohistochemistry was used to verify Chr2 expression in dopamine terminal fields in the dBNST, as well as providing an anatomic appreciation of circuit morphology, as previously described (Melchior et al., 2015). Male and female mice were anesthetized (isoflurane inhalation) and transcardially perfused with PBS followed by 4% PFA solution in PBS. Brains were then removed, submerged in 4% PFA for an additional 24 h, and subsequently transferred to 30% sucrose in PBS for 72 h. Coronal sections (40 μ m) were obtained on a Leica CM3050S (Leica Instruments) cryostat and stored in PBS for immunohistochemistry. Sections were permeabilized in 0.3% Triton (Sigma Millipore) in PBS (PBS-Tx) for 2 h, blocked in 5% normal goat serum (Vector Laboratories) in PBS-Tx, and incubated in primary antibody in the blocking solution for 24–48 h. Primary antibodies include chicken anti-GFP (1:500 μ g/ μ l; Aves Labs) and rabbit anti-TH (1:1000 μ g/ μ l; Sigma Millipore). Sections were rinsed and transferred to secondary antibody in blocking solution for 1.5 h. Secondary antibodies include fluorescein-labeled goat anti-chicken IgY (Aves Labs, 1:250 μ g/ μ l) and goat

anti-rabbit AlexaFluor-594 IgG (1:250 μ g/ μ l; Invitrogen). Sections were mounted on 1 mm slides with Vectashield (Vector Labs) mounting medium, and images were obtained with an Axio Imager M2 fluorescent microscope (Carl Zeiss). Images were processed in Adobe Photoshop.

RNAscope. Assays for FISH of mRNA were performed as described previously (Fetterly et al., 2019; Salimando et al., 2020). Probes used were mm-Drd1-C1, mm-Drd2-C2, mm-Drd2-C3, and 3-plex negative control probe, obtained from Advanced Cell Diagnostics, and assays performed according to the company's protocol for labeling fresh frozen tissue. For tissue preparation, C57Bl6 mice ($n = 5$; 3 M/2F), aged 8–14 weeks, were anesthetized with isoflurane, brains rapidly removed, frozen in Tissue Tek OCT compound (Sakura Finetek) and stored at -80°C . Coronal slices (16 μ m) containing the dBNST were obtained on a CM3050S cryostat (Leica Microsystems), adhered to a charged glass microscope slide, and refrozen at -80°C until performing the hybridization protocol. Fixation, dehydration, hybridization, and fluorescent labeling of dBNST slices were performed according to the Advanced Cell Diagnostics protocol for hybridization of fresh frozen tissue. Following hybridization, slices were mounted in Aqua-Poly Mount (Polysciences) and coverslipped for imaging. Images were obtained with a Carl Zeiss 710 confocal microscope; for quantification, composite images of dBNST were acquired at $40\times$ (water immersion) magnification. Negative control images were used to determine the brightness and contrast parameters that minimized autofluorescence, and these adjustments were then applied to experimental images. Images were analyzed using Fiji/ImageJ (National Institutes of Health). The ROI was defined as the lateral half of each dBNST, such that the anterior-lateral, oval, and juxtacapular nuclei of the BNST were included in cell counting. D1 and D2 receptor mRNA transcripts were readily identified as fluorescent, round, fraction delimited spots over the surrounding DAPI-labeled nuclei; DAPI labeling distinguished individual cells.

Fiber photometry. WT male C57BL/6J mice (The Jackson Laboratory) were delivered at 8 weeks of age and acclimatized for at least 2 weeks before surgery. Mice were group-housed 2–5 per cage and maintained on a 12:12 h light cycle with light on at 7:00 A.M. Mice had access to food and water *ad libitum*. Following surgery and throughout behavioral testing, mice were singly housed. For surgeries, mice were anesthetized with isoflurane (initial dose = 3%; maintenance dose = 1.5%), and injected intracranially with AAV5-hSyn-GCaMP7f (Addgene). Targeted unilateral injections of 300 nl virus were made into the dBNST (AP: 0.14, ML: 0.88, DV: -4.24) at a 15.03° angle, with a perfusion rate of 50 nl/min. For fiber photometry experiments, a 400 μ m fiber optic cannula (Doric Lenses) was implanted 0.02 mm above the virus injection, and fixed to the skull using a dual-cure resin (Patterson Dental). Postexperimental expression of viral constructs and cannula placement was validated with endogenous fluorescent histology as described previously (Harris et al., 2018; Perez et al., 2020). Single $5\times$ images were taken with a Zeiss 710 (Carl Zeiss) confocal microscope.

The cocaine conditioned place preference (CPP) procedures were conducted as previously described (Mantsch et al., 2010; Perez et al., 2020). Mice were handled and acclimated to the fiber patch cord for 2 weeks before the experiment. On day 1, the pre-conditioning testing day, mice were allowed to freely move between the sides of the CPP apparatus for 20 min, and the time spent on each side was recorded. Mice showing a $> 65\%$ preference for one side of the apparatus were excluded from further analyses ($n = 2$). Next, mice were conditioned to cocaine. During the conditioning phase, mice received daily alternating injections of either cocaine (15 mg/kg, i.p.) or saline and then were immediately restricted to a compound-paired side of the apparatus for 20 min. After conditioning, CPP expression was determined by allowing mice to freely move between the sides of the chamber for 20 min. CPP was defined as an increase in time spent on the cocaine-paired side from baseline.

Optical detection of GCaMP fluorescence was acquired using a RZ5P fiber photometry workstation (Tucker-Davis Technologies) as previously described (Jaramillo et al., 2020; Salimando et al., 2020). Dual excitation wavelengths (470 nm blue light and 405 nm violet light) were generated by LEDs (ThorLabs), passed through a fluorescence minicube (Doric Lenses), which is coupled to a fiber-optic patch cable (400 μ m, 0.48 NA) connected to the implanted fiber-optic ferrule. Fluorescent

emission from the tissue was back-projected through the patch cable and minicube onto a femtowatt photoreceiver (Newport), such that both optical excitation and detection of GCaMP fluorescence occur concurrently. Fluorescent activity detected in the 470 and 405 nm wavelengths were modulated at frequencies of 210–220 and 330 Hz, respectively, and power output maintained at 20 mA with a DC offset of 3 mA for both light sources. All signals were acquired at 1 kHz, lowpass filtered at 3 Hz, and saved for online analysis. Analysis of the signal was performed with a custom-written MATLAB code. Both signal channels (470 and 405 nm) were monitored continuously throughout recordings, with the 405 nm signal used as an isobestic control. Signals detected with 405 nm wavelength light are not calcium-dependent and are indicative of ambient fluorescence and motion artifacts introduced by movement of the fiber-optic cable. Accordingly, a least-squares linear fit was applied to the 405 nm signal to align it to the 470 nm signal, producing a fitted 405 nm signal. A change in fluorescence (ΔF) was calculated as (470 nm signal – fitted 405 nm signal) and the GCaMP-dependent fluorescence was determined by normalizing ΔF to the fitted 405 nm signal such that $\Delta F/F = (470 \text{ nm signal} - \text{fitted } 405 \text{ nm signal})/\text{fitted } 405 \text{ nm signal}$. GCaMP transients were defined as a local maxima of the $\Delta F/F_0$ trace with a peak prominence that met a predetermined threshold value applied across all treatments and recordings.

Experimental design and statistical analysis. All data are represented as mean $\pm$ SEM for each group, and all statistical analyses were performed using Prism 8 software (GraphPad software). We used both male and female mice within this study. When sex was not found to be a statistically significant variable, data from male and female mice were combined within experimental groups for analysis. For voltammetry experiments, planned comparisons between the dBNST and caudate in DA release and uptake measures were analyzed using an unpaired two-tailed *t* test. The cumulative effects of autoreceptor agonists on stimulated release were analyzed using a one-way ANOVA with baseline, quinpirole, and UK 14304 time points used as groups; Tukey's multiple comparisons test was used to determine differences across groups. Planned comparisons of the respective signal inhibition produced by quinpirole versus UK 14304 were analyzed with a paired two-tailed *t* test. Similarly, the cumulative effects of cocaine on stimulated signal half-life were assessed using a one-way ANOVA across cocaine drug concentration groups, with Tukey's multiple comparisons test to determine differences across groups. Autoreceptor pharmacology effects across optical stimulation parameters were assessed using a two-way repeated-measures ANOVA; stimulation parameter was the within-subjects factor, and drug effect was the between-subjects factor. Differences between groups were tested using a Sidak's *post hoc* multiple comparisons test. For RNA *in situ* data quantification, planned comparisons of the proportion of total cells which express D1 versus D2 receptor mRNA were analyzed using a paired two-tailed *t* test of grouped data. For whole-cell electrophysiology experiments, the grouped effects of optical stimulation on input resistance were analyzed, versus baseline, with a paired two-tailed *t* test. Similarly, raclopride effects on signal amplitude, as well as cocaine effects on signal amplitude and cocaine effects on signal half-life were analyzed, versus baseline, with a paired two-tailed *t* test. Evoked EPSC amplitudes across stimulation pulses were analyzed with a one-way ANOVA; Tukey's multiple comparisons test was used to determine differences across stimulation pulse number. For *in vivo* fiber photometry experiments, planned comparisons between groups were assessed using paired *t* tests for single-factor comparisons or a mixed-effects analysis when comparing a single factor across multiple sessions. When significant main effects were obtained using mixed-effects analysis, Tukey's multiple comparisons test was used *post hoc* to determine differences between groups. For all analyses, significance levels were set at $\alpha = 0.05$.

Results

Electrically stimulated catecholamine release in the *ex vivo* dBNST

In order to understand how dopamine signaling modulates dBNST activity, we began by using FSCV to measure electrically

stimulated catecholamine release profiles in dBNST slices *ex vivo*. Dopamine and norepinephrine innervation converge in the dBNST, resulting in overlapping terminal fields, and these catecholamines have identical electroactive profiles, which make them indistinguishable by cyclic voltammetry. Here we applied local electrical stimulation to elicit catecholamine release. We recorded responses from the dBNST as well as from the neighboring caudate putamen, in the same slices (Fig. 1A), to compare dBNST catecholamine release properties with the traditional high-dopamine signals obtained in the striatum (Calipari et al., 2012b). In the caudate, we detected pronounced catecholamine release, which was elicited with a single-pulse stimulation, consistent with previous research (Calipari et al., 2012b); however, in the dBNST, we found that stimulation trains, 10 pulses at 20 Hz frequency, were necessary to elicit measurable catecholamine signals. Nonetheless, such stimulation trains produced clear and distinct transient responses in the dBNST (Fig. 1B); these transients were time-locked to the stimulation and showed demarcated peaks of current at 0.6 V and -0.2 V on the voltage ramp, which are the signature oxidation and reduction voltages for catecholamines (Wightman et al., 1988). Further, we found that these electrically stimulated responses were consistent in amplitude across successive stimulations for several hours, when measured once every 10 min. These data support that stimulation trains are sufficient to elicit measurable catecholamine release in the dBNST, *ex vivo*, consistent with previous reports in the ventral BNST (Harris et al., 2018; Schmidt et al., 2019).

When comparing catecholamine signals obtained in the dBNST with those from the caudate ($n = 46$ slices across 32 male animals; 46 slices, 32 M), we found that dBNST signals were scaled down in amplitude but longer in duration (Fig. 1C). The average catecholamine release measured in the dBNST was 495 ± 29.8 nM (Fig. 1D), which was less than half (45%) of the magnitude of signals in the caudate (1100 ± 58.6 nM, unpaired $t_{(90)} = 9.20$, $p < 0.0001$). Similarly, the average uptake rate in the dBNST was 202 ± 17.5 nM/s (Fig. 1E), which was significantly slower than the caudate (1955 ± 105.4 nM/s, unpaired $t_{(90)} = 16.42$, $p < 0.0001$). Often release magnitude can influence uptake rates whereby increased release recruits more available transporter and increases the rate of clearance. Maximal uptake rates ($V_{\max}$) can be determined from large signals, such as those in the caudate, where dopamine release saturates the available DATs, thus showing "maximal" DAT function in the region (Fig. 1C). With the relatively low release profile of signals in the dBNST, it cannot be assumed that maximal transporter function is achieved in the observed uptake rates. However, there was a positive correlation between release and uptake rate in the dBNST ($r_{(44)} = 0.52$, $p = 0.0002$; Fig. 1F), as there was in the caudate ($r_{(44)} = 0.68$, $p < 0.0001$; Fig. 1G), such that the larger signals had increased uptake rates, which would be consistent with active transporter function (Miles et al., 2002). Nevertheless, the uptake rate in the dBNST was approximately one-tenth of that in the caudate, which resulted in a noticeably longer duration of the signal extrasynaptically. In a separate experiment, we applied 10 pulse stimulation trains in both the caudate ($n = 3$ slices, 2 M) and dBNST ($n = 6$ slices, 6 M) and found that the signal half-life in the dBNST (2.63 ± 0.39 s) was nearly 4 times the duration of caudate signals (0.69 ± 0.10 s; $t_{(7)} = 3.4$, $p = 0.01$). Together, these data suggest that catecholamine signaling in the dBNST is tuned for lower release and longer duration compared with that in the caudate.

Figure 2. Dorsal BNST catecholamine autoreceptor pharmacology. **A**, Grouped data timeline showing the cumulative effects of the D2 autoreceptor agonist quinpirole (Quin, arrow) and the α_2 adrenergic autoreceptor agonist UK 14304 (UK, arrow), applied in series. Each autoreceptor agonist provided distinct inhibition of the stimulated release magnitude over the course of the experiment. **B**, Representative traces of the dBNST signal at baseline (BL, black) and following bath application of Quin (blue) and subsequent application of UK (Quin + UK, red). Each autoreceptor agonist reduced the amplitude of catecholamine release. **C**, Grouped data showing the amount of inhibition resulting from Quin (blue) was greater than inhibition from UK (red). **D**, Estimated contribution of dopamine (DA, blue) and norepinephrine (NE, red) to the total catecholamine signal in the dBNST, based on magnitude of autoreceptor agonist effects. The majority of the catecholamine signal in the dBNST is DA. **E–H**, Reversing the order of autoreceptor agonist application results in increased effects of UK on stimulated catecholamine signals. **E**, Timeline showing the cumulative effects of UK and Quin application on stimulated catecholamine signals. **F**, Representative traces of stimulated catecholamine signals at BL (black), following application of UK (red), and subsequent application of Quin (UK + Quin, blue). **G**, Grouped data showing the respective signal inhibition produced by UK and Quin. **H**, The estimated contribution of DA is greater than NE in the dBNST. **I–L**, Autoreceptor agonist pharmacology in the ventral BNST. **I**, Timeline of Quin and UK application on stimulated catecholamine signals in the ventral BNST. **J**, Representative traces of ventral BNST catecholamine signals at BL (black), following application of Quin (blue) and subsequent application of UK (Quin + UK, red). **K**, Grouped data showing the respective signal inhibition produced by Quin and UK in the ventral BNST. **L**, Estimated contribution of DA and NE to catecholamine signals in the ventral BNST; catecholamine signals in the ventral BNST are primarily noradrenergic. Grouped data presented as mean, error bars indicate $\pm$ SEM; individual data points overlain on bar graphs. $*p < 0.05$.

Catecholamine autoreceptor pharmacology in the dBNST

Next, we wanted to examine the relative contributions of dopamine and norepinephrine to the electrically stimulated catecholamine signals in the dBNST. Dopamine terminals and norepinephrine terminals each express autoreceptors (D2 dopamine receptors and α_2 adrenergic receptors, respectively), which act as a negative feedback mechanism to regulate transmitter release. Activating these autoreceptor populations can potentially inhibit stimulated catecholamine release (Gillsbach et al., 2009; Ford, 2014). We wanted to examine the relative contributions of dopamine and norepinephrine to our total signal; therefore, we bath-applied, in series, the D2/D3 dopamine autoreceptor agonist quinpirole (10 μ M) and the α_2 -adrenergic receptor agonist UK 14304 (10 μ M) and measured the inhibition of electrically stimulated catecholamine release. In the dBNST, we found that application of each autoreceptor agonist resulted in clear inhibition of the catecholamine signal (Fig. 2A). Grouped data ($n = 7$ slices, 5 M) show that quinpirole caused a rapid and significant inhibition of release, reducing the signal to $45.5 \pm 1.9\%$ of baseline amplitude (Fig. 2B). The maximal effect occurred primarily within the initial 10 min interval (between stimulations)

following drug application. The amplitude of stimulated release then remained consistent for the next 30 min. Subsequently, we applied UK 14304, and a second inhibition of stimulated release occurred, reducing the signal to $18.0 \pm 1.7\%$ of baseline over the course of 30 min. A one-way ANOVA found a significant effect of autoreceptor agonists on release amplitude ($F_{(2,6)} = 1170$, $p < 0.0001$) across time points 60 min (baseline), 100 min (quinpirole), and 140 min (quinpirole + UK 14304). Tukey's multiple comparisons tests showed significant effects of quinpirole on baseline amplitude, as well as significant effects of UK 14304 on the amplitude of signals with quinpirole already on board ($p < 0.0001$, respectively). We next compared the amount of inhibition produced by quinpirole versus UK 14304 in the dBNST (Fig. 2C). Quinpirole produced a $54.3 \pm 1.9\%$ inhibition of the signal, which was significantly larger than the subsequent $29.5 \pm 2.2\%$ inhibition produced by UK 14304 ($t_{(6)} = 6.64$, $p = 0.0006$). Based on the autoreceptor pharmacology, we estimate that dopaminergic innervation contributes the majority ($\sim 54\%$) of the electrically evoked catecholamine transient in the dBNST, whereas norepinephrine release accounts for $\sim 30\%$ of the signal (Fig. 2D).

In separate experiments, we reversed the order of autoreceptor agonist application such that UK 14304 was applied first and quinpirole application followed. Again, UK 14304 produced a slow inhibition over the course of 60 min, whereas quinpirole application rapidly inhibited the signal, reaching maximum inhibition within 20 min (Fig. 2E). Grouped data ($n=6$ slices, 5 M) show that UK 14304 reduced the signal to $60.1 \pm 1.9\%$ of baseline, whereas quinpirole application further reduced the signal to $12.2 \pm 1.2\%$ of baseline (Fig. 2F). A one-way ANOVA found a significant effect of autoreceptor agonists on release amplitude ($F_{(2,5)} = 899$, $p < 0.0001$) across time points 60 min (baseline), 120 min (UK 14304) and 150 min (UK 14304 + quinpirole). Tukey's multiple comparisons tests showed significant effects of UK 14304 on baseline amplitude, as well as significant effects of quinpirole on the amplitude of signals with UK 14304 already on board ($p < 0.0001$, respectively). UK 14304 produced a $40.2 \pm 2.2\%$ inhibition of the signal, which was not significantly different from the subsequent $47.7 \pm 3.1\%$ inhibition produced by quinpirole ($t_{(5)} = 1.44$, $p = 0.21$; Fig. 2G). Based on the autoreceptor pharmacology for this order of drug application, the estimated dopaminergic contribution is $\sim 48\%$ and the norepinephrine contribution is $\sim 40\%$ of the signal (Fig. 2H), which represents a quantitative shift in values but still supports a larger contribution of dopamine to the dBNST catecholamine field.

In control experiments, we performed the same experimental design in the ventral BNST, an area believed to primarily contain noradrenergic innervation (Fig. 2I–L). In these experiments, 20 pulse electrical stimulation trains were necessary to elicit comparative levels of catecholamine release, as previously reported (Harris et al., 2018). In the ventral BNST ($n=3$ slices, 3 M), quinpirole had no effect on stimulated catecholamine release ($101.5 \pm 8.0\%$), whereas UK 14304 reduced the signal to $20.8 \pm 1.1\%$ of baseline (Fig. 2I,J). A one-way ANOVA found a significant effect of autoreceptor agonists on release amplitude in the ventral BNST ($F_{(2,2)} = 73.8$, $p = 0.0047$) across time points 60 min (baseline), 100 min (quinpirole), and 140 min (quinpirole + UK 14304). Tukey's multiple comparisons tests did not detect a significant effect of quinpirole on baseline amplitude ($p = 0.95$); however, there was a significant effect of UK 14304 ($p = 0.02$) on signals with quinpirole already on board. UK 14304 inhibited the catecholamine signal $79.2 \pm 1.1\%$, which was significantly more effective than quinpirole ($t_{(2)} = 8.87$, $p = 0.01$; Fig. 2K), and supports that the catecholamine contribution to the ventral BNST is primarily noradrenergic (Fig. 2L). These data also support the idea that quinpirole effects in the dBNST are likely specific to dopamine terminals, and thus represent a more accurate measure of dopaminergic contribution to the total catecholamine signal, whereas the increased inhibition observed when UK 14304 was applied before

Figure 3. Cocaine effects on dBNST catecholamine signals, *ex vivo*. **A**, Representative traces demonstrating the effects of bath-applied cocaine on stimulated catecholamine release in the dBNST. Baseline (BL, black) signals showed increased release and duration in the presence of cocaine (1 μ M, maroon; 10 μ M, blue). **B**, Grouped data show that cocaine increased the catecholamine signal half-life in a dose-dependent manner. * $p < 0.05$ versus baseline. Data fit with a nonlinear regression line (black). **C**, Representative traces demonstrate that the DAT-specific uptake inhibitor, GBR 12909 (1 μ M, gray), increased catecholamine release and duration in the dBNST, similar to cocaine. **D**, Grouped data show that GBR 12909 (1 μ M, gray) nearly doubled the catecholamine signal half-life in the dBNST. * $p < 0.05$. Grouped data presented as mean with individual points overlain; error bars indicate $\pm$ SEM.

quinpirole, versus after quinpirole, suggests that UK 14304 may have inhibitory actions on dopamine terminals.

Cocaine effects on dBNST catecholamine signals

Next, we measured the effects of cocaine on electrically stimulated catecholamine signals in the dBNST. Similar to the NAC, cocaine markedly increases catecholamine signaling in the dBNST of rats *in vivo* (Carboni et al., 2000; Jadzic et al., 2019). Because the dBNST contains both dopamine and norepinephrine innervation, and cocaine inhibits both DAT and NET (Gu et al., 1994), we wanted to measure the local effects of cocaine on stimulated catecholamine signals in the dBNST, *ex vivo*. Additionally, the basal uptake rates in the dBNST were considerably slower compared with the striatum (Fig. 1), so we were interested in the degree to which cocaine could mediate further decreases in the uptake rates of catecholamine signals in this region. We applied 10 pulse electrical stimulation trains in dBNST slices once every 10 min. Once release amplitude became stable across stimulations, cocaine was bath-applied in doses (0.3, 1, 3, 10, and 30 μ M), in a cumulative manner. Signals were allowed to equilibrate to each dose before the next dose was added.

We found that cocaine augmented stimulated catecholamine signals in the dBNST ($n=8$ slices, 8 M), and this occurred in a dose-dependent manner (Fig. 3A). Increases in release amplitude occurred across the lower doses (0.3–3 μ M), whereas increases in signal duration occurred across all doses, becoming greater as the concentration of cocaine was increased (Fig. 3B). We used the catecholamine signal half-life as a measure of cocaine-induced increases in signal duration. Cocaine significantly increased the catecholamine signal half-life (one way ANOVA: $F_{(5, 42)} = 11.52$, $p = 0.0006$) resulting in

Figure 4. Dopamine receptor mRNA expression across dBNST neurons. **A**, Dopamine mRNA transcript binding in the dBNST. D1 receptor (red) and D2 receptor (green) mRNA transcript expression is robust in the caudate region around the dBNST, whereas, within the dBNST, receptor transcript expression is comparatively less abundant. **B**, Higher magnification (40 $\times$, gray box in **A**) of dBNST dopamine receptor transcript expression shows that D1 and D2 receptor mRNA is detected in numerous dBNST neurons, as denoted by transcripts overlapping with DAPI staining (blue). **C**, High magnification (63 $\times$) shows that D1 and D2 receptor mRNA expression is largely segregated across separate populations of dBNST neurons. **D**, Quantification of D1 and D2 receptor mRNA expression across neurons in the lateral half of the dBNST ($n = 9$ unilateral dBNST across 5 animals). D1 (red) and D2 (green) receptor transcripts were detected in $\sim 30\%$ of neurons, and were largely segregated to distinct populations (2.9% of cells with overlapping expression; yellow). There was a similar proportion of expression of D1 (17.6%) and D2 (13.7%) transcripts across cells ($p = 0.13$), with $\sim 65\%$ of dBNST neurons showing no expression of dopamine receptor transcripts (unlabeled, blue).

a $223.7 \pm 40.6\%$ increase in signal half-life at the highest dose tested (30 μM). Tukey's multiple comparisons found that each dose of cocaine significantly increased signal half-life compared with baseline (1.95 ± 0.21 s), cocaine 0.3 μM (2.79 ± 0.35 s, $p = 0.02$), cocaine 1 μM (4.20 ± 0.62 s, $p = 0.018$), cocaine 3 μM (5.35 ± 1.01 s, $p = 0.044$), cocaine 10 μM (5.78 ± 1.17 s, $p = 0.049$), and cocaine 30 μM (6.97 ± 1.15 s, $p = 0.014$).

In separate experiments, we bath-applied the DAT-specific uptake inhibitor GBR 12909 (1 μM , $n = 6$ slices, 6 M). We found that GBR 12909 also increased the magnitude of catecholamine signals in the dBNST, in a similar manner to the effects of cocaine (Fig. 3C). Specifically, we found that GBR 12909 (1 μM) nearly doubled the signal half-life in the dBNST (Fig. 3D; baseline 2.63 ± 0.39 s, GBR 5.19 ± 0.51 s), which was a significant increase in signal duration (paired $t_{(5)} = 7.14$, $p = 0.0008$). Thus, 1 μM cocaine (215%) and 1 μM GBR (197%) had similar effects on catecholamine signal half-life, which supports a role for DAT inhibition in cocaine effects on dBNST catecholamine signaling. However, as cocaine inhibits both DAT and NET, and NET can readily take up dopamine as well as norepinephrine (Gu et al., 1994; Carboni et al., 2006), the relative influence of DAT versus NET on cocaine-mediated inhibition of catecholamine uptake in the dBNST remains to be explicitly determined.

Dopamine receptor mRNA expression across dBNST neurons

To begin to explore the distribution of dopamine receptors in the dBNST, we examined the native fluorescence of dBNST neurons in both a D1-tdTomato and a D2-eGFP mouse reporter line ($n = 1$ for each mouse line; data not shown). We identified a small subset of D1-tdTomato-expressing cells scattered throughout the dBNST (with no clear association with the oval nucleus), whereas we noted that D2-eGFP expressing cells were highly abundant throughout the dBNST, consistent with a recent report (Lu et al., 2021). However, as different cell types may express presynaptic D2 receptors on distal terminals (outside of the dBNST), we were interested in determining whether the D2-eGFP signal we noted represented somatodendritic D2 expression within the dBNST, or peripheral D2 expression in these cells. We took coronal sections containing bilateral dBNST from 5 mice (3 M/2 F), and performed RNAscope FISH labeling for D1- and D2-dopamine receptor mRNA, and imaged each section with confocal microscopy. In general, we found that, in the neighboring caudate regions, D1 and D2 mRNA transcript expression was highly abundant and mostly segregated across neurons, as expected (Gagnon et al., 2017). Conversely, within the dBNST, dopamine receptor mRNA transcript expression was comparatively more subtle (Fig. 4A). However, at higher magnification, it was clear that both D1 and D2 mRNA transcripts were expressed across dBNST neurons (Fig. 4B,C), in a

primarily nonoverlapping manner. In general, we found that dopamine receptor transcript expression was more abundant in the lateral half of the dBNST, a region containing the anterior-lateral nucleus, the oval nucleus, and the juxtacapsular nucleus, with the most prominent expression occurring in cells in the ventral lateral corner of the dBNST. Using cell counting (~ 800 cells per dBNST), we quantified the distribution of dopamine receptor transcripts across neurons in the lateral half of the dBNST (Fig. 4D). In total, we found that $17.6 \pm 2.6\%$ of neurons in the lateral dBNST expressed D1 receptor transcripts, whereas $13.7 \pm 2.1\%$ of neurons expressed D2 receptor transcripts, which were not significantly different in proportion ($t_{(8)} = 1.68$, $p = 0.13$). The cellular distribution of D1 and D2 transcripts was largely nonoverlapping; however, $2.9 \pm 0.5\%$ of cells had associated transcripts of both receptor types. Thus, our data suggest that $\sim 30\%$ of neurons in the lateral dBNST express mRNA for D1 and/or D2 dopamine receptors, in primarily distinct populations, whereas $\sim 65\%$ of dBNST neurons were found to be unlabeled for the relevant dopamine receptor transcripts.

Optogenetically stimulated dopamine signals in the dBNST

Previous studies have examined how dopamine modulates synaptic activity in the dBNST (Kash et al., 2008; Krawczyk et al.,

Figure 5. Optically stimulated dopamine signals in the dBNST. **A**, Histologic assessment of viral injections targeting dopamine neurons in the VTA. **A–C**, Red represents TH expression. Green represents ChR2 expression. **B**, Viral injections targeting dopamine neurons in the CLi and the vPAG. **C**, Viral injections in dopamine A10 and A10dc cell groups (A and B) results in robust and targeted ChR2 expression in dopamine terminal fields in the dBNST. **D**, Representative cyclic voltammetry trace of a dopamine signal elicited with a 20 pulse, 20 Hz optical stimulation train (blue). Top, Current increased in response to optical stimulation and lasted ~10 s. Inset, Current peaks at 0.6 V and -0.2 V reflect the oxidation and reduction voltages for dopamine. Bottom, Color plot represents current (z axis) as a measure of voltage (y axis) and time (x axis). Scale bar: y = 100 nm dopamine, x = 1 s. **E**, Optically stimulated dopamine release magnitude was consistent across successive 20 pulse stimulations, applied every 10 min (20p, black line). Stereotyped reductions in release magnitude occurred when stimulation trains were reduced to 10 pulses (10p), 5 pulses (5p), or a single pulse (1p); release magnitude was consistent across repeated stimulations, applied every 5 min, for 10p and 5p stimulation parameters (black line). Application (Drug, arrow) of the D2 autoreceptor agonist quinpirole (quin, red) inhibited release across all stimulation parameters, whereas application of the D2 autoreceptor antagonist raclopride (Rac, purple) increased release with longer duration stimulation trains (20p). Data are grouped average, error bars indicate $\pm$ SEM.

2011a, 2013). However, these studies used bath-applied dopamine, which circumvents endogenous signaling and the spatio-temporal aspects of transmitter release, which may encode specific aspects of neuronal signaling. Here, we were interested in investigating how endogenous dopamine signals alter neuronal function in the dBNST. Thus, we generated an optogenetic strategy to target stimulation specifically to dopamine fields in the dBNST. We used a BAC-derived *TH:Cre* mouse line (GENSAT) and injected virus (AAV5-Ef1 α -DIO-hChR2-eYFP) encoding Cre-dependent expression of ChR2 into several dopamine cell groups that are known to project to the dBNST. Specifically, the dBNST receives innervation from cells within the A10 dopamine cell groups, including the VTA, as well as from a continuum of TH-expressing cells located along the rostral and CLi extending dorsally into the vPAG and DR regions of the A10dc dopamine cell groups (Hasue and Shammah-Lagnado, 2002; Meloni et al., 2006; Zahm and Trimble, 2008). Thus, each animal received viral injections into three separate regions (VTA, CLi, and vPAG) to maximize ChR2 expression in dBNST dopamine terminal fields. Following 6 weeks for viral

incubation, animals were utilized for histologic and physiological assessment of ChR2 expression and function.

Using immunohistochemistry, we colabeled coronal slices for TH, the precursor for dopamine, as well as ChR2 expression, and assessed the accuracy and validity of our injections with respect to ChR2 expression in the dBNST (Fig. 5A–C). We found that bilateral injections into the VTA led to ChR2 expression throughout the region, with little to no expression seen in the neighboring substantia nigra compacta dopamine cells (Fig. 5A). In the posterior midbrain, we found that unilateral injections along the midline, across two depths, was sufficient to infect dopamine neurons in the CLi and the vPAG regions, with minimal expression seen in dopamine cells located laterally in the A8 reticulorubral fields (Fig. 5B). Together, these data support that our injection targets were accurate, and the spread of virus outside the ROIs was minimal. Further, the relatively specific targeting of ChR2 expression within midbrain injection locations supports that norepinephrine neurons, which are located in the hindbrain, are excluded from ChR2 expression. Analysis of the dBNST showed robust ChR2 expression in fibers throughout the region

(Fig. 5C), and there was minimal expression of ChR2 seen in dopamine terminal fields in the neighboring caudate nucleus, which supports that ChR2 expression occurred in dopamine neurons that target the extended amygdala (Lin et al., 2020), including the dBNST.

Next, we measured optically stimulated dopamine release in dBNST slices using FSCV. Optical stimuli (473 nm, LED) were transmitted through a 40 $\times$ microscope lens targeting the dBNST, and were delivered as 5, 10, or 20 pulse stimulation trains with a frequency of 20 Hz. Cyclic voltammetry recordings were performed in the mediolateral dBNST. We found that optical stimulation trains produced clear dopamine signals in the dBNST (Fig. 5D). Signals showed oxidation and reduction peaks at 0.6 V and -0.2 V, which are the signature voltages for dopamine redox reactions, and are identical to catecholamine signals resulting from electrical stimulation of the dBNST (Fig. 1). The average amplitude ($n = 12$ slices, 10 M) of stimulated dopamine release resulting from a 10 pulse optical stimulation train was 223 ± 30 nM dopamine, which was less than the total catecholamine release that occurred from a 10 pulse electrical stimulation train (495 ± 29.8 nM; $t_{(56)} = 4.49$, $p < 0.0001$). Optically stimulated dopamine release was 45% of the magnitude of electrically stimulated catecholamine release, which would be expected from the inference that dopamine constitutes approximately half of the electrically stimulated signal (Fig. 2). The average uptake rate for optically stimulated dopamine release (using 20p stimulations to maximize release amplitude) was 153 ± 21.8 nM/s, which was not significantly different from the uptake rate noted for electrically stimulated catecholamine signals in the dBNST (202 ± 17.5 nM/s; $t_{(57)} = 1.39$, $p = 0.17$).

To measure the stability of optically stimulated dopamine release, we ran a protocol in which we measured 20 pulse-stimulated release once every 10 min for an hour, followed by 10 pulse-stimulated release once every 5 min, then 5 pulse-stimulated release once every 5 min and finally a single-pulse stimulation (Fig. 5E). We found that optically stimulated dopamine release was consistent across successive stimulations for all stimulation parameters. Further, the change in magnitude of release when stimulation trains were reduced from 20-10 to 5 pulses was remarkably consistent across slices. Reducing the stimulation train to 10 pulses reduced the magnitude of dopamine release to $68.5 \pm 1.6\%$ of baseline 20 pulse stimulated release, while reducing the stimulation train to 5 pulses reduced the dopamine signal to $45.2 \pm 1.8\%$ of baseline 20 pulse stimulated release, with very little variability across slices. The reduction in release amplitude produced by 10 and 5 pulse stimulation trains allowed for the interval between stimulations to be reduced to 5 min, while still providing consistent, stereotyped dopamine release on each stimulation.

Next, we wanted to assess optically stimulated dopamine signals for responsiveness to drugs targeting the dopamine D2 autoreceptor. In separate slices, we ran the same protocol across stimulation parameters but bath-applied either quinpirole or the D2 autoreceptor antagonist raclopride during baseline collections with 20 pulse stimulations (Fig. 5E, Drug, arrow). A two-way ANOVA of drug response found that quinpirole (10 μ M, three slices, 3 M) significantly reduced optically stimulated dopamine release across all stimulation parameters compared with control slices ($n = 9$; $F_{(1,127)} = 1312$, $p < 0.0001$). Conversely, raclopride (10 μ M, three slices, 3 M) caused a significant increase in optically stimulated dopamine release compared with control slices ($F_{(1,126)} = 147.7$, $p < 0.0001$), with the primary effect occurring during the 20 pulse stimulation parameter. This was

expected, as dopamine release must be of sufficient duration to engage autoreceptor feedback inhibition (Bello et al., 2011); thus, D2 autoreceptor antagonists show more pronounced effects on dopamine release with longer duration stimulation parameters, such as the 20 pulse (1 s) stimulations used here. Together, these data support that optogenetically targeting dopamine terminals in dBNST allows for repeated optical stimulation of endogenous dopamine release in dBNST slices *ex vivo*, and this release was stable over time and across different stimulation parameters. In general, we found that optically stimulated signals were cleaner, more consistent, and more responsive than electrically stimulated signals in the dBNST.

Dopamine-mediated hyperpolarization responses in dBNST neurons

Next, we performed whole-cell electrophysiological recordings of dBNST neurons in *ex vivo* slices, and measured the cellular responses to optically stimulated dopamine release in optogenetic mice (9 M/11 F). Optical stimulation trains were 10 pulses at 20 Hz (500 ms duration), with a minimum 5 min interval between stimulations to allow for consistent dopamine release amplitudes across stimulations (Fig. 5E). We blind-patched individual dBNST neurons and found that, in a subset of cells, optically stimulated dopamine release induced novel cellular responses. Specifically, 31% of cells showed a clear slow outward current that was triggered by optical stimulation, and had a time course that was very similar to optically stimulated dopamine release (Fig. 6A,B). Further, when recording in current-clamp mode (at resting membrane potential), these slow currents translated into a hyperpolarization of the cell membrane that also followed a similar time course to the duration of dBNST dopamine release (Fig. 6C).

Grouped data (Fig. 6D) showed the average peak amplitude of outward current in response to optically stimulated dopamine release was 6.79 ± 0.88 pA ($n = 19$ cells), whereas, in current-clamp mode, this translated to a peak hyperpolarization amplitude of -4.74 ± 0.67 mV ($n = 14$ cells). In several instances, we increased the number of pulses in the optical stimulation train to 20 pulses (20 Hz, 1000 ms duration), which would result in increased dopamine release amplitude (Fig. 5); however, this did not affect the amplitude of whole-cell responses, suggesting a ceiling effect of receptor activation occurs with 10 pulse optically stimulated dopamine release. The signal half-life for optically stimulated dopamine-mediated current responses was 2.87 ± 0.35 s ($n = 16$ cells), whereas the half-life of hyperpolarization responses was 3.18 ± 0.24 s ($n = 14$ cells); thus, the duration of the hyperpolarization response was not significantly different from the current response ($t_{(28)} = 0.72$, $p = 0.47$; Fig. 6E). In a subset of dopamine responsive cells, we bath-applied the D2/D3 dopamine receptor antagonist raclopride (2 μ M) and measured the effect on the amplitude of hyperpolarization responses ($n = 4$ cells). Raclopride significantly decreased the amplitude of optically stimulated hyperpolarization responses by 77% ($t_{(3)} = 3.97$, $p = 0.029$; paired t test; Fig. 6F), suggesting that optically stimulated dopamine-mediated hyperpolarization responses are largely dependent on D2/D3 dopamine receptor activation.

With regards to the similarities between the dopamine release signal and the cellular responses, we assessed the time from stimulation onset (10 pulse train) to the peak response and found that optically stimulated dopamine release peaked at 0.9 ± 0.05 s ($n = 12$ slices), while the peak current response occurred at 1.03 ± 0.08 s ($n = 17$ cells) and the peak voltage response at 1.35 ± 0.08 s ($n = 14$ cells) following optical stimulation onset. A one-

D2/D3-type Response

Figure 6. Dopamine-mediated hyperpolarization responses in dBNST neurons. **A**, Representative trace of dopamine release in response to optical stimulation (10 pulses, 20 Hz; blue). **B**, Representative averaged trace of slow outward current (pA) in response to optically stimulated dopamine release ($n = 7$ cells). **C**, Representative averaged trace of hyperpolarizing voltage (mV) response to optical stimulation at resting membrane potential, normalized for grouped average ($n = 7$ cells). **D**, Grouped data showing the absolute peak amplitude of current (pA) and voltage (mV) responses across cells. **E**, Grouped data showing the signal half-life for current (pA; **B**) and voltage (mV; **C**) responses across cells. **F**, The D2/D3 receptor antagonist raclopride ($2 \mu\text{M}$) significantly reduced the peak hyperpolarizing voltage (mV) amplitude in response to optical stimulation. $*p < 0.05$. **G**, Input resistance as a percentage of baseline resistance is significantly decreased following optical stimulation in cells with a slow current response (SC^+) compared with cells with no slow current response (SC^-). $*p < 0.05$. Bar graphs represent grouped mean with individual points overlain; error bars indicate $\pm$ SEM.

way ANOVA found a significant difference in rise time across groups ($F_{(2,40)} = 9.1$; $p = 0.0006$) with the hyperpolarization response being significantly slower to peak than dopamine release (Tukey's multiple comparisons test; $p = 0.0006$). Thus, the order of operations appears to be that dopamine release induces membrane currents which result in hyperpolarization, with the temporal difference between peak extrasynaptic dopamine release and peak cellular hyperpolarization being ~ 450 ms. Next, we looked at signal duration and found that the half-life of optically stimulated dopamine release was 1.80 ± 0.16 s ($n = 12$ slices; Fig. 5) and the half-life of optically stimulated outward current responses was 2.87 ± 0.35 s ($n = 19$ cells), which was significantly longer ($t_{(26)} = 2.53$, $p = 0.018$). Thus, the cellular response had a 60% longer half-life than the dopamine release signal.

In an effort to determine the functional consequences of these dopamine-mediated outward currents, we performed a test to determine whether membrane input resistance was altered by endogenous dopamine release. We applied a voltage step (20 mV, 1 s duration) at baseline and immediately following the optical stimulation, where the outward current reaches its peak amplitude. We compared the changes in input resistance from baseline between cells that showed a slow outward current (SC^+) versus cells that showed no slow outward current (SC^-) in response to optical stimulation (Fig. 6G). Optically stimulated dopamine-mediated slow outward currents reduced the membrane input resistance to $86.1 \pm 3.4\%$ of baseline ($n = 7$ cells), whereas cells that showed no slow current response ($n = 4$ cells) had an input resistance of $101.7 \pm 1.8\%$ of baseline; thus, the slow current responses significantly reduced the membrane input resistance ($t_{(9)} = 3.24$, $p = 0.01$).

Glutamate corelease from optically stimulated dopamine terminals in the dBNST

A number of recorded cells showed fast EPSCs in response to optical stimulation of dopamine terminals in the dBNST, indicative of glutamate signaling. Glutamate corelease from optically stimulated dopamine terminals in the dBNST (specifically from DR/vPAG afferents) has been reported previously (Li et al., 2016; Matthews et al., 2016). These fast responses were distinguishable from the slow current responses detected in cells (Fig. 7A). Further, glutamate corelease was detected in cells that did not show slow current responses, and vice versa, suggesting that these cellular responses were not interdependent. In a sample of 51 cells (9 M/8 F animals), we found that 65% of all recorded cells showed optically induced EPSCs; in comparison, 31% of cells showed optically induced slow currents. Of the cells that had slow current responses, 63% also had optically stimulated EPSCs. Thus, the occurrence of detecting EPSCs was higher than the detection of D2/D3-mediated slow currents, and the percentage of cells showing EPSCs was roughly the same regardless of whether those cells had slow current responses.

We also found that the magnitude of glutamate corelease was variable across cells, with some cells displaying robust EPSCs at each pulse of the optical stimulation train (10 pulses, 20 Hz), while in other cells, the responses were more abbreviated in magnitude or were not present at all. We set a threshold for glutamate corelease such that the first pulse elicited an EPSC of > 10 pA, and at least 3 (of 10) pulses elicited detectable EPSCs. Subsequently, we plotted the EPSC amplitude across the 10 pulses of the stimulation train for all cells that met the threshold. We found that the first pulse of the stimulation train generally produced the highest magnitude EPSCs, -35.9 ± 3.02 pA ($n = 51$ cells), with the EPSC amplitude being lower across the

Figure 7. Glutamate corelease from optically stimulated dopamine terminals in the dBNST. **A**, Representative trace of optically stimulated dopamine-induced cellular response highlighting the glutamate corelease upon stimulation (red box). Inset, Example trace of a cell with strong glutamate corelease with clear EPSCs detected at each pulse of the stimulation train (blue). **B**, Grouped data showing the magnitude of EPSCs detected across the 10-pulse stimulation train for all cells that showed detectable glutamate corelease. The magnitude of first pulse EPSCs was greater than the EPSC magnitude for each subsequent pulse in the stimulation train. $*p < 0.001$. **C**, There was no correlation between the amplitude of the first pulse EPSC (when present) and the magnitude of slow current responses, suggesting that these events are not interdependent.

successive pulses of stimulation (Fig. 7B). The magnitude of the first pulse EPSC was significantly larger than the EPSCs produced in subsequent pulses in the stimulation train (one-way ANOVA; $F_{(9,381)} = 23.22$; $p < 0.0001$; Tukey's multiple comparisons test; $p < 0.0001$ vs each subsequent stimulation pulse EPSC amplitude). Our stimulation parameters (20 Hz) result in a 50 ms interpulse interval. Therefore, we measured the paired-pulse ratio of the first 2 stimulation pulses in the train. Grouped data showed the paired-pulse ratio of the first two pulses to be 0.57 ± 0.04 ($n = 50$ cells). We plotted the magnitude of the first pulse fast EPSC with the magnitude of the evoked slow current responses (Fig. 7C) and found that there was a low, nonsignificant, negative correlation between EPSC magnitude and slow current magnitude ($r_{(14)} = -0.40$, $p = 0.13$), with the two largest slow current responses showing no evoked EPSCs. This further supports that glutamate corelease and dopamine signaling may represent separate modes of communication from dopamine inputs within the dBNST.

Cocaine augments dopamine-mediated inhibitory responses in dBNST neurons

We next examined how cocaine alters cellular responses to optically stimulated dopamine release in the dBNST. We previously showed that bath-applied cocaine dose-dependently increased the half-life of electrically stimulated catecholamine release in the dBNST (Fig. 3). This effect is consistent with cocaine inhibition of DAT and NET (Gu et al., 1994), which inhibits catecholamine uptake rates and prolongs the extrasynaptic catecholamine signal. In optogenetic animals (4 M/8 F), we sampled the dBNST neuronal population with whole-cell patch-clamp recordings to identify neurons that responded to optically stimulated dopamine release (10 pulses, 20 Hz, 5 min intervals). When we identified cells that showed the D2/D3 receptor mediated responses, we collected baseline signals and then bath-applied cocaine (1 μM) to the slice. We found that cocaine increased the duration of optically stimulated slow current responses (Fig. 8A) and/or membrane hyperpolarization responses (Fig. 8B), with a clear flattening of the signal decay slope. This effect would be consistent with cocaine inhibiting dopamine terminal transporters, reducing the uptake rate, and prolonging the stimulated dopamine release signal. Grouped data showed that cocaine did not alter the peak amplitude of current responses ($t_{(4)} = 0.36$, $p = 0.74$; Fig. 8C). However, the slow current signal half-life was significantly increased by cocaine from 2.52 ± 0.23 s at baseline

to 10.09 ± 1.87 s after cocaine ($t_{(4)} = 3.94$, $p = 0.017$; Fig. 8D). Similarly, cocaine did not increase the peak amplitude of optically stimulated hyperpolarization responses ($t_{(6)} = 0.39$, $p = 0.71$; Fig. 8E). Yet, the hyperpolarization response half-life was extended from 4.06 ± 1.05 s at baseline to 8.61 ± 1.44 s following cocaine ($t_{(6)} = 4.49$, $p = 0.004$; Fig. 8F). Thus, our data show that cocaine (1 μM) increased stimulated catecholamine release half-life to 215% of baseline (Fig. 3), and the half-life of downstream dopamine-mediated membrane hyperpolarization responses to 212% of baseline (Fig. 8F), showing that modulation of the presynaptic dopamine signal translates well to the postsynaptic cellular responses.

On rare occasions, across all cellular recordings (Figs. 6D, 8E), we came across an optically responsive cell that was also firing action potentials spontaneously. More often than not, this endogenous spiking was transient, lasting 5–10 min. However, in these few instances, we saw that optically stimulated dopamine-mediated hyperpolarization was sufficient to inhibit endogenous spiking. In Figure 8G, we show an example of a cell that briefly started firing action potentials on the initial exposure to cocaine while still maintaining a resting membrane potential of -60 mV. Optically stimulated dopamine release induced a transient membrane hyperpolarization that paused endogenous cell spiking for a duration consistent with the temporal profile of the endogenous dopamine signal. This provides a nice example of the potential impact of dopamine signaling on local micro-circuit activity within the dBNST, highlighting how dopamine signaling can modulate endogenous activity in real time.

Cocaine administration reduces *in vivo* calcium transient activity in dBNST neurons

Acute cocaine administration induces robust increases in extracellular dopamine in the dBNST *in vivo*, in a manner similar in ratio to the NAc (Carboni et al., 2000). Here, we have demonstrated a mechanism of inhibitory dopamine signaling in dBNST neurons and show that cocaine significantly increases the duration of this inhibitory signaling. In addition, we have observed that these cellular responses are rapid and sensitive to the influence of extrasynaptic dopamine levels. Cocaine targets DAT, inhibiting dopamine uptake, and it is believed that, *in vivo*, this results in the accumulation of extrasynaptic dopamine (Jones et al., 1998; España et al., 2008) and increased tonic activation of D2 dopamine receptors (Volkow et al., 2019). Further, D2 receptors have a

Figure 8. Cocaine augments dopamine-mediated inhibitory responses in dBNST neurons. **A**, Representative trace showing cocaine effects on optically stimulated (blue; 10 pulses at 20 Hz) slow current responses; data reduced to 100 Hz sampling frequency to highlight differences in slow current. Cocaine (red) extended the duration of slow currents compared with baseline (black, $n = 2$ cells averaged). **B**, Representative trace showing that cocaine (red) extends the duration of optically stimulated hyperpolarization responses compared with baseline (black, $n = 6$ cells averaged). **C**, Cocaine did not change the peak amplitude of slow current responses (pA). **D**, Grouped data showing that cocaine significantly increased the slow current (pA) half-life compared with baseline ($n = 5$ cells). $*p < 0.05$. **E**, Cocaine did not alter the peak amplitude of hyperpolarization responses (mV). **F**, Grouped data showing that cocaine significantly increased the half-life of hyperpolarization responses (mV) compared with baseline ($n = 7$ cells). $*p < 0.01$. **G**, Example trace of a cell firing endogenous action potentials in response to cocaine; the optically stimulated dopamine-mediated hyperpolarization response paused endogenous spiking. Bar graphs represent grouped mean with individual point overlain; error bars indicate $\pm$ SEM.

higher affinity for dopamine (dissociation constant, $K_d \approx 100$ nM) than D1 dopamine receptors ($K_d \approx 1$ μ M) (Richfield et al., 1989) and thus would be disproportionately sensitive to increases in basal dopamine tone. Therefore, we wanted to determine what effect cocaine has on dBNST neuronal population activity *in vivo*.

To do this, we injected a virus encoding the fluorescent calcium indicator GCaMP7f into the dBNST to express GCaMP endogenously across dBNST neurons (Fig. 9A). GCaMP fluorescence provides a proxy of intracellular calcium-mediated neuronal activation; thus, total GCaMP fluorescence is thought to represent net calcium activity across a population of neurons within the field of illumination (Dana et al., 2019). We implanted fiber optic ferrules, unilaterally, and positioned them to illuminate and record GCaMP fluorescence in the dBNST (Fig. 9B). In order to assess cocaine reinforcement, GCaMP-expressing animals ($n = 13$ M) were run through a previously established cocaine CPP protocol (Perez et al., 2020). During the 20 min pretest session, the amount of time mice spent in each visually distinct side of a modified CPP chamber was recorded. Mice were then conditioned by pairing a cocaine injection (15 mg/kg, i.p.) with 20 min restricted access to one side of the chamber or a saline injection with 20 min restricted access to the alternate side of the chamber. Mice received one pairing session per day, alternating days for cocaine or saline pairing, for a total of four

pairing sessions for each treatment (8 d total; Fig. 9A). Following conditioning, mice were given free access to both rooms of the chamber (20 min), and the time spent on the cocaine-paired side was measured. In addition, during the first and fourth conditioning sessions, for both cocaine and saline, we performed *in vivo* fiber photometry and recorded GCaMP activity in the dBNST during the pairing sessions (20 min). GCaMP fluorescence was detected by emitting a 470 nm wavelength LED through the fiber optic to excite the GCaMP fluorophore and measuring changes in total GCaMP fluorescent signal. In addition, a 405 nm wavelength light was emitted to control for background, nonspecific fluorescent activity and motion artifacts (isosbestic control) (Fig. 9A).

We found that cocaine significantly increased the locomotor activity in cocaine-paired sessions compared with saline-paired sessions (mixed-effects analysis, $F_{(1,89,28.35)} = 53.72$, $p < 0.0001$; Fig. 9C). Tukey's multiple comparisons test found that each cocaine session had significantly greater locomotor activity than each saline session, with no group differences between the two cocaine sessions or between the two saline sessions. The increase in locomotor activity is consistent with the classic effect of cocaine on locomotor function (Chen et al., 2006). However, despite this increased motor function, cocaine significantly decreased the frequency of GCaMP transients recorded in the dBNST compared with saline administration (mixed-effects

Figure 9. Cocaine administration reduced calcium transient activity in dBNST neurons during acquisition of cocaine CPP. **A**, Cocaine CPP experimental design. Cocaine (blue) or saline (white) was administered intraperitoneally and paired with a distinct side of the CPP chamber across four training sessions for each treatment (top). Before training, animals had surgeries to express GCaMP in dBNST neurons (bottom left) and had a chronically implanted fiber optic ferrule positioned over the dBNST. *In vivo* fiber photometry (bottom right) was used to record endogenous GCaMP activity on the first and fourth training sessions for cocaine and saline (top left, green). **B**, *Post hoc* analysis of optical fiber placements in the dBNST for fiber photometry recordings of GCaMP activity. **C**, Grouped data showing cocaine administration significantly increased locomotor activity during the first and fourth training sessions compared with the corresponding training sessions with saline administration. **D**, Cocaine significantly decreased the frequency of GCaMP activity in the dBNST during the first and fourth training session compared with saline administration. **E**, Cocaine place conditioning increases time spent on the cocaine-paired side during the post-conditioning test (POST) compared with the pre-conditioning (PRE) baseline. Bar graphs represent grouped mean with individual points overlain; error bars indicate $\pm$ SEM. $^{**}p < 0.0001$.

analysis, $F_{(2,22,22,92)} = 15.88$, $p < 0.0001$; Fig. 9D). Tukey's multiple comparisons test found that each cocaine session had a significantly lower frequency of recorded GCaMP transients compared with each saline session, with no group differences between the two cocaine sessions or between the two saline sessions. This supports the idea that cocaine-induced elevations in catecholamine signaling may have a predominantly inhibitory effect on regional population activity in the dBNST. Following conditioning, in the post-conditioning test, mice spent more time in the cocaine-paired side compared with the pre-conditioning test (paired t test, $t_{(12)} = 7.58$, $p < 0.0001$; Fig. 9E), showing a 38% increase in time spent in the cocaine-paired side. Thus, the decrease in dBNST calcium activity occurred during the development of cocaine place preference, suggesting that decreased dBNST activity may contribute to cocaine reinforcement.

Discussion

Using *ex vivo* FSCV and whole-cell electrophysiology, we profiled characteristics of endogenous catecholamine release in the dBNST and the cellular responses to endogenous dopamine release in this region. We show that dBNST dopamine release is inhibited by presynaptic D2 autoreceptors, and that endogenous dopamine elicits rapid D2 dopamine receptor-dependent cellular inhibition in a subpopulation of dBNST neurons. Further, we demonstrate that cocaine increases the half-life of both dopamine release and dopamine-mediated cellular inhibition in the dBNST. Finally, we show that intraperitoneal cocaine

administration decreases the activity of dBNST neurons *in vivo*. Together, these data support that dBNST endogenous dopamine signaling is sensitive to cocaine and thus could provide a pathway for drug/alcohol-reward related dopamine signaling to modulate stress-related dBNST circuitry.

Characteristics of dBNST catecholamine signaling

Our studies build on previous results demonstrating dBNST catecholamine detection (Li et al., 2016) by providing a pharmacological assessment of some of the basic tenants that shape signal profiles, namely, autoreceptor-mediated release inhibition and transporter-mediated reuptake and signal termination (Sulzer et al., 2016). We show that activation of dopamine (D2) or norepinephrine ($\alpha 2$) autoreceptors produces separable phases of catecholamine release inhibition, which would be consistent with anatomically overlapping dopamine and norepinephrine terminal fields in the dBNST (Freedman and Cassell, 1994). Further, we show that dopamine autoreceptors inhibited the total catecholamine signal to a greater extent (54%) than norepinephrine autoreceptors (30%), and this contrasts with the ventral BNST, where norepinephrine autoreceptors contributed the entirety of inhibition (79%). Together, these data support the current dBNST/ventral BNST model of catecholamine innervation (Fox and Wightman, 2017), and suggest that, in the dorsal region, dopamine innervation may be a more prominent source of catecholamine.

Stimulated catecholamine signals in the dBNST were considerably lower in magnitude and slower in uptake rate compared with signals in the caudate, and perhaps are more similar in

profile to catecholamine signals observed in the PFC, which are lower in magnitude but maintained for longer durations (Garris et al., 1993; Garris and Wightman, 1994). However, VTA dopamine neurons that project to the PFC do not express D2 autoreceptors, and have very low expression of DAT (Lammel et al., 2008). While it is difficult to disentangle catecholamine uptake in the dBNST (Carboni et al., 2006), here we show that dBNST catecholamine signals are sensitive to cocaine. Further, GBR12909, which is highly specific for DAT over NET, also produced a similar degree of uptake inhibition, which supports that dBNST dopamine terminals express functional levels of DAT (Healey et al., 2008; Matthews et al., 2016). Thus, despite the relatively slow basal uptake rates for dBNST catecholamine signals, cocaine can more than double the half-life in *ex vivo* preparations. This provides a mechanism for a range of psychostimulants that target DAT (Calipari et al., 2012a) to locally increase dopamine tone in the dBNST *in vivo* (Carboni et al., 2000), thus putting dBNST dopamine signaling in play during drug reward (Dumont et al., 2005; Kim et al., 2013).

Rapid dopamine-mediated cellular inhibition

Dopamine transmission has long been thought to regulate behavioral responses to environmental stimuli, yet the relatively slow release profile of dopamine transmission coupled to the long duration of GPCR-mediated cellular signaling pathways (Klein Herenbrink et al., 2016) has clouded interpretation of how dopamine transmission may integrate synaptic function to modulate circuit activity in real time. As such, much emphasis has been on deciphering how slower fluctuations in dopamine tone may modulate subthreshold cellular “states” whereby dopamine receptors are engaged prior to converging cortical signaling in response to environmental stimuli (Gerfen and Surmeier, 2011; Manvich et al., 2019). However, dopaminergic neurons within the VTA and the DR/vPAG regions show a similar pattern of phasic activation in response to salient stimuli (Schultz, 1998; Cho et al., 2017; Lin et al., 2020), which induces transient elevations in dopamine in the dBNST (Park et al., 2012, 2013). The phasic aspects of dopamine transmission within the dBNST underscores the need to better understand how rapid fluctuations in dopamine signaling may influence cellular and circuit activity in this region, and that was the goal of this study.

The use of optogenetics to induce endogenous dopamine release has recently demonstrated that phasic dopamine transients can induce rapid, and long-lasting, changes in cellular excitability in the NAc (Lahiri and Bevan, 2020). Our work extends this demonstration of rapid dopaminergic actions to D2 receptor-expressing neurons in the dBNST. We show that endogenous dopamine signals hyperpolarize dBNST neurons on a time scale that closely mimics endogenous dopamine release (Fig. 8G). This effect is similar to those observed by Marcott et al. (2014) in which they artificially overexpressed G-protein gated inwardly rectifying K^+ (GIRK) channels in striatal MSNs, and found a real-time effect of D2 receptor activation on cell hyperpolarization. This also highlights that, within the complexity of GPCR signaling cascades, some cellular effects may be rapid; for example, inhibitory $G_{i/o}$ receptor complexes can be coupled to membrane GIRK channels (Kano et al., 2019) such that dissociation of the $G\beta\gamma$ -subunit upon GPCR activation can rapidly activate GIRK channels independently of $G\alpha$ -subunit modulation of adenylyl cyclase activity.

While endogenous expression of GIRK channels is very low in striatal MSNs, GIRK subunits are heavily expressed in the dBNST, where they are thought to modulate 5-HT receptor-

mediated cellular inhibition (Levita et al., 2004). GIRK channels are also expressed on subsets of VTA dopamine neurons, where they are coupled to D2 receptors (Beckstead et al., 2004; McCall et al., 2017) and provide a similar time scale of inhibition to what we report here. Future directions will investigate whether GIRK channels, or another K^+ -channel subtype (Lahiri and Bevan, 2020), mediate the outward currents induced by D2 receptor activation in dBNST. Further research into the nature of dopamine signaling in the dBNST *in vivo* (i.e., tonic vs phasic) will also be important in determining the role of these real-time cellular effects, as the demonstration of rapid responses to phasic dopamine release does not exclude that more prolonged shifts in tonic dopamine activity may also hold a cell in a more hyperpolarized state. Indeed, prolonged elevations of dBNST dopamine, via the local actions of cocaine, are the basis for our hypothesis as to how cocaine may be reducing dBNST neuronal population activity. We also did not detect sex differences in the cellular responses to dopamine; however, our groups were underpowered for analysis of sex as a variable, and dBNST dopamine signaling has been suggested to mediate sexually dimorphic behaviors (Yu et al., 2021).

Inhibition of dBNST activity *in vivo*

Kim et al. (2013) investigated dBNST neuronal effects on anxiety-like behaviors and found that either pharmacological or non-specific optogenetic inhibition of the dBNST was anxiolytic. Further, they targeted D1 receptor-expressing neurons in the dBNST with ChR2 and showed that these neurons had interneuron properties, forming local synaptic connections with other dBNST cells, and that ~80% of these connections were inhibitory. Thus, the combination of D1 receptor-expressing neurons locally inhibiting dBNST neurons (Krawczyk et al., 2011b, 2013) and D2 receptor-expressing neurons showing direct inhibition by stimulated dopamine release suggests that elevated dopamine signaling would produce a net inhibition of dBNST activity. Dopamine receptor activation within the dBNST can also reduce blood corticosterone levels in mice (Di et al., 2020), further highlighting a mitigating role of dBNST dopamine signaling in stress responses. While mRNA analysis of D2 receptor-expressing neurons in the dBNST showed a similar propensity of D2- versus D1-expressing neurons in the dBNST, evidence from D2-eGFP cell reporter mice suggest that we may be underrepresenting the role of D2 receptor expressing cells in the dBNST (Lu et al., 2021). Nonetheless, these studies indicate that drug-induced elevations in dopamine signaling permit multiple modes of inhibition of dBNST activity in a manner that may be anxiolytic and drive pro-reinforcement behavior.

Here we show that intraperitoneal cocaine administration significantly decreases dBNST neuronal activity *in vivo*. This effect is similar to cocaine actions on neuronal activity in the NAc (Schramm-Sapota et al., 2006; Calipari et al., 2016). While overall NAc neuronal activity was significantly decreased, subtype-specific analysis showed that cocaine decreased D2-MSN activity and increased D1-MSN activity, effects that would both contribute to inhibition of dBNST neuronal populations. Further, the reduced activity of NAc MSNs in response to cocaine during training coincided with reward learning-associated activity in the NAc in post-cocaine testing (Calipari et al., 2016). Indeed, we show that reduced dBNST neuronal activity in response to cocaine administration during training resulted in a cocaine CPP in these animals, further supporting that reduced dBNST activity during drug administration may contribute to drug reinforcement. Notably, cocaine inhibits

reuptake of dopamine, norepinephrine, and serotonin (Gu et al., 1994; Shen et al., 2004), all of which contribute to cocaine reward (Sora et al., 2001; Hall et al., 2004) and all of which modulate dBNST signaling (Marcinkiewicz et al., 2016); therefore, parsing out the relative contributions of each monoamine species to the inhibitory actions of cocaine on dBNST activity will be important for understanding how different modes of peptide modulation in this region contribute to cocaine reward.

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