Supplemental Information for:

Circuit Architecture of VTA Dopamine Neurons Revealed by Systematic Input–Output Mapping

Kevin T. Beier[1,2], Elizabeth E. Steinberg[2], Katherine E. DeLoach[1], Stanley Xie[1], Kazunari Miyamichi[1,5], Lindsay Schwarz[1], Xiaojing J Gao[1,6], Eric J. Kremer[3,4], Robert C. Malenka[2]*, and Liqun Luo[1]*

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

DNA Constructs

AAV-CAG-FLEx^{loxP}-G, *AAV-CAG-FLEx^{loxP}-TC*. *pAAV-CAG-FLEx^{FRT}-G, pAAV-CAG-FLEx^{FRT}-TC*, and *CAV-FLEx^{loxP}-Flp* were constructed as reported previously (Schwarz et al., 2015).

pAAV-hSyn1-FLEx^{FRT}-mGFP (pAAV-hSyn1-FLEx^{FRT}-mGFP-2A-synaptophysin-mRuby) was also constructed using standard molecular cloning methods. GFP was PCR amplified from *pCAG-GFP* (addgene #11150), with primers designed to append the palmitoylation site from GAP43 for membrane targeting to the 5' end of the gene. The 5' primer contained an AscI site, and 3' primer contained the 5' part of a T2A sequence. Synaptophysin-mRuby (gift from Paul Temkin) was PCR amplified using a 5' primer containing the 3' part of a T2A sequence with 20 base pair overlap with the mGFP fragment, and 3' primer containing a SalI site. These fragments were then combined, and a single insert containing mGFP-2A-synaptophysin-mRuby was constructed using the 5' mGFP primer 2 and 3' synaptophysin-mRuby primers. This fragment was then subcloned into the SalI and AscI sites of *pAAV-CAG-FLEx^{FRT}-TC* (to make *pAAV-CAG-FLEx^{FRT}-mGFP-2A-synaptophysin-mRuby*). Primers were as follows:

5' mGFP primer 1:
GAACCAAACAGGTTGAAAAGAATGATGAGGACCAAAAGATCATGGTGAGCAAGGGC
GAGGAG
5' mGFP primer 2:
CTATACGAAGTTATAGGCGCGCCACCATGCTGTGCTGTATGAGAAGAACCAAACAGGT
TGAAAAG
3' mGFP:
CCACGTCGCCGCAGGTCAGCAGGCTGCCCCTGCCCTCCTTGTACAGCTCGTCCATGC
5' synaptophysin-mRuby:
GCTGACCTGCGGCGACGTGGAGGAGAACCCCGGCCCCATGGACGTGGTGAATCAGC
TG
3' synaptophysin-mRuby:
GCTATACGAAGTTATGTCGACTTACCCTCCGCCCAGGCCGGCGAAC

Once completed, the *FLEx^{FRT}* cassette was PCR amplified from *pAAV-CAG-FLEx^{FRT}-mGFP-2A-synaptophysin-mRuby*, and subcloned into the BamHI and EcoRI sites of *pAAV-hSyn1-mCherry* (gift from Michael Lochrie) to make *pAAV-hSyn1-FLEx^{FRT}-mGFP-2A-synaptophysin-mRuby*. Primers used were as follows:

5' FLEx^{FRT}: AGTCGAGAAGGTACCGGATCCTGTGCTGTCTCATCATTTTGG
3' FLEx^{FRT}: GATAAGCTTGATATCGAATTCATCGATCGGCCGCATATAA.

Viral Tracing

For rabies tracing, 100 nL of a 1:1 volume mixture of *AAV$_5$-CAG-FLEx^{loxP}-TC and AAV$_8$-CAG-FLEx^{loxP}-G* was injected into the VTA of 4-6 week old mice. Two weeks later, RV*dG* was injected into the same brain location. After recovery, mice were housed in a BSL2 facility for 5 days to allow for rabies spread and GFP expression.

For TRIO experiments, the same procedure was performed as above, except that *CAV-Cre* was also injected into a VTA output site and 500 nL of a 1:1 volume mixture of *AAV$_5$-CAG-FLEx^{loxP}-TC* and *AAV$_8$-CAG-FLEx^{loxP}-G* were injected into the VTA of wild-type mice.

For cTRIO, *CAV-FLEx^{loxP}-Flp* was injected into a VTA output site, and 500 nL of a 1:1 volume mixture of *AAV$_5$-CAG-FLEx^{FRT}-TC* and *AAV$_8$-CAG-FLEx^{FRT}-G* was injected into the VTA of *DAT-Cre* mice.

For axon arborization experiments, *CAV-FLEx^{loxP}-Flp* was injected into a VTA output site, and *AAV$_{DJ}$-hSyn1-FLEx^{FRT}-mGFP* was injected into the VTA of *DAT-Cre* mice.

For injections into VTA output sites for TRIO, cTRIO, and axon arborization experiments,

250 nL of *CAV-Cre* or *CAV-FLEx^{loxP}-Flp* was injected into NAcLat (relative to Bregma and in mm, AP +1.45, LM 1.75, DV –4.0), 250 nL into NAcMed (AP +1.78, L0.4, DV –4.1), 500 nL into CeA (AP –1.43, LM 2.5, DV –4.5), and 1 µL into mPFC (two injections of 500 nL, one at AP +2.15, LM 0.27, DV –2.1 and another at AP +2.15, L0.27, DV –1.6).

The titers of these viruses, based on quantitative PCR analysis, were as follows: *AAV$_5$-CAG-FLEx^{loxP}-TC*, 2.4 x 10^{13} genome copies (gc)/mL; *AAV$_8$-CAG-FLEx^{loxP}-G*, 1.0 x 10^{12} gc/mL; *AAV$_5$-CAG-FLEx^{FRT}-TC*, 2.6 x 10^{12} gc/mL; *AAV$_8$-CAG-FLEx^{FRT}-G*, 1.3 x 10^{12} gc/mL; *AAV$_{DJ}$-hSyn1-FLEx^{FRT}-mGFP*, 2.9 x 10^{13} gc/mL, *CAV-Cre*, 2.5 x 10^{12} gc/mL, *CAV-FLEx^{loxP}-Flp*, 5.0 x 10^{12} gc/mL. The titer of RV*dG* was estimated to be 5.0 x 10^8 colony forming units (cfu)/mL based on serial dilutions of the virus stock followed by infection of the 293-TVA800 cell line.

Histology and Imaging

Animals were transcardially perfused with phosphate buffered saline (PBS) followed by 4% formaldehyde. Brains were dissected, post-fixed in 4% formaldehyde for 12–24 hours, and placed in 30% sucrose for 24–48 hours. They were then embedded in Tissue Freezing media and stored in a –80ºC freezer until sectioning.

For rabies tracing analysis, consecutive 60-µm coronal sections were collected onto Superfrost Plus slides and stained for NeuroTrace Blue (NTB, Invitrogen). For NTB staining, slides were washed 1x5 min in PBS, 2x10 min in PBST, incubated for 2–3 hours at RT in (1:500) NTB in PBST, washed 1x20 min with PBST and 1x5min with PBS. Sections were additionally stained with DAPI (1:10,000 of 5 mg/mL, Sigma-Aldrich), which was included in the last PBST wash of NTB staining. Whole slides were then imaged with a 5x objective using a Leica Ariol slide scanner with the SL200 slide loader.

For analysis of DA neuron output, every 60-µm coronal section was collected sequentially into PBS. Sections were washed 2x10 min in PBS and blocked for 2–3 hours at room temperature (RT) in 10% normal goat serum (NGS) in PBS with 0.3% Triton-X100 (PBST). Primary antibody (chicken anti-GFP, Aves Labs, 1:1000) was diluted in 5% NGS in PBST and incubated for four nights at 4^{o}C. After 3x10 min washes in PBST, secondary antibodies were applied for two nights at 4^{o}C (donkey anti-chicken Alexa-488, 1:250, Jackson ImmunoResearch), followed by 3x10min washes in PBST. Sections were additionally stained with DAPI. All images were acquired using a 5x objective on the Leica Ariol slide scanner, and processed using NIH ImageJ software.

For starter cell identification, sections were unmounted after slide scanning, blocked in PBST with 0.3% Triton-X100 and 10% NGS for 2–3 hours at room temperature, and incubated in rat anti-mCherry antibody (1:2000, Life sciences) and rabbit anti-TH antibody (1:1000, Millipore) at 4^{o}C for four nights. After primary antibody staining, sections were washed 3x10 min in PBST, and secondary antibodies (donkey anti-rat Cy3 and donkey anti-rabbit 647, Jackson ImmunoResearch) were applied for two nights at 4^{o}C, followed by 3x10min washes in PBST and remounting. Confocal z-stacks were acquired using a 20x objective on a Zeiss LSM 780 confocal microscope. For Figure S1, imaging and quantification were done as previously described (Lammel et al., 2015).

Data Analysis for Whole-Brain Tracing

The 22 regions chosen for analysis included the major inputs to the VTA that fell outside of the excluded region near the injection site. For VTA transsynaptic tracing and TRIO/cTRIO analysis, GFP-positive input neurons were manually counted from every third 60-µm section through the entire brain, except near the VTA, as specified in Figure S2 (posterior 2.7 mm to 3.9 mm from bregma). As each brain differed in total numbers of input neurons, we normalized neuronal number in each area by the total number of input neurons counted in the same brain, as previously described (Weissbourd et al., 2014). For 21 brain regions (all except for the LDT),

every third section was counted. For the LDT, as the nucleus is small, every other section was counted. If the total number of inputs in a region was less than 5, every section was counted. Counts were then multiplied by the scale factor (3 for most regions, 2 for the LDT). We did not adjust for the possibility of double-counting cells in any of our quantifications, which likely results in slight over-estimates, with the amount of over-estimation depending on the size of the cell in each region quantified (Weissbourd et al., 2014).

Anatomical Regions and Their Abbreviations

For quantifications of brain regions, boundaries were based on the Mouse Brain Atlas in Stereotaxic Coordinates, Franklin and Paxinos, 3rd ed (Franklin & Paxinos, 2007). The anterior cortex included all cortical regions located anterior to, and including, the first section containing the corpus callosum. Subdivisions of the nucleus accumbens shell (NAcMedS, NAcLatS) were identified primarily on the basis of NeuroTrace staining, and for output tracing, were assisted by DAT antibody staining (rabbit anti-DAT, ab18548, abcam, 1:1000). Other region definitions that included multiple subregions listed in the Franklin-Paxinos atlas, 3rd ed. (Franklin & Paxinos, 2007), are as follows:

AC – anterior cingulate cortex (ACC); infralimbic cortex (IL); insular cortex (Ins); motor cortex (MO; anterior portion); orbital cortex (Orb); prelimbic cortex (PL); somatosensory cortex (SS, anterior portion).

CeA – central amygdala lateral, medial, and capsular nuclei

DR – as defined in Weissbourd et al., 2014.

EAM – extended amygdala, anterior amygdaloid area

LDT – laterodorsal tegmental area, dorsomedial tegmental area, dorsal tegmental nucleus, Barrington's nucleus, ventral tegmental nucleus, subpeduncular tegmental nucleus

PO – medial preoptic area, lateral preoptic area, lateral anterior hypothalamic area, anterior hypothalamic area, striohypothalamic nucleus

Septum – triangular septal nucleus, lateral septum, dorsal fornix, septofimbrial nucleus, medial septum, septohypothalamic nucleus, septohippocampal nucleus, lambdoid septal zone

VP –interstitial nucleus of posterior limb of anterior commissure (IPAC), substantia innominata, horizontal diagonal band, nucleus of the vertical diagonal band

Abbreviations for brain regions made throughout the paper are listed below, in alphabetical order:

AC – anterior cortex
BNST – bed nucleus of the stria terminalis
CeA – central amygdala
DCN – deep cerebellar nucleus
DR – dorsal raphe
DStr- dorsal striatum
EAM – extended amygdala
EP – entopeduncular nucleus (GPi)
GP – globus pallidus (GPe)
LDT – laterodorsal tegmentum
LH – lateral hypothalamus
LHb – lateral habenula
MHb – medial habenula
NAcCore- nucleus accumbens, core
NAcMedS – nucleus accumbens, medial shell
NAcLatS – nucleus accumbens, lateral shell
PBN – parabrachial nucleus

PO – pre-optic area
PVH – paraventricular hypothalamus
VP – ventral pallidum
VTA – ventral tegmental area
ZI – zona incerta

Rabies Tracing Combined with In Situ Hybridization (ISH)
For each input region, we alternated sections between probes, with an average of every 2nd section with the same probe in the PVH and LH, and every 4th section for the DR. The brain regions analyzed conform to the regions defined as in Mouse Brain Atlas in Stereotaxic Coordinates, Franklin and Paxinos, 3rd ed. (Franklin & Paxinos, 2007), except for the DR, which was as previously defined (Weissbourd et al., 2014). Sections covered the whole region expressing the relevant gene in the region being analyzed.

PCR Primers for ISH Probes
The probes for Oxytocin, Vasopressin, VGluT1/2//3, and GAD1/2 are the same as used previously (Weissbourd et al., 2014). For the other probes, the following primers were used to amplify the templates for ISH probes. T3 polymerase recognition site is indicated by underline.
Neurotensin
5'-AGAAGAAGATGTGAGAGCCCTG
5'-AATTAACCCTCACTAAAGGGCTGCTTTGGGTTAATAACGCTC
Orexin
5'-CTGCTGCTGCTGCTACTGCT
5'-AATTAACCCTCACTAAAGGGGGGAAGTTTGGATCAGGACA
Tph2
5'-GTATTGAGAATGTGGTGCAGGA
3-AATTAACCCTCACTAAAGGGCACTCAGTCTACATCCATCCCA
Tyrosine Hydroxylase
5'-GATTGCAGAGATTGCCTTCC
5'-AATTAACCCTCACTAAAGGGCCTGTGGGTGGTACCCTATG

Axon Arborization Analysis
After sectioning and antibody staining, sections were mounted and imaged, as for input tracing analysis. For each region, five 60-μm sections were chosen for analysis from within a defined anterior-posterior segment (all coordinates are relative to bregma in mm): anterior 1.41 to 0.85 for nucleus accumbens and striatum, 0.37 to 0.01 for BNST and VP, 1.87 to 1.53 for mPFC, and posterior 0.95 to 1.79 for the CeA. For the septum, 10 sections were analyzed, 5 each from 1.41 to 0.85 and 0.37 to 0.01. The entire regions within each section were taken, and boundaries were defined based on DAPI staining and on the Franklin and Paxinos mouse brain atlas, 3rd ed (Franklin & Paxinos, 2007). For analysis, the background was first subtracted, and the mean of local background after subtraction was multiplied by a constant value (4, for all samples but one sample targeting NAcLat-projecting neurons that had sparser labeling, where 3 was used). This value was then set as the threshold, with pixels above this gray-scale value being interpreted as positive signal from VTA-DA neuron axons. The threshold value was kept constant for all sections analyzed within a brain. The total area and area fraction covered by the above-threshold axon signal was measured for each region and averaged across the 5 (or 10) sections.

Analysis of Spatial Distribution of Starter Cells in the VTA
To assess the spatial distribution of starter cells in our experiments, nine 60-μm coronal sections through the VTA were quantified (corresponding to one tissue section per atlas section in the

Franklin and Paxinos Atlas). These sections were collected in order through the VTA of experimental animals and processed as described above in Histology and Imaging. Starter cells were considered as all cells that were both GFP^+ and TC^+. The location of these cells was manually mapped onto digital reference images from the Franklin-Paxinos atlas using the Cell Counter plugin in ImageJ. x, y, and z coordinates were obtained for each starter cell in each experiment. Diagrams displaying the spatial distribution of starter cells (Figure 3C, S3F, S4) are collapsed along the anterior–posterior (z) axis, and therefore underestimate the true distinction in spatial distribution of starter neurons. The spatial distribution for the starter cells was calculated for the x and y dimensions separately.

As we injected two AAVs to express TC and G separately, it was possible that some cells expressed one gene, but not the other. Cells that expressed TC, but not G, would therefore appear red and green and would be considered a starter cell, but as they would lack expression of G, no inputs would be labeled from these "false" starter cells. Corrections for false starter cells were made using the observed spread of *AAV_5-CAG-FLEx^{loxP}-TC* and *AAV_8-CAG-FLEx^{loxP}-G*. Native mCherry fluorescence was used to visualize TC; for G, 60-µm tissue sections were stained for the rabies glycoprotein (mouse anti-rabies glycoprotein, Millipore, 1:500, followed by donkey anti-mouse, 1:500, Jackson ImmunoResearch). The distance from the targeted center of the injection site in the VTA to the edge of observed fluorescence for three sections located near the injection site, and three sections located approximately 600 µm posterior to the injection site was quantified. False starter cells were considered as those that expressed TC, but not G. The Euclidian distance from the center of injection to the edge of spread was then calculated for each AAV, and as the radius of spread from the injection site as visualized in both anterior and posterior sections approximated a sphere, a virtual sphere was created with a radius equal to the spread distance of *AAV_8-CAG-FLEx^{loxP}-G* (which was smaller than that of *AAV_5-CAG-FLEx^{loxP}-TC*). These values (for either 0.1 µL, in the case of *DAT-Cre* and *GAD2-Cre* input tracing, or 0.5 µL, for TRIO and cTRIO) were then used to exclude starter cells that were located outside of the sphere of TC and G co-expression. The false starter cell correction, as well as the center of mass (CoM; the mean location of starter cells) and standard deviation of the starter cell spatial distribution calculations were made using custom MATLAB code based on the x, y, and z coordinates for each starter cell.

For input quantification, not every section was counted for starter cell counting: while the VTA spanned about twenty sections of 60-µm thickness, only nine sections containing the VTA were quantified, as this corresponded to the number of unique sections containing the VTA that are represented in the Franklin-Paxinos atlas. In this way, starter cells from one 60 µm section would correspond to one digital image. Therefore, when total numbers of starter cells were reported (Table S1), the total number of starter cells for each brain was multiplied by 20/9.

Electrophysiological Recordings from Adult Mouse Midbrain Slices

Mice were deeply anaesthetized with pentobarbital (200 mg/kg ip; Ovation Pharmaceuticals, Deerfield, IL). Coronal midbrain slices (200 µm) were prepared after intracardial perfusion with ice-cold artificial cerebrospinal fluid (ACSF) containing elevated sucrose (in mM): 50 sucrose, 125 NaCl, 25 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgCl_2$, and 2.5 glucose (oxygenated with 95% O_2/5% CO_2). After 60 min of recovery, slices were transferred to a recording chamber and perfused continuously at 2–4 ml/min with oxygenated ACSF (22.5 mM sucrose) at 30 ºC. Picrotoxin (50 µM, Sigma) was added to block inhibitory currents mediated by $GABA_A$ receptors. Patch pipettes (3.8-4.4 MΩ) were pulled from borosilicate glass (G150TF-4; Warner Instruments) and filled with internal solution containing (in mM): 117 $CsCH_3SO_3$, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 TEA, 4 MgATP, 0.3 NaGTP, 5 QX314, and 0.1 Spermine (pH 7.3, 270–285 mOsm). Labeled DA neurons were visualized by infrared-differential interference contrast (IR-DIC) video microscopy and epifluorescence (Olympus) for detection of retrobeads

(red) and GFP.

Labeled DA neurons were visualized with a 40X water-immersion objective on an upright fluorescent microscope (BX51WI; Olympus) equipped with infrared-differential interference contrast video microscopy and epifluorescence (Olympus) for detection of retrobeads. ChR2 was stimulated by flashing 473 nm light (2-ms pulses; 0.1 Hz; 12 mW) through the light path of the microscope using a ultrahigh-powered light-emitting diode (LED) powered by an LED driver (Prizmatix, Modiin Ilite) under computer control. The light intensity of the LED was not changed during the experiments and the whole slice was illuminated. A dual lamp house adaptor (Olympus) was used to switch between the fluorescence lamp and LED light source

A total of 13 mice were used for recording experiments (5 for mPFC, 5 for LHb, and 3 for anterior cortex).

Behavioral Experiments

Subjects: 15 male C57Bl/6J mice (Jackson Laboratories, #664), 8–10 weeks old at the start of the experiment, were group-housed in a light-regulated colony room (12 hr light/dark cycle; lights on at 7:00 am). Food and water were available *ad libitum* throughout the experiment.

Surgical procedures for behavioral experiments: Standard stereotaxic procedures were used to infuse virus, and implant optical fibers and guide cannulas in two separate surgeries under ketamine/dexmedetomidine anesthesia. All coordinates are relative to skull surface and bregma in mm. In the first surgery, *AAV_5-hsyn1-eYFP* (n=8) or *AAV_5-hsyn1-ChR2-eYFP* (n=7) (UNC viral vector core, $3.5x10^{12}$ and $4.6x10^{10}$ viral particles/ml, respectively) was infused into 4 separate sites (2 per hemisphere) in the anterior cortex. Coordinates for viral injections were as follows: AP +2.1; ML ± 2.2; DV –2.1; 0.3 µL and AP +1.95; ML ± 0.3; DV –2.1; 0.2 µL. Virus was delivered at a rate of 0.1 µL/min and the injection needle was left in place for 5 minutes after each infusion before it was slowly removed. Following surgery, mice were returned to group housing after being allowed to recover on a heating pad. 8 weeks later, mice underwent a second surgery to implant optical fibers (made in-house with 0.39 NA, 200 µm diameter optical fiber, Thorlabs) targeted just dorsal to the VTA and guide cannulas (26 ga, Plastics One) targeted to the lateral NAc. The following coordinates were used: optical fibers; AP –3.1; ML ± 1.2; DV –4.1; 10° angle within the coronal plane and cannulas; AP +1.45; ML ± 1.55; DV –3.0; no angle. Implants were secured to the skull with metal screws (Antrin Miniature Specialists) and Geristore dental epoxy (DenMat). Although only used for drug infusions in ChR2-eYFP-expressing mice, all mice received cannula implants in order to maintain consistency between groups. Following the second surgery mice were single-housed for the remainder of the experiment. Mice were allowed to recover for >10d before behavioral procedures commenced.

Behavioral apparatus: Experimental sessions were conducted in operant conditioning chambers (24 cm W x 20 cm D x 18 cm H, Med Associates Inc.) contained within sound-attenuating cubicles. The left side of the chamber was fitted with 5 nosepoke ports, each with an LED light at the rear. A video camera (Med Associates Inc.) was positioned at the top of the sound-attenuating cubicle that provided a top-down view of the entire conditioning chamber. Offline video analysis with tracking software (Biobserve) allowed locomotion—measured as total distance traveled during the 1hr session— to be quantified during some training sessions. Prior to behavioral sessions, mice were gently attached to patch cables made in-house with optical fiber (0.39 NA, 200 µm diameter, Thorlabs) via a ceramic split sleeve (Precision Fiber Products). The patch cables were also connected to bilateral rotary joints (Doric Lenses), which permitted free rotation while transmitting blue light from an upstream 473 nm blue laser (Laserglow). Optical stimulation was controlled by a computer running Med PC IV software (Med Associates Inc.), which also recorded responses at all nosepoke ports and initiated and terminated video recording.

ICSS acquisition: Prior to the first behavioral session, mice were familiarized with cereal treats (Fruit Loops, Kellogg) in their home cage. On the first training day, all nosepokes were baited with crushed cereal treats to facilitate initial investigation. The start of the session was indicated to the mouse by the illumination of a white house light. Session length was 60 min, during which time mice were free to respond at any nosepoke port. 4 ports were designated "active" ports, and a response at these ports produced 2s of optical stimulation at a particular frequency (1, 5, 10 or 20 Hz); the LED at the back of the corresponding port was concurrently illuminated to provide a visual cue signaling the presence of optical stimulation. Responses made within the 2s stimulation period were recorded but had no consequence. Responses at a 5th "inactive" nosepoke port were recorded but did not result in either optical stimulation or cue light presentation. Testing occurred once per day for 5 days. Peak light output during photostimulation was estimated to be ~2.75 mW at the tip of the implanted fiber (~22 mW/mm^2), and ~5 mW/mm^2 at the targeted tissue 200 µm from the fiber tip. The power density estimates were based on the light transmission calculator at www.optogenetics.org/calc.

Drug infusions: A subset of high-responding mice from the ChR2-eYFP group (n=5) was used for drug infusion studies. These mice received 4 additional ICSS training sessions prior to the initiation of drug infusions in order to establish baseline response rates. On infusion days, mice received bilateral 0.25 µL infusions of either flupenthixol (F114, Sigma, dissolved in saline) or saline vehicle via a 33 ga infusion cannula (Plastics One) that was inserted into the implanted guide cannula and extended a further 1.5 mm into the brain, for a final depth of 4.5 mm below skull surface. Infusions were made at a rate of 0.25 µL/min with a syringe pump (Harvard Apparatus), and the infusion cannula was left in place for an additional minute to permit drug diffusion. After each infusion, mice were returned to their home cage for 10 minutes to allow drugs to take effect before behavioral sessions began. Behavioral sessions where flupenthixol was administered were always preceded by at least one sham infusion session (where all infusion procedures were followed except that the infusion cannula was cut so as not to project past the end of the guide cannula, and no liquid was infused) and a saline infusion session. Three saline infusion sessions were conducted in total (24 hrs before each flupenthixol infusion); responding in these sessions was equivalent (1-way repeated measures ANOVA, $p>0.05$) so data from these sessions were averaged to yield a single saline measurement.

Histology for mice used for behavioral studies: Mice were deeply anesthetized with sodium pentobarbital and perfused transcardially with 4% formaldehyde. Mice were perfused with infusion cannulae inserted into guide cannulae to facilitate post-hoc identification of infuser tip placement. Brains were removed, post-fixed for 24 hours, and sectioned at room temperature on a vibratome. Free-floating 60-µm coronal sections were then processed for TH immunohistochemistry. Sections containing the VTA were also processed for eYFP immunohistochemistry. Sections were incubated in a blocking solution containing bovine serum albumin and Triton X-100 (each 0.2%) in phosphate-buffered saline (PBS) for 20 min. Normal donkey serum (10%) was added to blocking solution for a further 30 min incubation. Sections were then incubated overnight with primary antibodies diluted in blocking solution. Following 3 washes with blocking solution, sections were incubated in PBS containing 2% normal donkey serum for 10 minutes. Sections were then incubated for 2 hours with secondary antibodies diluted in PBS, washed with PBS several times, mounted on microscope slides in phosphate-buffered water, and coverslipped with Fluoromount G mounting medium containing DAPI nuclear counterstain (Southern Biotech). Concentrations and sources for antibodies were as follows: rabbit anti-TH 1:1500 (Millipore, #AB152), sheep anti-GFP 1:3000 (Novus Biologicals, NB110-75114), donkey anti-rabbit AlexaFluor647 and donkey anti-sheep AlexaFluor488, both

1:200 (Invitrogen). Sections were visualized on a Nikon A1 confocal microscope to determine optical fiber and cannula placements. Although optical fiber and cannula placement varied slightly between animals (see Figure S6), no subjects were excluded.

Data Analysis: Behavioral data were analyzed with SigmaStat software. Parametric tests (one-way repeated measures ANOVA followed by Holm-Sidak post-hoc tests) were used in cases where data met assumptions of normality and equal variance. In cases where data did not meet these assumptions, non-parametric tests (Friedman's repeated-measures ANOVA on ranks followed by Tukey post-hoc tests for within-group comparisons; Mann-Whitney rank sum tests with Bonferroni correction for between-group comparisons) were used.

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