Supplemental Experimental Procedures

Animals. All animal experiments were approved by the Stanford University IACUC committee, protocol #10747. All mice used in this study were on a C57BL/6J background. In addition to wildtype mice (Jackson, strain 664), Ai9 tdTomato cre reporter mice (Jackson, strain 7909), TH-GFP transgenic mice (MMRRC, stock #000292-UNC) and D1-tdTomato transgenic mice (Jackson, strain 16204) were used where indicated. Only heterozygote transgenic mice, obtained by backcrossing to C57BL/6J wildtypes, were used for experiments. All mice were 2-4 months old. Males were used for TRIO and output tracing. Electrophysiology experiments and behavior were performed in both males and females (counterbalanced across conditions) with no significant effects of sex observed.

Injection and Implant Surgeries. Mice were anesthetized in an isoflurane induction chamber at 3-4% isoflurane, and then injected with buprenorphine (0.05 mg/kg, s.q.) and carprofen (5 mg/kg, s.q.) prior to the start of surgery. Anesthesia was maintained using 1-2% isoflurane. Hair was trimmed from the scalp and the skin was cleaned with a povidone-iodine solution (Betadine) prior to incision. The scalp was opened using a sterile scalpel and holes were drilled in the skull at the appropriate stereotaxic coordinates (relative to bregma: +0.75 AP, 1.5 ML, -2.8 DV for DMS, +0.25 AP, 2.5 ML, -3.4 DV for DLS, -3.1, 0.8 ML, -4.7 DV for DMS-projecting SNc, and -3.1, 1.3 ML, -4.2 DV for DLS-projecting SNc.) Viruses were infused at 100-200 nl/min through a 33-gauge injection needle using a syringe pump (World Precision Instruments). Red Retrobeads IX (Lumafluor, diluted 1:2 in sterile saline, 80 nl) were infused using the same syringe pump system run at 80 nl/min. The needle was left in place for 5 min following the end of the injection, then slowly retracted to avoid leakage up the injection tract. For fiber photometry experiments, a fiber optic cannula was then implanted over the SNc through the same hole as made for the virus injection. To reduce autofluorescence artifacts and maximize light collection, cannulae (special ordered from Doric Lenses) were fabricated using 0.48 NA 400μM BFH48-400 fiber, non-fluorescent epoxy and metal 2.5mm ferrules. Cannulae were fixed to the skull using a thin later of opaque Metabond dental cement (Parkell, S396 Radiopaque L-powder), followed by Flow-it ALC light curing dental cement (Pentron Clinical, N11VH). After surgery, mice

were allowed to recover until ambulatory on a heated pad, then returned to their homecage.

Viruses. Viruses were injected where indicated in the main text at the following volumes at titers: CAV-cre, 2.5 x 10^{12} vg/ml, 250 nl; AAVDJ-hSyn1-mGFP-T2A-mRuby, 2.9 x 10^{13} vg/ml, 250 nl; AAV5-CAG-FLeX-TC, 2.6 x 10^{12} vg/ml, 325-500 nl; AAV8-CAG-FLeX-G, 1.3 x 10^{12} vg/ml, 325-500 nl; RV*dG*-GFP, 5 x 10^{8} pfu/ml, 500 nl; AAV5-CaMKIIα-ChR2(H134R)-EYFP, 4 x 10^{12} vg/ml 250-500 nl; AAV5-EF1α-DIO-GCaMP6f, 1.9 x 10^{13} vg/ml, 1000 nl.

Transcardial Perfusions. Mice received lethal i.p. injections of Beuthanasia (Merck), a combination of sodium pentobarbital and sodium phenytoin, to induce a smooth and rapid onset of unconsciousness and death. Once unresponsive to a firm toe pinch, an incision was made up the middle of the body cavity. An injection needle was inserted into the left ventricle of the heart, the right atrium was punctured and solution (NMDG cutting solution for electrophysiology, or PBS followed by 4% PFA for traditional histology, or PBS followed by CLARITY monomer solution for CLARITY) was infused as the mouse was exsanguinated. The mouse was then decapitated and its brain was quickly removed for further experimentation.

CLARITY. Mice were perfused with CLARITY monomer solution containing 1% acrylamide, 0.0125% bis-acrylamide, 4% PFA, and 0.25% VA-044 diluted in PBS and left for two overnights at 4°C to allow the monomer to fully penetrate the tissue. Then, the samples were degassed and moved to a 37°C water bath for 6-7 hours to polymerize. After polymerization, excess hydrogel was poured off and the brains were rinsed with PBS, then transferred to clearing solution containing 4% SDS and 200mM boric acid in water, adjusted to pH 8.5 with NaOH. Whole brains were incubated in clearing solution, shaking, at 37°C for 4-5 weeks (until brains were clear). Clearing solution was changed once per week. After clearing, brains were stored in PBS + 0.02% sodium azide at room temperature until ready for COLM imaging. For imaging, brains were equilibrated in Focusclear (CelExplorer Labs).

Tissue Sectioning

After perfusion, brains were fixed overnight at 4°C in 4% PFA, then transferred to solution of 30% sucrose in PBS, where they were stored for at least two overnights at 4°C before sectioning. For TRIO, tissue was embedded in OCT (Tissue-Tek) and sectioned on a cryostat at 60 μm and mounted on slides for counterstaining. All other tissue was sectioned on a freezing microtome at 40 μm, stored in cryoprotectant (25% glycerol and 30% ethylene glycol in PBS, pH 6.7) at 4°C, then stained as free-floating sections before mounting.

Histology and Immunostaining.

TRIO sections were counterstained with Neurotrace 435/455 Blue Fluorescent Nissl Stain and DAPI (Life Technologies, Cat. No. N-21479, D1306) on slides. Slides were rinsed with PBS for 5 minutes, permeabilized with 0.3% Triton X in PBS (PBS-T) for 20 minutes, stained with Neurotrace (1:500 in PBS) for 2 hours at room temperature, stained with DAPI (300nM) for 20 minutes, then rinsed with PBS for 5 minutes and coverslipped with Fluoromount G mounting medium (Southern Biotech). Tyrosine hydroxlase (TH) staining was performed where indicated in the text. Staining was performed on free floating sections, which were blocked with 3% normal donkey serum in PBS-T for 1 hour at room temperature, then stained with 1:500 primary antibody (Aves Labs, Cat No. TYH) in blocking solution at 4°C overnight. Secondary staining was performed using 1:500 goat anti-chicken Alexa Fluor 647 secondary antibody (Life Technologies, Cat. No. A-21449). Anti-GFP staining was performed to amplify signals from all sections containing mGFP and GCaMP6f by blocking in 3% normal donkey serum in PBS-T for 1 hour at room temperature, then using 1:500 primary antibody conjugated directly to either Alexa Fluor 488 or Alexa Fluor 647 (Life Technologies, Cat. No. A-21311, A-31852) in blocking solution at 4°C overnight.

Traditional Imaging.

Axon collateralization mapping sections and TRIO sections were imaged on a epifluorescent slide scanner system (Leica, DM6000 B with Ariol SL200 slide loader) with a 5x air objective using Neurotrace or DAPI counterstaining to focus the images. All other images were taken using a confocal microscope (Leica, TCS SP5) with 5x or 10x air

immersion objectives, or 40x or 63x oil immersion objectives. In sections where fluorescence intensity was quantitatively compared, all acquisition settings were held constant.

Axon Collateralization Analysis.
Images of mGFP expression were acquired from a systematic image series from each mouse corresponding to the full anterior-posterior span of the striatum (5-6 sections per brain). All images were acquired using identical settings and were analyzed using ImageJ. Regions of interest were defined using the DAPI reference channel. Images were then background subtracted (rolling ball radius of 50 pixels), thresholded and binarized (pixel values >30 = black). The projection fraction was calculated as (black area in region of interest) / (total black area in all regions).

COLM Imaging and 3D volume rendering. Whole brain imaging was performed using COLM (Tomer et al., 2014) or second generation COLM (Tomer et al., in preparation), as described in detail (Tomer et al., 2014). The images were acquired along the dorsal-ventral axis. Whole brain volume rendering movies were prepared using 4x4 fold downsampled data in the x-y dimensions or using full resolution data in the case of Movie S5. Data stitching was performed using an adapted Terastitcher based pipeline (Bria and Iannello, 2012) and volume rendering using Amira (FEI) and ImageJ software.

Cell Counting. Cell counting was performed in a semi-automated fashion using Imaris software (Bitplane). For each brain region counted, Imaris spot detection parameters were defined that most accurately counted GFP-labeled cell bodies as determined by manual verification. Brain regions were defined with reference to the Allen Mouse Brain Reference Atlas (http://mouse.brain-map.org/static/atlas), using the Paxinos and Franklin atlas (Paxinos and Franklin, 2004) as a secondary resource (e.g. for NAc core vs. shell). For subdivision of the dorsal striatum into dorsomedial and dorsolateral portions, an in-house atlas was created (Fig. S1C-D). Regional boundaries were defined *a priori* without biasing by reference to labeling in our experiments.

Slice Electrophysiology. Three weeks after injections, mice were transcardially perfused with a room-temperature NMDG slicing solution (containing in mM: 92 *N*-methyl-D-glucamine, 2.5 KCl, 30 $NaHCO_3$, 1.2 NaH_2PO_4-H_2O, 20 HEPES, 25 glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, adjusted to pH 7.4 with HCl) and the brain tissue was sliced in the same NMDG solution using a vibratome (VT1200S, Leica). Slices containing the striatum were fixed in 4% PFA and saved for verification of the ChR2 and retrobead injection sites. 300μm coronal slices containing the substantia nigra were allowed to recover for 10 minutes at 33°C in NMDG solution, then another 20 minutes at 33°C in a modified HEPES artifical cerebrospinal fluid (containing in mM: 92 NaCl, 2.5 KCl, 30 $NaHCO_3$, 1.2 NaH_2PO_4-H_2O, 20 HEPES, 25 glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate), then another 15 minutes at room temperature in the modified HEPES solution. Finally, slices were transferred to standard artificial cerebrospinal fluid (aCSF; containing in mM: 125 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 26 NaHCO3, 1.25 NaH2PO4-H2O, 11 glucose) bubbled with 95%O_2/5%CO_2 and stored at room temperature until recording. Whole-cell patch clamp recordings were performed in the same aCSF solution at 30-32°C. Signals were amplified with a Multiclamp 700B amplifier, acquired using a Digidata 1440A digitizer, sampled at 10 kHz, and filtered at 2 kHz. All data acquisition and analysis were performed using pCLAMP software (Molecular Devices). Neurons were visually identified for patching using an upright microscope (Olympus BX51WI) equipped with IR-DIC optics, filter sets for visualizing YFP and RFP, and a CCD camera (RoleraXR, Q-Imaging). Resistance of the patch pipettes was 2.5–4MΩ when filled with intracellular solution. To record action potentials and I_h currents, we used intracellular solution containing the following (in mM): 150 K-gluconate, 5 NaCl, 0.2 EGTA, 10 HEPES, 1$MgCl_2$, 2 Mg-ATP, 0.3 Na-GTP, adjusted to pH 7.3 with KOH. To measure I_{leak} and I_h, we held neurons at -40mV and stepped for 500ms to -120mV. The average of three sweeps was taken, then I_{leak} was calculated as the change in current between the baseline before the voltage step was applied and the current at ~40 ms after the voltage step was applied. I_h was calculated from the change in current between ~40 and 498 ms after the voltage step was applied. To record EPSCs and IPSCs, neurons were voltage-clamped at -70mV. To record EPSCs, we added picrotoxin (50μM) to the extracellular solution and used intracellular solution containing the following (in mM): 120

$CsMeSO_3$, 15 CsCl, 8 NaCl, 0.2 EGTA, 10 HEPES, 2 Mg-ATP, 0.3 Na-GTP, 10 TEA (tetraethylammonium), 5 QX-314 (lidocaine N-ethyl bromide), adjusted to pH 7.25 with CsOH. To record IPSCs, we added NBQX (5μM) and APV (50μM) to the extracellular solution and used intracellular solution containing the following (in mM): 130 CsCl, 1 EGTA, 10 HEPES, 2 Mg-ATP, 0.3 Na-GTP, 10 TEA (tetraethylammonium), 5 QX-314 (lidocaine N-ethyl bromide), adjusted to pH 7.25 with CsOH. Miniature EPSCs and IPSCs were recorded in the presence of TTX (1μm). To isolate monosynaptic IPSCs from the DMS and DLS, we added TTX (1μm) and 4-AP (100μM) to the extracellular solution. To stimulate ChR2 expressed in axon terminals from the striatum, 5ms blue light pulses (475nm, ~10mW/mm^2) were generated using a Spectra X LED light engine (Lumencor) and delivered to the slice via a 40x/0.8 water immersion objective focused onto the recorded neuron. Pulses were delivered once every 30 seconds. Response sizes were calculated by baseline-subtracting and averaging 10-15 traces together, then calculating the peak amplitude in a 50ms window after the light pulse. Neurons that did not show a peak amplitude in this window that exceeded 5 SD of the baseline noise were counted as non-responders. To measure qIPSC amplitude, we replaced extracellular calcium with 2mM strontium to induce asynchronous release. We then calculated the average amplitude of events occurring between 50ms and 250ms after the blue light pulse. The amplitude of each event was measured from a local baseline just before the event (from 5-20ms prior to the start of the event to the start) to avoid artifacts arising from the decaying transient of the initial synchronous release.

Reward Behavior. Mice were water-restricted to ~1mL/day (maintained at >85% ad libitum weight) and trained to press a lever for 25μl of 20% sucrose water on a 1:1 continuous reinforcement schedule. Med Associates operant boxes (ENV-307A-CT) were arranged with an extra-tall sucrose port on one wall (to allow for head entry with the implant attached) and active levers both to the right and to the left of the port. Mice were required to retrieve each reward before the onset of the next trial. Mice were trained on this schedule to criterion performance of >60% available rewards earned in one hour, which required 4-8 days. Mice were then advanced to testing on a random ratio (RR10)

schedule in which each lever press had a 10% chance being rewarded. This schedule was chosen to allow for increased total number of trials per session and for within-session comparisons of rewarded and non-rewarded trials. One mouse was not advanced to testing due to complications of surgery becoming evident during training. All sessions from start of task habituation and training were performed with the mouse plugged in to a 400 μm BFH48-400 patch cord using a metal ferrule sleeve, regardless of whether signals were being recorded in order to habituate the mouse to tethering.

Shock Exposure Behavior. Mice were placed in an operant chamber (Med Associates, different from the reward training chamber, but of the same dimensions) with a shock floor, with their fiber optic implant plugged in to a 400 μm BFH48-400 patch cord using a metal ferrule sleeve. Fifteen mild shocks (0.4 mA, 0.5 s) were delivered at random, about once per minute (RI60 schedule). Photometry signal was recorded throughout session and behavior was recorded by overhead camera. Freezing, defined as the complete absence of movement except as required for respiration, was manually scored. Activity burst, defined as abrupt increase in velocity of movement, was manually timed until return to baseline movements in cage. Behavioral scoring was completed for a subset of animals by a single experimenter blind to mouse group and photometry results.

Fiber Photometry. As in Gunaydin et al. (2014), we measured bulk fluorescence from deep brain regions using a single optical fiber for both delivery of excitation light and collection of emitted fluorescence. The fluorescence output of the calcium sensor is modulated by varying the intensity of the excitation light, generating an amplitude-modulated fluorescence signal that is demodulated to recover the original calcium sensor response. This 'upconversion' of the calcium signal avoids contamination of the signal by changes in ambient light levels with behavior , as well as avoiding low-frequency 'flicker noise' in our photodetector. We have extended this method to the case of multiple excitation wavelengths delivered over the same fiber, each modulated at a distinct carrier frequency, to allow for simultaneous multicolor measurements. Two excitation LEDs (490nm 'blue' and 405nm 'violet', Thor Labs M490F1 and M405F1) were controlled via an RP2.1 real-

time processor (Tucker Davis Technologies) running custom software. Blue excitation was sinusoidally modulated at 211Hz and passed through a GFP excitation filter (Thor Labs, MF469-35); violet excitation was modulated at 531Hz and passed through a 405nm bandpass filter (Thor Labs, FB405-10). These carrier frequencies were chosen to avoid contamination from overhead lights (120Hz and harmonics) and cross-talk between channels (the bandwidth of GCaMP6f was observed to be <15Hz), while remaining within the 30-750Hz bandwidth of the photoreceiver. Excitation light from each LED was coupled into a 0.39NA, 200um-core fiber (chosen to avoid overfilling the patchcord to the animal), collimated, then combined by a 425nm long-pass dichroic mirror (ThorLabs, DMLP425). The excitation light was coupled into a high-NA (0.48), low-autofluorescence 400μm patch cord (manufactured by Doric Lenses,, using BFH48-400 fiber from ThorLabs) using a fixed-focused high NA (0.51) coupler/collimator (Thor Labs, F240FC-A). Each LED was set to 30μW at the far end of the patch cord, which was terminated with a 2.5 mm ferrule. The far end of the patch cord and the 2.5mm metal optical implant ferrule were cleaned with isopropanol before each recording, then securely attached via a metal or zirconia sleeve. The GCaMP6f emission signal was collected through the patch cord and collimator, passed through a GFP emission filter (Thor Labs, MF525-39) and focused onto a femtowatt photoreceiver (Newport, Model 2151) using a lens (Edmund Optics, Cat. No. 62-561). The photoreceiver signal was sampled at 6.1 kHz, and each of the two modulated signals generated by the two LEDs was independently recovered using standard synchronous demodulation techniques implemented on the RP2.1 real-time processor: the detector output was routed to two product detectors, one using the selected channel's modulation signal as a reference, and the other using a 90-degree phase-shifted copy of the same reference. These outputs were low-pass filtered (corner frequency of 15Hz), and added in quadrature. This dual-phase detection approach makes the output insensitive to any phase delay between the reference and signal. The resulting fluorescence magnitude signals were then decimated to 382 Hz for recording to disk and further filtered using an ~2Hz low-pass filter before analysis. Behavioral variables, such as lever presses, reward port entry times and shock times, were fed into the real-time processor as TTL signals from the operant chambers. Files were then exported for analysis to MATLAB (MathWorks) using a custom

script. A least-squares linear fit was applied to the 405nm control signal to align it to the 490nm signal. The ΔF/F time series was then calculated for each behavioral session as ((490nm signal - fitted 405nm signal) / fitted 405nm signal). Peristimulus time histograms (PSTHs) were created using the TTL timestamps that had been fed in from the operant chambers. Peak reward ΔF/F was calculated as the maximum ΔF/F value in the rewarded port entry PSTH in a one second period around the reward retrieval (time 0). No reward ΔF/F was calculated as the ΔF/F at time 0 in the non-rewarded port entry PSTH. Peak ΔF/F during shock was calculated as the most extreme ΔF/F value (positive or negative) observed during the shock period (time 0-0.5 seconds). Mean ΔF/F at 1-5 seconds post shock was calculated as the mean ΔF/F value at time 1-5 seconds, where the shock occurred at time 0-0.5 seconds.

Statistics. Unpaired t-tests were used for comparisons between two groups (DMS- and DLS-projecting SNc dopamine neurons). Two-way ANOVAs were used to assess how the properties or responses of DMS- and DLS-projecting SNc dopamine neurons were affected by other factors (e.g. input area). When a statistically significant effect was observed using a two-way ANOVA, post-hoc testing with correction for multiple comparisons was performed using Sidak's or Tukey's multiple comparisons test. Statistics were performed using Prism 6 (GraphPad) software.

Supplementary Tables

Table S1. Percent Ipsilateral and Contralateral Inputs to DMS- and DLS-projecting SNc Dopamine Neurons, Related to Figures 1 and S1
N= 4 brains per condition. Shown are means ± SEM.

	% Ipsilateral Inputs		% Contralateral Inputs	
Input Area	DMS-projecting	DLS-projecting	DMS-projecting	DLS-projecting
M1/2	1.66 ± 0.17	1.90 ± 0.20	3.04 ± 0.97	2.33 ± 0.58
S1	0.99 ± 0.07	1.21 ± 0.08	0.91 ± 0.36	0.52 ± 0.22
Cortex (Other)	0.67 ± 0.08	0.78 ± 0.16	2.27 ± 0.70	1.42 ± 0.13
NAc core	7.88 ± 2.03	3.49 ± 1.01	1.90 ± 1.39	1.15 ± 0.73
NAc med shell	4.23 ± 0.74	1.87 ± 0.62		
NAc lat shell	2.83 ± 0.33	3.12 ± 0.16		
DMS	18.09 ± 3.67	11.72 ± 1.33	0.84 ± 0.39	0.63 ± 0.29
DLS	27.90 ± 2.12	34.78 ± 3.81		
FS (IPAC)	0.93 ± 0.45	2.10 ± 0.38	0.42 ± 0.15	0.07 ± 0.07
GPe	4.47 ± 0.28	6.01 ± 1.20	0.17 ± 0.1	0.66 ± 0.66
SI/VP	2.04 ± 0.12	2.45 ± 0.20	5.33 ± 1.79	2.91 ± 1.70
BNST	1.87 ± 0.43	2.21 ± 0.39	0.34 ± 0.13	0.40 ± 0.31
CeA	1.44 ± 0.35	1.83 ± 0.38	0.24 ± 0.13	0.10 ± 0.07
Hypothalamus	12.11 ± 1.55	11.37 ± 1.50	29.61 ± 0.92	23.49 ± 6.74
Thalamus	2.13 ± 0.17	1.63 ± 0.32	4.89 ± 1.37	3.23 ± 0.72
LHb	0.10 ± 0.03	0.31 ± 0.16	2.79 ± 0.69	1.52 ± 0.18
SC	1.83 ± 0.24	1.50 ± 0.30	4.48 ± 0.56	2.81 ± 0.59
IC	0.28 ± 0.10	0.10 ± 0.10	1.25 ± 0.81	0.63 ± 0.30
SNc	1.80 ± 0.49	1.65 ± 0.57	1.30 ± 0.53	1.96 ± 1.14
SNr	1.61 ± 0.28	1.83 ± 0.41	1.40 ± 0.76	2.86 ± 0.66
VTA	2.93 ± 0.52	2.27 ± 0.56	3.60 ± 2.08	5.82 ± 2.41
PAG	2.64 ± 0.58	2.45 ± 0.64	11.52 ± 2.98	11.74 ± 2.20
Dorsal Raphe	0.41 ± 0.22	0.62 ± 0.15	0.73 ± 0.43	7.53 ± 1.26
MRN	4.41 ± 1.10	3.38 ± 0.87	11.09 ± 3.12	22.83 ± 5.13
PPN	1.44 ± 0.32	0.66 ± 0.22	0.81 ± 0.51	1.70 ± 0.90
PB	0.72 ± 0.10	0.76 ± 0.33	9.86 ± 4.15	2.75 ± 1.45

Supplemental References

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