Intact-Brain Analyses Reveal Distinct Information Carried by SNc Dopamine Subcircuits

Graphical Abstract

Highlights

- Intact, inclusive approaches to classifying neuronal cell types
- Differential brain-wide circuit incorporation of SNc dopamine neuron subpopulations
- Opposite valence encoding of shock by projection target-defined SNc neurons
- Independently controlled information streams from the SNc to the DMS and DLS

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In Brief
Exploring the mammalian brain with an array of intact-brain circuit interrogation tools—including CLARITY, COLM, optogenetics, viral tracing, and fiber photometry—reveals that neurons in the SNc region present different biophysical properties, wiring of inputs and outputs, and activity during behavior, despite signaling through the same neurotransmitter.

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Intact-Brain Analyses Reveal Distinct Information Carried by SNC Dopamine Subcircuits

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SUMMARY

Recent progress in understanding the diversity of midbrain dopamine neurons has highlighted the importance—and the challenges—of defining mammalian neuronal cell types. Although neurons may be best categorized using inclusive criteria spanning biophysical properties, wiring of inputs, wiring of outputs, and activity during behavior, linking all of these measurements to cell types within the intact brains of living mammals has been difficult. Here, using an array of intact-brain circuit interrogation tools, including CLARITY, COLM, optogenetics, viral tracing, and fiber photometry, we explore the diversity of dopamine neurons within the substantia nigra pars compacta (SNC). We identify two parallel nigrostriatal dopamine neuron subpopulations differing in biophysical properties, input wiring, output wiring to dorsomedial striatum (DMS) versus dorsolateral striatum (DLS), and natural activity patterns during free behavior. Our results reveal independently operating nigrostriatal information streams, with implications for understanding the logic of dopaminergic feedback circuits and the diversity of mammalian neuronal cell types.

INTRODUCTION

Dopamine (DA) is a neurotransmitter that is crucial for many biological processes relevant to health and disease and is thought to regulate (among other behaviors) voluntary movement, reinforcement learning, and motivation (Bromberg-Martin et al., 2010). Seminal early studies into the information encoded by midbrain DA neurons suggested that a key function of DA is to transmit reward prediction error signals (Mirenowicz and Schultz, 1996; Schultz et al., 1997; Waelti et al., 2001), a hypothesis concordant with temporal difference learning models (Montague et al., 1996, 2004; Steinberg et al., 2013; Sutton, 1988). However, not all midbrain DA neurons appear to encode similar information in their activity patterns (Bromberg-Martin et al., 2010; Lammel et al., 2014; Roeper, 2013). For example, DA neurons have been observed that differ in their responses to aversive stimuli, leading to the hypothesis that the DA neurons, which increase their firing in response to these stimuli, may signal salience rather than value (Brischoux et al., 2009; Bromberg-Martin et al., 2010; Matsumoto and Hikosaka, 2009), though controversy on this point remains (Cohen et al., 2012; Fiorillo et al., 2013, 2013; Ungless et al., 2004).

The concept that there are diverse subsets of midbrain DA neurons transmitting distinct signals naturally leads to the question of whether these subsets are functionally incorporated into different circuits with different roles in the brain. Explorations of DA neurons in the ventral tegmental area (VTA) have revealed that these neurons can be divided into distinct categories based on their projection targets, which include the prefrontal cortex, nucleus accumbens (NAC) core, NAC medial shell, NAC lateral shell, and amygdala. When divided by projection target, different VTA DA neuron classes express varying levels of the DA transporter (DAT), DA D2 autoreceptors, GIRK channels, and HCN channels mediating the Ih current (Lammel et al., 2008, 2011; Margolis et al., 2008), all of which could affect the dynamics of signals represented and transmitted. Furthermore, projection target-defined subpopulations are located in different subregions of the VTA, their excitatory synapses are differentially modulated by rewarding and aversive stimuli, and they receive distinct inputs, which can elicit opposite behaviors when selectively recruited (Lammel et al., 2008, 2011, 2012).

Distinctions among DA neurons have also increasingly been drawn between DA neurons within the VTA and those within the substantia nigra pars compacta (SNC). For example, a recent viral circuit tracing study showed that VTA and SNC DA neurons receive different proportions of inputs from key brain regions (Watabe-Uchida et al., 2012). In particular, this study noted that SNC neurons receive a large proportion of their inputs from the dorsal striatum. In contrast, studies attempting to functionally characterize direct striatal projections to midbrain DA neurons using optogenetics and slice recordings that have failed
Figure 1. Whole-Brain Mapping of Inputs to DMS-Projecting and DLS-Projecting SNc DA Neurons

(A) Viral injection strategy for whole-brain input mapping based on output (TRIO). CAV-cre is injected into the striatum (DMS or DLS), and AAVs expressing cre-dependent TC and G are injected into the SNc. Two weeks later, RVdG-GFP is injected into the SNc, where it infects TC-expressing cells and spreads one synapse upstream from G-expressing cells.
to identify such connections (Chuhma et al., 2011; Xia et al., 2011) or found them to be very weak (Bockisch et al., 2013). The functional connectivity from striatum to SNc thus remains an open question.

In addition to differences from VTA neurons, there are hints in the literature that SNc DA neurons alone could be further divisible into functionally distinct subsets. One pioneering study demonstrated that DA neurons located more medially within the SNc express higher levels of K-ATP channels, which mediate burst firing (Schiemann et al., 2012). Another study used recordings in awake monkeys to demonstrate a correlation between the depth at which a DA neuron was recorded along the electrode (indicating a more ventromedial location in the midbrain) and the likelihood that it would decrease rather than increase its firing in response to an aversive cue (Matsumoto and Hikosaka, 2009).

How might such differences among SNc DA neurons relate to circuit wiring and function? One intriguing hypothesis is that distinct locations within the SNc give rise to projections targeting distinct locations within the dorsal striatum, such as dorsomedial striatum (DMS) and dorsolateral striatum (DLS); distinct SNc subfields could furthermore be set up to receive, process, and transmit functionally distinct streams of information from elsewhere in the brain. Such circuit organization would have powerful implications. For example, given the high medial SNc expression of K-ATP channels that enable bursting, the prediction could be made that the striatal target field of the medial SNc would receive strong novelty-driven bursts of DA during exploratory behavior that the striatal target field of the lateral SNc could not receive.

However, other lines of research suggest that DAergic projections to the DMS and DLS carry similar information streams because restriction of DA signaling to either subregion has similar effects (Darvas and Palmiter, 2009, 2010). In fact, SNc DA neurons could in principle even be similar to VTA DA neurons in terms of the valence of information represented because mice DA neurons could in principle even be similar to VTA DA neurons (B). Low-magnification (5×) images of DMS- and DLS-projecting SNc ‘‘starter cells,’’ from which tracing likely occurred. Green shows RVdG-GFP expression, red shows TC expression, and blue shows (TH) immunostaining.

We achieved unbiased global visualization of SNc inputs and outputs using a combination of CLARITY and CLARITY-optimized light-sheet microscopy (COLM) (Chung et al., 2013; Tomer et al., 2014), along with a variation of rabies-based circuit mapping (Schwarz et al., 2015); the latter method (TRIO) operates similarly to previously published rabies-based circuit mapping technologies, utilizing a GFP-expressing rabies virus (RVdG-GFP) that both lacks the glycoprotein needed to spread transynaptically and is pseudotyped with EnvA (so that it can infect only neurons expressing TVA, an avian receptor protein normally absent in mammalian cells [Wickersham et al., 2007]). Glycoprotein and TVA can then be expressed using cre-dependent vectors to direct the rabies virus to infect and spread to connected inputs from a cre-defined subset of neurons. In TRIO, cre is delivered from the retrograde CAV-cre virus (Hnasko et al., 2006; Soudais et al., 2001), which transduces axon terminals and thereby defines “starter cells” (from which rabies tracing will occur) by virtue of their projection target. We injected CAV-cre into either the DMS or DLS and then injected AAVs expressing cre-dependent rabies glycoprotein (G) and TC, a high efficiency version of the TVA receptor fused to mCherry (Miyamichi et al., 2013), into the SNc. Two weeks later, we injected RVdG-GFP into the SNc (Figure 1A). Control experiments revealed that resulting putative starter cells in the SNc, as defined by neurons that expressed both RVdG-GFP and TC, were predominantly DAergic (TH+; 96% ± 3% after CAV-cre injection into the DMS, n = 4 mice; 98% ± 2% after CAV-cre injection into the DLS, n = 4 mice; ***p < 0.001 and ****p < 0.0001. Other abbreviations: motor cortex (M1/2), somatosensory cortex (S1), fundus of the striatum/interstitial nucleus of the posterior limb of the anterior commissure (FS/IPAC), substantia innominata/ventral pallidum (SI/VP), superior colliculus (SC), inferior colliculus (IC), substantia nigra pars compacta (SNc), substantia nigra pars reticulata (SNr), ventral tegmental area (VTA), pedunculopontine nucleus (PPN), and parabrachial nucleus (PB).

Results

Unbiased Brain-wide Mapping of Inputs to Striatum Subfield-Projecting SNc Neurons

To directly explore the hypothesis that distinct SNc neuron populations receive and deliver separable information streams, we began with an unbiased approach for globally mapping the input/output relationships of SNc neurons. We mapped inputs onto two subpopulations of SNc DA neurons defined by their outputs to the DMS and DLS. These regions of the dorsal striatum have been functionally distinguished by previous studies (Castané et al., 2010; Faure et al., 2005; Featherstone and McDonald, 2004; Yin and Knowlton, 2004; Yin et al., 2004, 2005a, 2009, 2005b), leading us to hypothesize that SNc projections to these areas might also participate in separable circuits.

We achieved unbiased global visualization of SNc inputs and outputs using a combination of CLARITY and CLARITY-optimized light-sheet microscopy (COLM) (Chung et al., 2013; Tomer et al., 2014), along with a variation of rabies-based circuit mapping (Schwarz et al., 2015); the latter method (TRIO) operates similarly to previously published rabies-based circuit mapping technologies, utilizing a GFP-expressing rabies virus (RVdG-GFP) that both lacks the glycoprotein needed to spread transsynaptically and is pseudotyped with EnvA (so that it can infect only neurons expressing TVA, an avian receptor protein normally absent in mammalian cells [Wickersham et al., 2007]). Glycoprotein and TVA can then be expressed using cre-dependent vectors to direct the rabies virus to infect and spread to connected inputs from a cre-defined subset of neurons. In TRIO, cre is delivered from the retrograde CAV-cre virus (Hnasko et al., 2006; Soudais et al., 2001), which transduces axon terminals and thereby defines “starter cells” (from which rabies tracing will occur) by virtue of their projection target. We injected CAV-cre into either the DMS or DLS and then injected AAVs expressing cre-dependent rabies glycoprotein (G) and TC, a high efficiency version of the TVA receptor fused to mCherry (Miyamichi et al., 2013), into the SNc. Two weeks later, we injected RVdG-GFP into the SNc (Figure 1A). Control experiments revealed that resulting putative starter cells in the SNc, as defined by neurons that expressed both RVdG-GFP and TC, were predominantly DAergic (TH+; 96% ± 3% after CAV-cre injection into the DMS, n = 4 mice; 98% ± 2% after CAV-cre injection into the DLS, n = 4 mice; ***p < 0.001 and ****p < 0.0001. Other abbreviations: motor cortex (M1/2), somatosensory cortex (S1), fundus of the striatum/interstitial nucleus of the posterior limb of the anterior commissure (FS/IPAC), substantia innominata/ventral pallidum (SI/VP), superior colliculus (SC), inferior colliculus (IC), substantia nigra pars compacta (SNc), substantia nigra pars reticulata (SNr), ventral tegmental area (VTA), pedunculopontine nucleus (PPN), and parabrachial nucleus (PB).
Additional controls demonstrated that the RVdG-GFP was properly EnvA-pseudotyped and that long-range tracing of inputs was cre dependent (Figures S1A and S1B). We began our analysis of GFP-labeled inputs to DMS- and DLS-projecting SNc DA neurons by optically clarifying the labeled brains (Chung et al., 2013; Tomer et al., 2014) and visualizing using COLM (Tomer et al., 2014). We reasoned that, although thin sectioning approaches used for analysis of anatomical tracing have yielded important insights, these are not ideal for visualization of brain-wide patterns. Thin sectioning results in particular caveats for the interpretation of negative results: slices may tear, fragment, or otherwise be damaged or lost, and strategies to minimize overcounting of somata split across adjacent sections (as counting only separated sections—e.g., every third or sixth—may underestimate cell counts, particularly in small brain regions. Whole-brain CLARITY/COLM circumvents these difficulties, allowing an intact global overview of brain-wide structural patterns.

Using CLARITY/COLM, we noted an interesting GFP expression pattern in the striatum (Movies S1, S2, S3, and S4). When inputs to DMS-projecting SNc DA neurons were labeled, we observed relatively strong labeling in the NAc and DMS (Movies S1 and S2) in comparison to the DLS, whereas when inputs to DLS-projecting SNc DA neurons were labeled, we observed relatively strong labeling in the DLS, particularly in the caudal tail of the striatum (Movies S3 and S4) in comparison to the DMS and NAc. Moreover, fine details of local neuronal architecture in GFP-labeled neurons could be observed in higher-resolution volume renderings (Movie S5).

To quantify these divergent input patterns to SNc subfields depending on their projection target, we followed up with detailed targeted slicing approaches and quantified the inputs from each brain region to DMS- and DLS-projecting SNc DA neurons as a proportion of the total inputs observed (Figures 1E and 1F and Table S1). Existing atlases used to define major brain regions were further refined to define the boundaries of the DMS and DLS (Figures S1C and S1D). Because the inputs to SNc DA neurons are almost entirely ipsilateral (DMS-proj 96.4% ± 0.9%, n = 4; DLS-proj 96.5% ± 0.8%, n = 4, p = 0.91), we focused on the injected hemisphere (contralateral inputs were examined separately; Figures S1E and S1F and Table S1), noting first that brain-wide inputs to SNc DA neurons, regardless of projection target, broadly matched those observed by Watabe-Uchida et al. (2012) using RVdG-GFP tracing from the SNc of DAT-cre mice. However, despite gross similarities between groups, a two-way ANOVA revealed a significant interaction between starter cell projection target and input area (p < 0.001). Multiple comparisons revealed significant differences in inputs from the DMS (p < 0.001) and the DLS (p < 0.0001; Figure 1F), indicating a marked preferential reciprocal connectivity of dorsal striatal subregions to their specific DAergic SNc input subregions. Injections in D1-tomato bacterial artificial chromosome (BAC) transgenic mice further revealed that striatal inputs to SNc DA neurons arise from DA D1 receptor-expressing neurons (Figure S1G; 92/97 DMS-projecting neurons and 210/214 DLS-projecting neurons), consistent with the idea that D1 (but not D2) striatal neurons project directly to the midbrain and with hypotheses regarding the patch/matrix organization of striatum (Crittenden and Graybiel, 2011). These anatomical studies suggested a fundamental distinction in circuit incorporation for projection-defined SNc subregions and provided a roadmap for a more detailed investigation of the circuit.

**Organization of SNc DA Neurons Projecting to Distinct Regions of Dorsal Striatum**

To quantify the spatial patterns of cre expression following injections of CAV-cre into the striatum, we injected CAV-cre into the DMS or DLS of Ai9 tdTomato cre reporter mice (Figure 2A). Viral spread in the striatum was contained within the targeted subregion (Figure S2A). In the SNc, two key observations were made. First, cre expression was largely restricted to DA neurons, identified by tyrosine hydroxylase (TH) immunostaining, following injection in both the DMS (344/360 neurons) and the DLS (722/772 neurons). Although a few scattered TH+ cells were labeled in the VTA in both groups, adjacent non-DAergic regions did not contain cre-expressing cells (Figure 2B). Second, the patterns of cre expression following DMS and DLS injection differed. DMS-projecting neurons were observed in the medial SNc, whereas DLS-projecting neurons were observed in the lateral SNc. This differing anatomical localization of DMS- and DLS-projecting SNc DA neurons supports the idea of parallel nigrostriatal subcircuits within the SNc.

We next asked whether DMS- and DLS-projecting SNc DA neurons projected only to the DMS or DLS, or instead collateralized substantially. To visualize the boundaries of SNc DA neuron axonal arborizations in the striatum, we injected CAV-cre into the striatum and an adeno-associated virus (AAV) for cre-dependent expression of membrane-bound GFP (mGFP) and synaptophysin-mRuby (SYP-mRuby) into the SNc (Beier et al., 2015 [in this issue of Cell]; Figure 2C). With this approach, axons were labeled in green, and putative presynaptic sites were labeled in red. In the SNc, both green and red labeling were visible as anticipated (Figure 2D). The areas of highest fluorescence colocalized with TH in a pattern concordant with the locations of DMS- and DLS-projecting SNc DA neurons observed in Ai9 mice (Figure 2B). In the striatum, axons of DMS-projecting SNc DA neurons remained within the DMS, and axons of DLS-projecting SNc DA neurons remained within the DLS (Figures 2E–2G and S2B).

To quantify this differential axon distribution, we acquired a systematic image series from each mouse corresponding to the full anterior-posterior span of the striatum (Figure S2B). Projection fraction was calculated as the axonal coverage area in one region (DMS or DLS) divided by the total area covered across the entire striatum (Figure 2H). The entire striatum was defined to include the NAc, but we observed negligible contributions of projections to any of the NAc subregions across all conditions (Figure S2C). No GFP-labeled axons were observed in the prefrontal cortex or amygdala (Figure S2D). In dorsal striatum, an overwhelming fraction of DMS-projecting axons was localized within the bounds of the DMS (DMS 0.98 ± 0.01, n = 2; versus DLS 0.02 ± 0.01, n = 2; two-way ANOVA, brain area × projection target interaction, p < 0.0001; post hoc Tukey’s multiple comparisons, p < 0.0001); similarly, DLS-projecting axons were localized largely within the bounds of the DLS (DMS 0.14 ± 0.02, n = 2; versus DLS 0.86 ± 0.02, n = 2; post hoc Tukey’s multiple comparisons, p < 0.0001). These data confirmed that the SNc...
Projections to the DMS and DLS are largely parallel, defining separable nigrostriatal subcircuits with the potential to convey distinct and independent signals to the DMS and DLS.

Distinct Properties of SNc DA Neurons Depending upon Projection Target

We next explored whether projection-defined SNc DA neurons could be distinguished by intrinsic electrophysiological properties. To mark neurons for recording, we injected red-fluorescent retrobeads into the DMS or DLS of TH-GFP mice. Retrobead-labeled SNc neurons were also primarily GFP+ (Figure 3C; 509/515 DMS-projecting neurons and 526/542 DLS-projecting neurons). Although the specificity of the TH-GFP line has been questioned (Lammel et al., 2015), we found that GFP expression is specific for TH+ neurons in the SNc (Figure S3). Retrobead injections remained well localized to the injection site (Figure 3A) and retrobead-containing neurons within the SNc were readily identifiable for patching (Figure 3B).

Whole-cell patch-clamp analysis of retrobead+ and GFP+ SNc neurons revealed similar membrane capacitance, resistance,
Figure 3. Intrinsic Properties of DMS-Projecting and DLS-Projecting SNc DA Neurons

(A) Retrobeads (red) injected into the striatum of TH-GFP mice. Ctx, cortex; Str, striatum. Scale bars are 0.5 mm.

(B) DIC and red fluorescent images of a patched retrobead-containing neuron. Dotted lines highlight the position of the recording electrode. Scale bars are 10 μm.

(C) Colocalization of retrobead-containing neurons (red) with TH-GFP-labeled neurons (green) in the SNc. Colocalization rates were ~99% and ~97% in DMS- and DLS-projecting neurons, respectively.

(D) Membrane capacitance (Cm) of DMS- and DLS-projecting SNc DA neurons. Error bars are SEM.

(E) Membrane resistance (Rm) of DMS- and DLS-projecting SNc DA neurons. Error bars are SEM.

(F) Left, example responses of DMS-projecting SNc neurons (orange) and DLS-projecting SNc neurons (blue) to a hyperpolarizing current injection. Right, Ih and Ileak current measurements in response to hyperpolarizing current injection from DMS- and DLS-projecting SNc DA neurons. Error bars are SEM. **p < 0.01.

(G) Average action potential waveforms from DMS- and DLS-projecting SNc DA neurons. Area of light shading is SEM.

(H) Phase plots of average action potential waveforms from DMS- and DLS-projecting SNc DA neurons. Area of light shading is SEM.

(I) Average action potential height from DMS- and DLS-projecting SNc DA neurons. Error bars are SEM.

(J) Average action potential half widths from DMS- and DLS-projecting SNc DA neurons. Error bars are SEM. *p < 0.05.

(legend continued on next page)
and leak currents of DMS-projecting and DLS-projecting DA neurons (Figures 3D and 3E; Cm, DMS-proj 69.24 ± 3.589 pF, n = 21 versus DLS-proj 71.94 ± 4.663 pF, n = 16, p = 0.64; Rm, DMS-proj 384.7 ± 41.09 MΩ, n = 21 versus DLS-proj 327.4 ± 30.03 MΩ, n = 16, p = 0.30; Ileak, DMS-proj 317.1 ± 25.51 pA, n = 21 versus DLS-proj 345.6 ± 35.81 pA, n = 16, p = 0.51). However, we identified distinctly larger Ih currents in DLS-projecting DA neurons (Figure 3F; Ih, DMS-proj 296.2 ± 29.25 pA, n = 21 versus DLS-proj 460.8 ± 49.69 pA, n = 16, p < 0.01). All recorded neurons displayed significant Ih currents and broad action potential waveforms consistent with reliable identification of DA neurons across groups. A small but significant difference was observed in action potential half-width of DMS- versus DLS-projecting neurons (Figures 3G–3J; AP heights, DMS-proj 63.34 ± 3.589 pA, n = 15; DMS-proj 69.24 ± 3.589 pF, n = 16, p = 0.51; AP half widths, DMS-proj 1.152 ± 0.06 ms, n = 21 versus DLS-proj 0.9938 ± 0.03 ms, n = 16, p < 0.05).

**SNc DA Neurons Are Embedded within a Largely Inhibitory Network**

Projection-defined SNc DA neurons exhibit different intrinsic properties, project to non-overlapping striatal subregions, and receive differential inputs, together suggesting fundamentally different roles in the circuit. However, since the nature of the signals carried by the differing afferents remained unclear, we next functionally investigated these afferents using slice electrophysiology. As a first assessment of global functional afferent input, we recorded miniature excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs) (Figure 3K). Although no significant differences were observed between DMS- and DLS-projecting SNc DA neurons, both the amplitudes and frequencies of mIPSCs were higher than for mEPSCs across both groups (Figures 3L and 3M; mEPSC amplitude, DMS-proj −15.27 ± 0.50 pA, n = 15 versus DLS-proj −15.79 ± 0.68 pA, n = 15; mIPSC amplitude, DMS-proj −29.69 ± 2.395 mV, n = 15 versus DLS-proj −26.71 ± 2.13 pA, n = 15; two-way ANOVA, effect of mEPSCs versus mIPSCs, p < 0.0001; mEPSC frequency DMS-proj 0.49 ± 0.16 Hz, n = 15 versus DLS-proj 0.27 ± 0.10 Hz, n = 15; mIPSC frequency, DMS-proj 7.11 ± 1.86 Hz, n = 15 versus DLS-proj 15.07 ± 4.29 pA, n = 15; two-way ANOVA, effect of mEPSCs versus mIPSCs, p < 0.0001). This finding indicates that SNc DA neurons in general are embedded within a largely inhibitory network, in agreement with our findings and the findings of Watabe-Uchida et al. (2012) that SNc DA neurons receive afferents from many areas known to have GABAergic projection inputs specifically from the striatal subregion expressing ChR2 (Figure 4A). Examples of injection sites for ChR2 and retrobeads are shown in Figure 4B, along with the resulting distributions of red and green fluorescence in SNc (green fibers were observed both in the SNc and in the underlying SNr, where many direct pathway striatal neurons project).

Because striatal projection neurons are GABAergic, the glutamate receptor antagonists NBOX (5 μM) and APV (50 μM) were included in the extracellular solution to isolate inhibitory postsynaptic currents (IPSCs), and a high-chloride internal solution was used to facilitate event detection. We also included the voltage-gated sodium channel antagonist TTX (1 μM) and potassium channel antagonist 4-AP (100 μM) to isolate monosynaptic inputs by preventing disynaptic disinhibitory responses through the GABAergic cells of the SNr (with TTX) while enabling ChR2 to drive neurotransmitter release in the absence of action potentials (with 4-AP (Petreanu et al., 2009)). In all mice, recorded neurons were identified that directly responded to blue light stimulation, with response rates varying by condition. Connections from DMS to DMS-projecting SNc DA neurons and connections from DLS to DLS-projecting SNc DA neurons were clearly favored (Figure 4C; X2 p < 0.0001), mirroring our TRIO results (Figure 1) and functionally confirming a striking reciprocal connectivity between dorsal striatal subregions and their DAergic inputs.

**DLS Inputs to SNc DA Neurons Are Stronger than DMS Inputs**

Broad anatomical methods, while useful, cannot resolve key aspects of function, such as distinguishing very strong from very weak connections. In contrast, targeted optogenetic experiments can provide information not only about connection probabilities, but also about input strength. We next found, via IPSC amplitude quantification for SNc neurons that responded to stimulation of striatal afferents, that responses detected in both DMS- and DLS-projecting SNc neurons were much larger when DLS inputs were stimulated (Figure 4D; ChR2 DMS/DMS-proj −205.27 ± 146.09 pA, n = 13; ChR2 DMS/DMS-proj −96.23 ± 69.28 pA, n = 3; ChR2 DLS/DMS-proj −1193.10 ± 324.30 pA, n = 22; ChR2 DLS/DLS-proj −273.96 ± 369.82 pA, n = 38; two-way ANOVA, effect of ChR2 injection site, p < 0.01). Additionally, DLS-projecting SNc neurons had larger responses to DLS stimulation than did DMS-projecting SNc neurons (Tukey’s multiple comparisons, p < 0.05).

The observed differences in IPSC amplitudes between SNc neurons receiving inputs from the DMS and DLS were not fully explained by differences in the numbers of inputs arising from the two striatal subregions (Figures 1 and 4C), suggesting

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(K) Example traces of excitatory (top) and inhibitory (bottom) miniature postsynaptic currents from DMS-projecting (orange) and DLS-projecting (blue) SNc DA neurons.

(L) Amplitudes of mEPSCs and mIPSCs recorded from DMS-projecting and DLS-projecting SNc DA neurons. Error bars are SEM. ****p < 0.0001.

(M) Frequencies of mEPSCs and mIPSCs from recorded DMS-projecting and DLS-projecting SNc DA neurons. Error bars are SEM. ****p < 0.0001.

See also Figure S3.
additional possible differences in quantal IPSC amplitude, the number of synapses per input cell, and/or release probability. To test for differences in quantal amplitude, we replaced calcium in the extracellular recording solution with strontium to induce asynchronous release and found that the average quantal IPSC amplitude was larger for inputs arising from the DLS versus DLS-projecting SNc DA neurons in response to stimulation of DMS or DLS ChR2-expressing terminals. Example traces of evoked asynchronous IPSCs are shown on the right, with the period of qIPSC event collection highlighted in a pop out box. The blue bar indicates the time of stimulation. Error bars are SEM. *p < 0.05.
the DMS (Figure 4E; ChR2 DMS/DMS-proj –28.86 ± 3.48 pA, n = 10; ChR2 DMS/DLS-proj –26.77 ± 2.75 pA, n = 8; ChR2 DLS/DMS-proj –36.64 ± 3.77 pA, n = 11; ChR2 DLS/DLS-proj –40.60 ± 4.94 pA, n = 10, two-way ANOVA, effect of ChR2 injection, n = 10, p < 0.05), providing an additional partial explanation for the observed differences in IPSC amplitude.

**Selective Opposite-Valence Encoding of Aversive Stimuli by DMS- versus DLS-Projecting SNc DA Neurons**

Parallel nigrostriatal SNc DA subcircuits clearly have the potential to deliver different kinds of information to the DMS and DLS. To formally test this intriguing possibility, we monitored activity from projection-defined SNc DA neurons during behavior using fiber photometry, a method for collecting population intracellular \([\text{Ca}^{2+}]\) fluorescent signals from a genetically encoded \(\text{Ca}^{2+}\) indicator such as GCaMP through a single chronic fiber optic implant (Gunaydin et al., 2014). We expressed GCaMP6f (Chen et al., 2013) in DMS- or DLS-projecting SNc DA neurons by injecting CAV-cre into the DMS or the DLS, followed by injection of a cre-dependent GCaMP6f construct into the SNc. Through a fiber optic implanted into SNc at the GCaMP6f injection site (Figure 5A), we delivered excitation light at 490 nm to stimulate GCaMP6f fluorescence in a \(\text{Ca}^{2+}\)-dependent manner and at 405 nm, an excitation isosbestic wavelength for GCaMP6f that allowed us to perform ratiometric measurements of GCaMP6f activity, thereby correcting for bleaching and artifactual signal fluctuations (Figures S4A–S4D).

We recorded the activity of DMS- or DLS-projecting SNc DA neurons following either appetitive or aversive stimuli. To record appetitive signals, we trained mice to lever press for a sucrose reward retrieved from a reward port. Each press had a 10% chance of delivering reward, a contingency that allowed us to record during both rewarded and unrewarded port entries within the same behavioral session for comparison. DMS- and DLS-projecting SNc DA neurons reacted similarly to rewarding outcomes (Figures 5C–5E); a similar peak \(\Delta F/F\) was observed on rewarded port entries for both sets of mice (DMS-proj 2.164 ± 0.549%, n = 7 versus DLS-proj 1.753 ± 0.247%, n = 9, p = 0.47). Peaks were not observed for non-rewarded port entries (Figures 5C, 5D, and 5F; DMS-proj 0.483 ± 0.195%, n = 7 versus DLS-proj –0.274 ± 0.083%, n = 9, p = 0.13).

To record responses to aversive stimuli, we subjected the same set of GCaMP6f-expressing mice to mild, unpredicted electrical shocks. Strikingly, DMS- and DLS-projecting SNc DA neurons responded oppositely to this aversive stimulus. DMS-projecting neurons showed a marked dip in activity at the time of shock, whereas DLS-projecting neurons showed an increase (Figures 5G–5I; peak \(\Delta F/F\) i.e., positive or negative extreme during shock, DMS-proj –2.628 ± 0.513%, n = 8 versus DLS-proj 1.527 ± 0.358%, n = 9, p < 0.0001). Additionally, DLS-projecting neurons were returned immediately to baseline, whereas more persistent changes were observed in DMS-projecting neurons (Figure 5J; mean \(\Delta F/F\) between seconds 1 and 5, DMS-proj 2.403 ± 0.525%, n = 8 versus DLS-proj –0.3146 ± 0.176%, n = 9, p < 0.001). Post hoc histology revealed that GCaMP6f-expressing axons were located in the DMS or DLS as expected, that fiber optic implants were placed appropriately in the SNc relative to GCaMP6f-expressing cell bodies, and that GCaMP6f-expressing cell bodies were TH+ (Figure S4E).

DISCUSSION

Here, we have developed and applied anatomical and functional methodologies to provide a deeper understanding of striatonigrostriatal circuitry. We began by demonstrating that novel circuit-tracing and -recording methodologies can be effectively combined with whole-brain CLARITY/COLM imaging (Chung et al., 2013; Tomer et al., 2014) to provide a uniquely informative overview of richly defined cell types and their global connectivity motifs within the intact experimental subject brain. Further development of high-throughput processing of these whole-brain datasets will be needed (Chung and Deisseroth, 2013; Kim et al., 2013, 2015) to fully capitalize on this opportunity for the rapid advancement of understanding structure-function relationships in the brain. For example, looking to the future, it will be vital to increase experimental-subject group sizes despite the immensely large datasets acquired for each experimental subject. Although our current methods successfully captured the large though complex connectivity differences observed here, larger group sizes would broaden the potential to identify biologically and potentially clinically important differences with smaller effect sizes, including, perhaps, effects of age, gender, and life experience.

Additionally, we have shown that, despite the utility of anatomical tracing techniques such as TRIO, following up findings from their use with other experimental modalities examining the functionality of identified connections is essential. By stimulating inputs from the striatum to SNc DA neurons optogenetically, we discovered that DLS inputs are an order of magnitude stronger than DMS inputs, a finding that could not have been predicted anatomically but is nevertheless likely to be crucial in thinking about SNc circuit function. Our observations of unequivocal inputs from the dorsal striatum to the SNc contrast with certain earlier reports, which failed to find connections using similar techniques (Chuhma et al., 2011; Xia et al., 2011). Although it is often difficult to explain negative findings, these previous studies used younger animals than did this study, which is consistent with a possible role of age- or experience-dependent plasticity; moreover, we have shown that striatal projections to DA neurons may be very difficult to find if the subregions of the striatum and the SNc are not well matched (highlighting the value of the CLARITY/COLM/TRIO approach). For example, if ChR2 expression were predominantly in DMS.
Our result regarding the in vivo activity of DMS- and DLS-projecting SNc DA neurons is concordant with the hypothesis put forward by Matsumoto and Hikosaka (2009) that DA neurons located more ventrolaterally within the midbrain increase firing in response to aversive stimuli. By targeting SNc DA neurons for observation based on their striatal projection target, we directly tested and substantially extended this hypothesis, demonstrating that the differences in the representation of aversive stimuli among subsets of SNc DA neurons relate to projection target. Additionally,
we observed a sustained activity increase in DMS-projecting SNc DA neurons following shock. We speculate that this population of DA neurons may also encode for a heightened sensitivity or motivational state following an unfamiliar outcome (unlike the reward stimulus, the mice had no previous experience of shock prior to test day) in order to promote future action-outcome learning.

Regarding anatomy of efferents alone, our finding that the projections from SNc DA neurons to the DMS and DLS arise from distinct locations within the SNc is not inconsistent with several previous findings. For example, in rats that had the DA neuron toxin 6-OHDA injected into the DLS, degeneration of DA neurons was observed primarily in the lateral SNc (Faure et al., 2005). Similarly, for mice in which DA neuron TH expression was restricted to neurons projecting to either the DLS or the ventromedial striatum (VMS), DLS-projecting neurons were located more laterally than VMS-projecting neurons (Darvas and Palminteri, 2009; 2010; see also Schieman et al., 2012).

Information transmitted along these separable activity streams will depend on intrinsic properties of the DA neurons and on their afferents. Indeed, combined with other recent lines of evidence (Lammel et al., 2008, 2011; Margolis et al., 2008), we have found that projection-defined subpopulations of DA neurons display different intrinsic properties. For example, increasing-amplitude $I_h$ currents are observed progressing medially to laterally within the midbrain DA system; DA neurons in the medial paranigral nucleus of the VTA express little to no $I_h$ current (Lammel et al., 2011), whereas DA neurons in the lateral SNc express the largest $I_h$ currents. Differences in $I_h$ currents are strongly correlated with action potential rebound delays and can influence pacemaking activity (Neuhoff et al., 2002), particularly in the case of calbindin-negative SNc DA neurons (which constitute the majority of the SNc DA neuron population).

Afferents to projection-defined SNc DA neurons also differ; in particular, we here identify new principles of afferent-projection complexity in SNc subcircuits (see Beier et al., 2015 for additional illuminating information regarding VTA subcircuits). A prominent finding is that the DMS and DLS are preferentially reciprocally connected with the very same DA neurons that project back to these areas. This information notably enriches the ascending spiral model proposed by Haber et al. (2000), which could not make definitive statements about striatal inputs onto DAergic versus non-DAergic midbrain cells. We also observed strong DLS projections to DMS-projecting DA neurons; this finding implies a novel route of lateral to medial information flow.

In summary, our identification of two distinct nigrostriatal DA circuits—differing in inputs, outputs, biophysical properties, and environmental information representations—both reveals independently controlled information representations streaming through SNc and provides a generalizable framework for brain-wide mapping of diverse populations of neurons defined by multiple independent types of features. Particularly in the case of DA neurons, this type of approach may improve our understanding of the circuit mechanisms underlying normal brain function, as well as diseases such as depression, addiction, and schizophrenia.

**EXPERIMENTAL PROCEDURES**

**Animals**

All experiments were approved by the Stanford University IACUC committee, protocol 10747. All mice (A19, TH-GFP, D1-tdTomato, and wild-type) were on a C57BL6/J background and were 2–4 months old.

**Stereotoxic Injections**

Viruses and retrobeads were injected and optical implants placed at the following coordinates, relative to bregma: DMS +0.75 AP, 1.5 ML, −2.8 DV; DLS +0.25 AP, 2.5 ML, −3.4 DV; medial SNc −3.1, 0.8 ML, −4.7 DV; lateral SNc −3.1, 1.3 ML, −4.2 DV.

**Histology**

TH staining was done with 1:500 primary antibody overnight at 4°C and 1:500 secondary antibody coupled to Alexa Fluor 647 or Alexa Fluor 674 overnight at 4°C. Counterstaining was done with Neurotrace 435/455 Blue Fluorescent Nissl Stain (1:500) and/or DAPI (300 nM).

**CLARITY**

Brains were perfused and incubated with CLARITY monomer solution containing 1% acrylamide, 0.0125% bis-acrylamide, and 4% PFA and then polymerized at 37°C for 6–7 hr. Brains were passively cleared in SDS Borate Buffer (pH 8.5) at 37°C for 4–5 weeks, equilibrated in Focusslear for imaging and imaged using COML methods (Tomer et al., 2014).

**Slice Electrophysiology**

300 μm coronal sections were prepared in an NMDG-based solution at room temperature. Striatal slices were fixed in 4% PFA and saved for verification of injection sites. Whole-cell recordings were performed in standard aCSF at 30–32°C. Where indicated, TTX (1 μM), 4-AP (100 μM), NBOX (5 μM), APV (50 μM), and picrotoxin (50 μM) were added. In some experiments, extracellular Ca$^{2+}$ was replaced with Sr$^{2+}$ to induce asynchronous release. K-glutamate internal was used for recording action potentials and $I_h$ currents. EPSCs were recorded using CeMeSO$_3$ internal and IPSCs were recorded using high chloride CsCl internal. 5 ms blue light pulses (475 nm, ~10 mW/mm$^2$) were used to stimulate ChR2.

**Reward Behavior**

Mice were trained to lever press for 20% sucrose reward on an RR10 schedule, earning a maximum of 40 25 μl rewards in a 1 hr session.

**Shock Behavior**

Mice received 15 mild foot shocks (0.4 mA, 0.5 s) on an RI60 schedule.

**Fiber Photometry**

A 490 nm LED was sinusoidally modulated at 211 Hz and passed through a GFP excitation filter. A 405 nm LED was modulated at 531 Hz and passed through a 405 nm bandpass filter. Both light streams were coupled to a high NA (0.48), large core (400 μm) optical fiber patch cord, which was mated to a matching brain implant in each mouse. GCaMP6f fluorescence was collected by the same fiber, passed through a GFP emission filter, and focused onto a photoreceiver. Custom software running on a real-time signal processor controlled the LEDs and independently demodulated the fluorescence brightness due to 405 nm and 490 nm excitation. The timing of behavioral variables was recorded by the same system. To calculate ΔF/ΔF, a least-squares linear fit was applied to the 405 nm signal to align it to the 490 nm signal, producing a fitted 405 nm signal that was used to normalize the 490 nm as follows: $ΔF/ΔF = (490$ nm signal − fitted 405 nm signal)/fitted 405 nm signal.

**Statistics**

Unpaired t tests were used for comparisons between two groups (DMS- and DLS-projecting SNc DA neurons). Two-way ANOVA was used to assess how the properties or responses of DMS- and DLS-projecting SNc DA 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 Tukey’s or Sidak’s multiple comparisons test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, one table, and five movies and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.07.014.

AUTHOR CONTRIBUTIONS

T.N.L. and K.D. designed experiments, with input from L.L. and K.T.B on viral tracing; K.D. and L.L. on COML implementation; L.L. and K.T.B. provided unpublished TROJ procedures. T.N.L. performed and analyzed all experiments with contributions from K.T.B on viral tracing, contributions from R.T. and A.K.C. on whole-brain COML imaging experiments and related image analysis, contributions from K.E.E. on image quantification, and contributions from C.S., T.J.D., and K.A.Z. on fiber photometry methods development, implementation, and combination with freely moving behavior. T.N.L. and K.D. wrote the paper with editorial input from all authors. K.D. supervised all aspects of the work.

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Figure S1. Proper EnvA-Pseudotyping of RVdG-GFP and Cre Dependency of Long-Range Tracing of Inputs, Divisions of the DMS and DLS Subregions of Dorsal Striatum, Contralateral Inputs to DMS- and DLS-Projecting SNc Dopamine Neurons, and Dopamine D1 Receptor-Expressing Identity of Striatonigral Inputs Identified by TRIO, Related to Figure 1

(A) Coronal slice showing the SNc of a mouse injected with only the RVdG-GFP virus. TH staining for dopamine neurons is shown in blue. The lack of GFP+ cells indicates that the RVdG-GFP virus was not able to infect cells in the absence of TC expression.

(B) Coronal slices showing the SNc and striatum of a mouse injected with all the TRIO viruses except for CAV-cre. Left, some GFP+ cells are observed locally in the SNc, indicating leak of the TC virus. This local background precludes conclusions about local connectivity in TRIO, but does not prohibit conclusions about long-range connectivity. TH staining for dopamine neurons is shown in blue. Right, no GFP+ neurons are observed in the striatum (or any other structures that provide long-range inputs to SNc dopamine neurons), indicating that long-range TRIO tracing of inputs here was indeed dependent on CAV-cre injections in the DMS or DLS.

(C) Example coronal atlas images showing the divisions between DMS (yellow) and DLS (magenta).

(D) Example images showing coronal sections of the dorsal striatum at three distinct locations along the anterior-posterior axis (locations similar to the atlas images shown in A).

(E) Example images of RVdG-GFP labeling of contralateral inputs to DMS- and DLS-projecting SNc dopamine neurons from various brain regions. M1, motor cortex. HY, hypothalamus. LHb, lateral habenula. VTA, ventral tegmental area. SNc, substantia nigra pars compacta. SNr, substantia nigra pars reticulata. PAG, periaqueductal gray. MRN, midbrain reticular nucleus.

(F) Whole-brain quantification of contralaterally labeled inputs to DMS- and DLS-projecting SNc dopamine neurons, expressed as a percentage of all contralateral inputs. Other abbreviations: motor cortex (M1/2), somatosensory cortex (S1), fundus of the striatum/interstitial nucleus of the posterior limb of the anterior (legend continued on next page)
commissure (FS/IPAC), globus pallidus external segment (GPe), substantia innominata/ventral pallidum (SI/VP), bed nucleus of the stria terminalis (BNST), central amygdala (CeA), superior colliculus (SC), inferior colliculus (IC), pedunculopontine nucleus (PPN), parabrachial nucleus (PB).

(G) Images showing strong overlap of RVdG-GFP labeling in the dorsal striatum with expression of the D1 dopamine receptor (labeled in a D1-tmt BAC transgenic mouse). Scale bars are 50 μm.
Figure S2. CAV-cre Injections Contained within Distinct Dorsal Striatal Subregions and Anterior-Posterior Extent and Specificity of Striatonigral Projections to DMS and DLS, Related to Figure 2

(A) CAV-cre was injected into striatum of Ai9 td-Tomato cre reporter mice. Left, CAV-cre injected into DMS. Right, CAV-cre injected into DLS. Dotted white line highlights the corpus callosum.Ctx. Cortex. Str, Striatum.

(B) Example images of patterns of mGFP expression in the striatal projections of DMS- or DLS-projecting dopamine neurons, showing the full range of slices from most anterior to most posterior. Green images show the original mGFP expression pattern. Black and white images to the right of each of the green images show the analyzed image after background subtraction and thresholding.

(C) Projection fraction of DMS- and DLS-projecting SNc dopamine neurons in NAc core, NAc medial shell, NAc lateral shell, DMS and DLS. Error bars are SEM. Data for the DMS and DLS are the same as shown in Figure 2G. This graph simply displays the full results, which included quantifications of the projection fractions in NAc subregions.

(D) Histology sections showing a lack of mGFP-labeled axons from DMS- or DLS-projecting dopamine neurons in the PFC and amygdala. No mGFP expression was observed despite GFP immunostaining to enhance the signal. DAPI counterstaining (light blue) shows the location of the tissue. Scale bars are 0.5 mm.
Figure S3. Specificity of the TH-GFP Mouse Line for TH Immunopositive Neurons in the SNc and Lateral VTA, but Not the Midline VTA, Related to Figure 3

(A) Low magnification (10x) images showing GFP expression (TH-GFP, green) and TH immunostaining (TH Ab, blue) in the midbrain (SNc and VTA) of TH-GFP mice. Scale bars are 0.5 mm.

(B) High magnification (40x) images showing TH-GFP expression (TH-GFP, green) and TH immunostaining (TH Ab, blue) in four regions of the midbrain – lateral SNc, medial SNc, lateral VTA, and midline VTA – which were quantified separately in C. Scale bars are 50 μm.

(C) Quantification of the percentage of GFP+ neurons that were also TH+ in the midbrains of TH-GFP mice (n = 3 mice). Pooled VTA data give a specificity of 71.8%, comparable to the specificity of 69% reported by Lammel et al. (2015).
Figure S4. Fiber Photometry Artifact Correction Using an Isosbestic Excitation Wavelength Control Signal, Expression of GCaMP6f in DMS- and DLS-Projecting Dopamine Neurons, and the Relationship of Recorded Fiber Photometry Signals with Behavior, Related to Figure 5

(A) Cultured neuron expressing GCaMP6f showing increased fluorescence upon 50 Hz electrical field stimulation when illuminated with 475 nm but not 400 nm light.

(legend continued on next page)
(B) Quantification of the fluorescence time course represented in A.

(C) Schematic of the bleaching and artifact correction workflow. Both the calcium-dependent and –independent signals are filtered. The calcium-independent (control) signal is then fitted to the calcium-dependent signal and subtracted from it to remove artifacts. A ΔF/F is further calculated by dividing by the control signal.

(D) Sample trace from a GCaMP6f-expressing mouse under 405 nm or 490 nm illumination. Red dotted lines indicate reward retrieval times. Note the non-responsiveness of the 405 nm signal to rewarding outcomes.

(E) Left, Histology showing the expression of GCaMP6f in terminals in the either the DMS or the DLS. Dotted line highlights the corpus callosum dividing the cortex (ctx) and striatum (striatum). Middle, Histology showing the expression of GCaMP6f in SNc cell bodies in relation to TH staining (blue) and fiber optic placement (dotted rectangle). Right, higher magnification images of GCaMP6f-expressing cell bodies in the SNc, showing healthy expression and overlap with TH staining (blue).

(F) The number of rewards earned by individual mice expressing GCaMP6f in DMS-projecting SNc dopamine neurons did not correlate with the photometry signals observed during reward collection.

(G) The number of rewards earned by individual mice expressing GCaMP6f in DLS-projecting SNc dopamine neurons did not correlate with the photometry signals observed during reward collection.

(H) The time spent freezing during the shock session by individual mice expressing GCaMP6f in DMS-projecting SNc dopamine neurons did not correlate with the photometry signals observed during shock delivery.

(I) The time spent freezing during the shock session by individual mice expressing GCaMP6f in DLS-projecting SNc dopamine neurons did not correlate with the photometry signals observed during shock delivery.

(J) The time of post-shock motor activity (running or jumping) by individual mice expressing GCaMP6f in DMS-projecting SNc dopamine neurons did not correlate with the photometry signals observed during shock delivery.

(K) The time of post-shock motor activity (running or jumping) by individual mice expressing GCaMP6f in DLS-projecting SNc dopamine neurons did not correlate with the photometry signals observed during shock delivery.
Cell
Supplemental Information

Intact-Brain Analyses Reveal Distinct Information Carried by SNc Dopamine Subcircuits

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**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, $2.5 \times 10^{12}$ vg/ml, 250 nl; AAVDJ-hSyn1-mGFP-T2A-mRuby, $2.9 \times 10^{13}$ vg/ml, 250 nl; AAV5-CAG-FLeX-TC, $2.6 \times 10^{12}$ vg/ml, 325-500 nl; AAV8-CAG-FLeX-G, $1.3 \times 10^{12}$ vg/ml, 325-500 nl; RVdG-GFP, $5 \times 10^{8}$ pfu/ml, 500 nl; AAV5-CaMKIIα-ChR2(H134R)-EYFP, $4 \times 10^{12}$ vg/ml 250-500 nl; AAV5-EF1α-DIO-GCaMP6f, $1.9 \times 10^{13}$ 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 NaHCO3, 1.2 NaH2PO4-H2O, 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 NaHCO3, 1.2 NaH2PO4-H2O, 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%O2/5%CO2 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, 1MgCl2, 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, 200µm-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
A least-squares linear fit was applied to the 405nm control signal to align it to the 490nm signal. The $\Delta F/F$ time series was then calculated for each behavioral session as $(490\text{nm signal} - \text{fitted 405nm signal}) / \text{fitted 405nm signal})$. Peristimulus time histograms (PSTHs) were created using the TTL timestamps that had been fed in from the operant chambers. Peak reward $\Delta F/F$ was calculated as the maximum $\Delta F/F$ value in the rewarded port entry PSTH in a one second period around the reward retrieval (time 0). No reward $\Delta F/F$ was calculated as the $\Delta F/F$ at time 0 in the non-rewarded port entry PSTH. Peak $\Delta F/F$ during shock was calculated as the most extreme $\Delta F/F$ value (positive or negative) observed during the shock period (time 0-0.5 seconds). Mean $\Delta F/F$ at 1-5 seconds post shock was calculated as the mean $\Delta F/F$ 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.

<table>
<thead>
<tr>
<th>Input Area</th>
<th>% Ipsilateral Inputs</th>
<th>% Contralateral Inputs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMS-projecting</td>
<td>DLS-projecting</td>
</tr>
<tr>
<td>M1/2</td>
<td>1.66 ± 0.17</td>
<td>1.90 ± 0.20</td>
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<tr>
<td>S1</td>
<td>0.99 ± 0.07</td>
<td>1.21 ± 0.08</td>
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<tr>
<td>Cortex (Other)</td>
<td>0.67 ± 0.08</td>
<td>0.78 ± 0.16</td>
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<td>7.88 ± 2.03</td>
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<tr>
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<td>4.23 ± 0.74</td>
<td>1.87 ± 0.62</td>
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<tr>
<td>NAc lat shell</td>
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<td>3.12 ± 0.16</td>
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<tr>
<td>DMS</td>
<td>18.09 ± 3.67</td>
<td>11.72 ± 1.33</td>
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<tr>
<td>DLS</td>
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<tr>
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<td>PB</td>
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Supplemental References
