Supporting Information

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SI Materials and Methods

Adeno-Associated Virus Preparation. For construction of Cre-inducible recombinant adeno-associated virus (AAV) vectors, the Arch-eGFP transgene was inserted between two pairs of incompatible lox sites (loxP and lox2722) in the reverse orientation. The resulting double-floxed reverse Arch-eGFP cassette was cloned into a modified version of the pAAV2-multiple cloning site vector carrying the EF1a promoter and the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) to enhance expression. The recombinant AAV vector was serotyped with AAV2 coat proteins. The final viral concentration was 2×10^{12} capsids/mL.

Lentivirus Preparation. Short-hairpin RNA (shRNA) was delivered and constitutively expressed by using the BLOCK-iT lentiviral RNAi expression system (Invitrogen). Each shRNA target was inserted downstream of the human U6 promoter, and subsequently the mCherry transgene was inserted between the EF1a promoter and the WPRE. The following shRNA sequences were used: Drd1a, 5′-GCATTGATTCCATCACCTTCGCGAACGAAGGTGATGGAATCAATGC-3′ and Drd2, 5′-GCATGCCAAGATTGTCAATCCCGAAGGATTGACAATCTTGGCATGC-3′.

Viral particles were prepared as reported previously (1). The number of infectious units of the virus was determined by quantitative real-time PCR (LightCycler 480; Roche). For in vivo experiments, each lentivirus was used at 5×10^{10} infectious units/mL.

Stereotactic Virus Injection and Optical Fiber Implantation in the Ventral Tegmental Area. Mice were anesthetized by using a ketamine/xylazine mixture (ketamine, 90 mg/kg; xylazine, 20 mg/kg) diluted in PBS (pH 7.4). One microliter of the purified double-floxed AAV:Arch-eGFP virus [AAV-double-floxed inverted open reading frame (DIO)-Arch] was injected unilaterally into the left ventral tegmental area (VTA) (from bregma: anterior–posterior, −3.2 mm; lateral, 0.5 mm; and dorsal–ventral, 4.0 mm). An optical fiber (200-nm core diameter, 0.22 N.A.; Doric Lenses) was also implanted into the left VTA (from bregma: anterior–posterior, −3.2 mm; lateral, 0.5 mm; and dorsal–ventral, 3.75 mm) and secured by using dental cement. One microliter of the purified lentivirus (Lenti:mCherry, Lenti:shD1R-mCherry or Lenti:shD2R-mCherry) was injected into eight positions inside the nucleus accumbens (NAc) (from bregma: anterior–posterior, 1 and 1.5 mm; lateral, ±1.2 mm; and dorsal–ventral, 2.5 and 3.0 mm).

In Vivo Electrophysiological Recording with Optical Stimulation. Single-unit recordings were made from anesthetized TH-Cre mice. Recording electrodes were filled with 1 M KCl solution and electrical impedance ranged between 2 and 5 MΩ. An optical fiber (110-μm core diameter, 0.22 N.A.) was set in close proximity to the glass recording electrode by using a double-electrode holder. The optorode was positioned in the VTA (from bregma: anterior–posterior, −3.2 mm; lateral, 0.5 mm; and dorsal–ventral, 3.8–4.4 mm). Electrical signals were AC coupled, amplified, band-pass filtered between 0.3 and 5 kHz, and digitized at 20 kHz. The optical fiber was coupled to a 532-nm diode pumped solid-state laser with ∼5 mW of output power. The optical stimulation was repeated 30–100 times for each recording. Unit recordings were evaluated by duration of action potentials, and all of those inhibited by optical stimulation matched the criteria for dopamine (DA) neuron spikes (2).

Immunohistochemistry. Mice were anesthetized and perfused with 4% (wt/vol) paraformaldehyde in PBS (pH 7.4). Brains were removed, postfixed overnight, stored in 30% (wt/vol) sucrose in PBS, and then dissected into slices with 50-μm thickness. The following primary antibodies were added to a PBST solution and incubated overnight at 4 °C: chicken polyclonal against GFP (Aves Labs; 1:500) and rabbit polyclonal against tyrosine hydroxylase (Millipore; 1:500). Sections were incubated with the following secondary antibodies at room temperature: Alexa Fluor 488-labeled anti-chicken IgY (Jackson ImmunoResearch; 1:500) and Alexa Fluor 546-labeled anti-rabbit IgG (Invitrogen; 1:1,000) and counterstained with DAPI. Images were taken with a confocal microscope (LSM700; Zeiss) and a fluorescence microscope (BZ-9000; Keyence).

In Situ Hybridization. Ten minutes after dark-room test conditioning, mice were anesthetized and perfused as described above. In situ hybridization was performed according to previous studies (3, 4). The following probes were used: Fos (GenBank NM_010234.2, nucleotides 138–1,300), Tac1 (GenBank NM_009311, nucleotides 95–995), Penk (GenBank NM_001002927, nucleotides 303–1,109), Drd1a (GenBank NM_010076, nucleotides 519–1,819), and Drd2 (GenBank NM_010077, nucleotides 95–1,392). The RNA probes were labeled with digoxigenin (DIG) or fluorescein. Fluorescein-labeled probes were secondarily immunolabeled with HRP-conjugated antifluorescein (Jackson ImmunoResearch; 1:4,000) and enhanced with tyramide signal amplication-plus-dinitrophenyl (Perkin-Elmer). The DNP signal was then immunostained with fluorescein-conjugated anti-DNP (Invitrogen; 1:500). After inactivation of HRP by 0.3% H_2O_2, DIG-labeled probes were secondarily labeled with HRP-conjugated anti-DIG (Roche; 1:300) and enhanced with TSA plus-Cy3 (Perkin-Elmer), and the sections were then counterstained with DAPI.

Real-Time PCR. The following specific primer pairs were used: Drd1a, 5′-TCTGGTTTACCTGATCCCTCA-3′ and 5′-GCCTCCTCCCTCTTCAGGT-3′; Drd2, 5′-TGAACAGGCGGAGAATGG-3′ and 5′-CTGGTGCTTGACAGCATCTC-3′; and Gapdh, 5′-TGACCACAGTCCATGCCATC-3′ and 5′-GACGGACACATTGGGGGTAG-3′. Total RNA was extracted from the lentivirus-injected regions by using an RNeasy micro kit (Invitrogen). First-strand cDNA was generated from 200 ng of total RNA with a Transcriptor First Strand cDNA Synthesis kit (Roche). Quantitative real-time PCR was performed by using SYBR Green I Master (Roche) with a LightCycler 480 (Roche).

Western Blotting. The NAc region surrounding the lentivirus injection site was dissected, homogenized in buffer [10 mM Tris·HCl, pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1× Complete Protease Inhibitor mixture (Roche), and 1× PhosSTOP (Roche)], and incubated at 4 °C for 20 min. The samples were then centrifuged at 15,000 × *g* at 4 °C for 20 min. The protein concentration of the supernatants was quantified by using the Bradford reaction. Equal amounts of total protein were subjected to SDS/PAGE (NuPAGE 4–12% Bis-Tris gel; Invitrogen), transferred to PVDF membranes, and Western blotted following standard protocols. After blocking, membranes were incubated with the following primary antibodies overnight at 4 °C: mouse monoclonal antibody against D1R (Abcam; 1:5,000) and rabbit polyclonal antibody against D2R (Millipore; 1:3,000). After having been washed, the membranes were incubated for 1 h at room temperature with peroxidase-conjugated anti-mouse IgG (light chain specific, Jackson ImmunoResearch; 1:20,000) for D1R, visualized with

Luminata Forte (Millipore), and quantified by using a Luminescence Image Analyzer (LAS-4000; GE Healthcare). For the detection of D2R, membranes were incubated with IRDye 800CW anti-rabbit IgG (LI-COR; 1:3,000) and then visualized and quantified by using an Odyssey Infrared Imaging system (LI-COR). Bands specific for D1R and D2R were determined by comparing immunoblots of the samples from striatum, cortex, and thalamus (Fig. S7 *B* and *C*). The striatum contains enormous amounts of D1R and D2R compared with the cortex and thalamus. Dense bands around 80 kDa from striatum samples appeared to be specific compared with bands from samples of the cortex and thalamus. These multiple bands were reported to be due to glycosylation of D1R and D2R (5, 6).

1. Tiscornia G, Singer O, Verma IM (2006) Production and purification of lentiviral vectors. *Nat Protoc* 1(1):241–245.
2. Ungless MA, Grace AA (2012) Are you or aren't you? Challenges associated with physiologically identifying dopamine neurons. *Trends Neurosci* 35(7):422–430.
3. Pinaud R, Mello CV, Velho TA, Wynne RD, Tremere LA (2008) Detection of two mRNA species at single-cell resolution by double-fluorescence in situ hybridization. *Nat Protoc* 3(8):1370–1379.
4. Watakabe A, Komatsu Y, Ohsawa S, Yamamori T (2010) Fluorescent in situ hybridization technique for cell type identification and characterization in the central nervous system. *Methods* 52(4):367–374.
5. Fishburn CS, Elazar Z, Fuchs S (1995) Differential glycosylation and intracellular trafficking for the long and short isoforms of the D2 dopamine receptor. *J Biol Chem* 270(50):29819–29824.
6. Karpa KD, Lidow MS, Pickering MT, Levenson R, Bergson C (1999) N-linked glycosylation is required for plasma membrane localization of D5, but not D1, dopamine receptors in transfected mammalian cells. *Mol Pharmacol* 56(5):1071–1078.

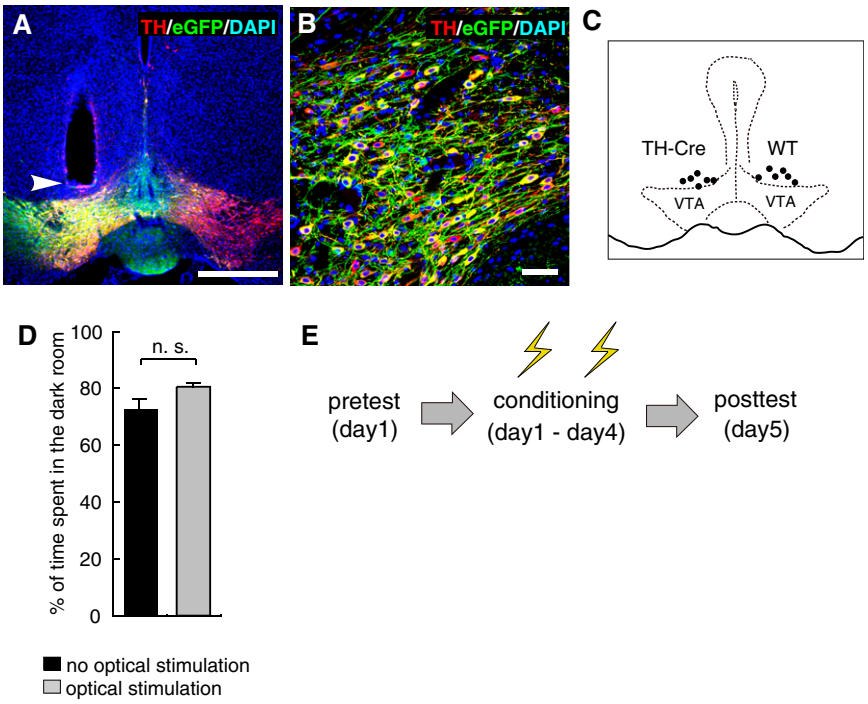

Fig. S1. Specific expression of Arch-eGFP in VTA DA neurons used in the dark-room preference test. (*A*) Representative photograph showing the expression of Arch-eGFP (green) and the tip of the optical fiber (arrowhead). (*B*) Magnified confocal image showing that the expression of Arch-eGFP highly overlapped that of TH (red). Of the GFP^+ cells, 96.9 ± 0.4% of them overlapped with TH, a marker for DA neurons, and 89.1 ± 1.5% of TH^+ cells were positive for GFP ($n = 3$ mice; 1,121 cells in total) (Scale bars, 500 μm in *A* and 50 μm in *B*.) (*C*) Histological analysis of locations of optical fiber tips shown as circles. (*D*) Percentage of time spent in the dark room after habituation of the WT mice. Optical stimulation in the dark room had no effect on preferential stay in the dark room ($t_6 = 1.997$, $P = 0.092$; *t* test, n.s., not significant, $n = 4$). (*E*) Time schedule of the dark-room preference test. During conditioning, mice received optical stimulation when they stayed in the dark room.

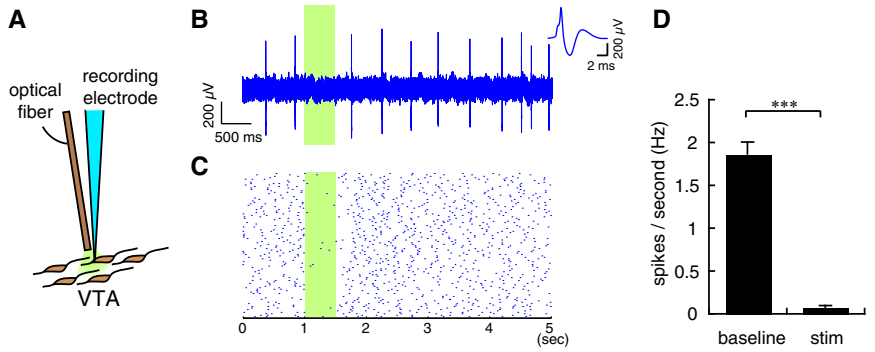

Fig. S2. In vivo electrophysiological recording for the inactivation of Arch-expressing DA neurons by optical stimulation. (*A*) Scheme for in vivo recording from DA neurons in the VTA and optical stimulation. (*B* and *C*) Single-unit recording (*B*) and raster plot (*C*) of an Arch-eGFP–expressing DA neuron. Firings were inhibited by optical stimulation (green rectangles). An averaged spike waveform of a typical DA neuron is shown in the *Inset*. (*D*) Firing rates of Arch-eGFP–expressing DA neurons. During optical stimulation, the firing rate was decreased to 0.07 ± 0.02 Hz (t_{12} = 13.55, ***P < 0.0001; paired t test, n = 13).

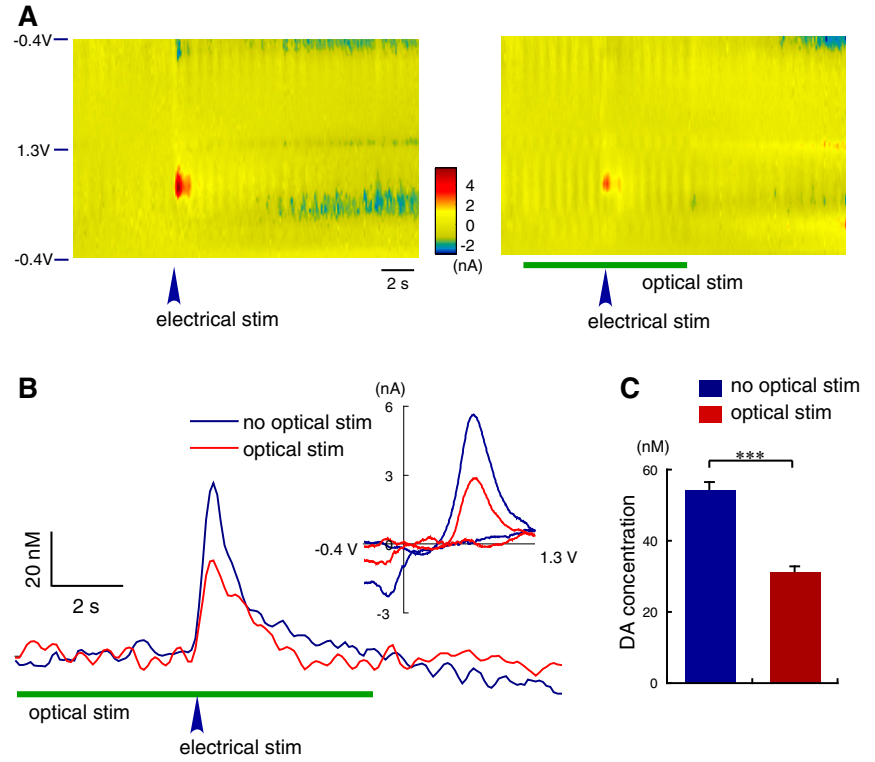

Fig. S3. Optical inactivation of DA neurons in the VTA suppresses evoked DA response in the NAc. (*A*) Background-subtracted voltammograms showing DA release in the NAc by electrical stimulation with (*Right*) and without (*Left*) optical stimulation of the VTA. The *y* axis is the applied potential (E_{app} versus Ag/AgCl reference electrode); and the *x* axis, the recording time. Current changes at the electrode are encoded in color. An increase in the DA level was seen during electrical stimulation with the applied potential of ~0.650 V (oxidation peak encoded as red). (*B*) Representative voltammetry traces obtained by electrical stimulations with or without optical stimulation. (*Inset*) Background-subtracted voltammogram taken from the peak of the reaction, indicating that the signal measured was DA on the basis of comparison with that of DA obtained in a solution. (*C*) Peak levels of DA release evoked by electrical stimulation. Optical stimulation of Arch-expressing DA neurons resulted in reduced peak DA levels evoked by electrical stimulation (t_{12} = 9.434, ***P < 0.001; t test, n = 7).

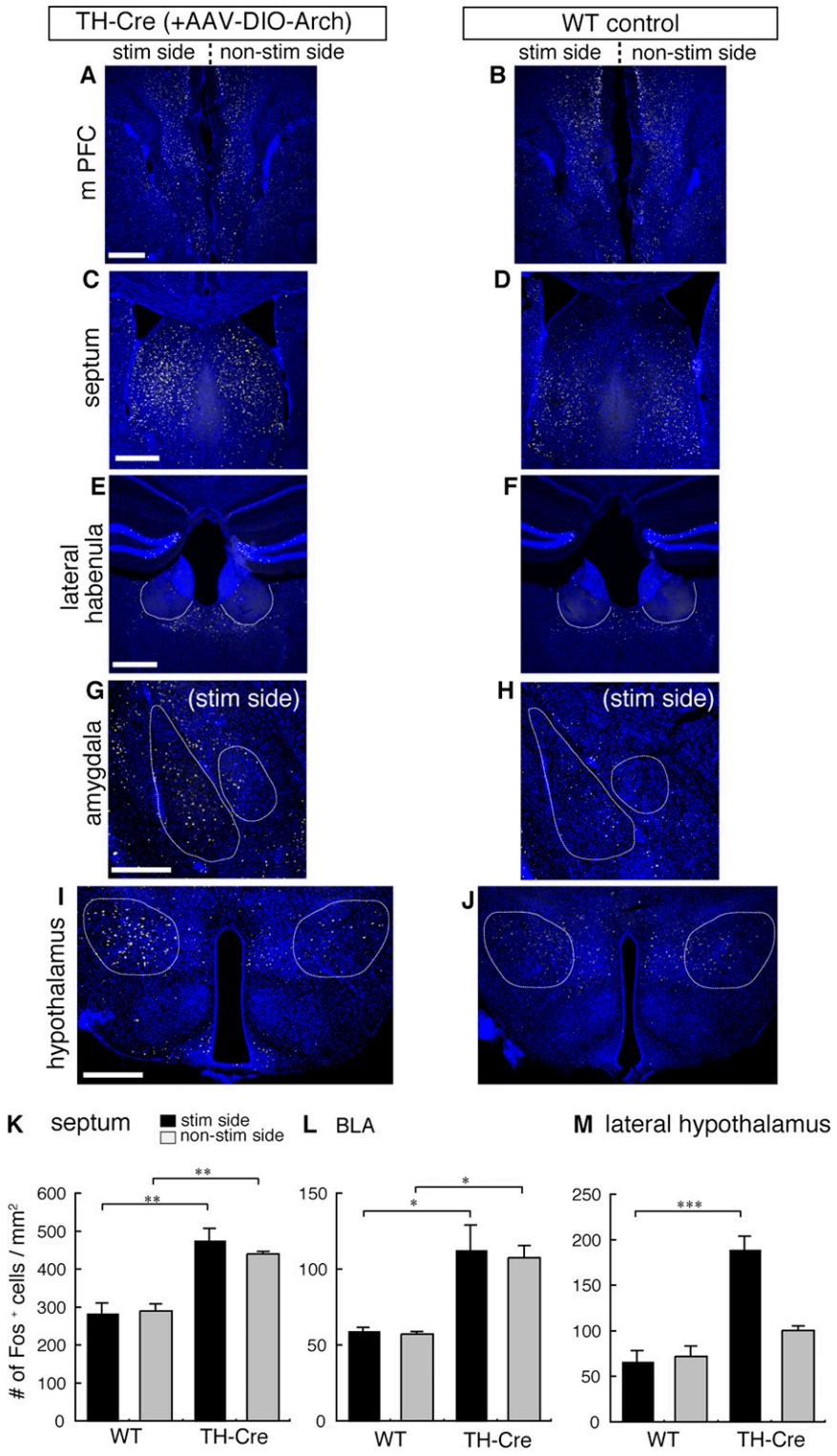

Fig. S4. Fos expression evoked by optical inactivation of VTA DA neurons. (*A–J*) Representative photographs for Fos expression (yellow) in the indicated brain regions of the TH-Cre and WT mice. The Fos expression in the medial prefrontal cortex (mPFC) and lateral habenula was not different between the TH-Cre and WT mice. (*K–M*) Numbers of Fos^+ cells per square millimeter in the indicated brain regions. The Fos expression in the bilateral septum (*K*; $F_{3,8} = 20.71$, $P = 0.0004$; post hoc test, $^{**}P < 0.01$, $n = 3$), in the bilateral basolateral amygdala (BLA) (*L*; $F_{3,8} = 10.81$, $P = 0.0035$; post hoc test, $^{*}P < 0.05$, $n = 3$), and in the stimulated side of the lateral hypothalamus (*M*; $F_{3,8} = 26.12$, $P = 0.0002$; post hoc test, $^{***}P < 0.001$, $n = 3$) of the TH-Cre mice was significantly increased.

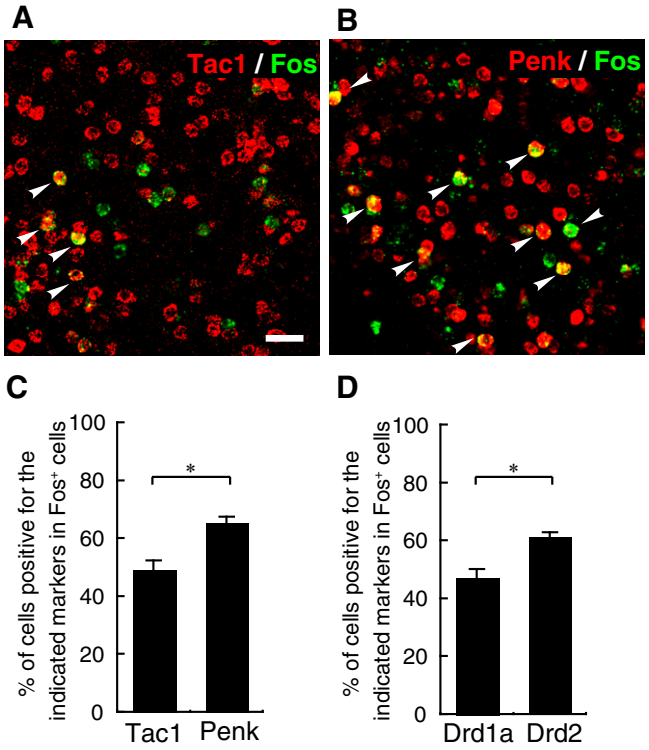

Fig. S5. Higher overlaps of Fos expression with indirect pathway-specific markers in the NAc. (*A* and *B*) Double in situ hybridization for Tac1/Fos (*A*) and Penk/Fos (*B*) in the NAc is shown at the ipsilateral side of optical stimulation. Arrowheads indicate double-positive cells. (Scale bar, 50 μm.) (*C* and *D*) Percentages of cells positive for the indicated markers among Fos^+ cells. In *C*, the overlap of the Penk/Fos expression was higher than that of the Tac1/Fos expression (49.1 ± 3.2% and 65.5 ± 1.9% of Fos^+ cells were positive for Tac1 and Penk, respectively. $t_4 = 4.359$, $^*P < 0.05$; *t* test, $n = 3$). In *D*, the overlap of the Drd2/Fos expression was higher than that of the Drd1a/Fos expression (47.3 ± 2.8% and 61.2 ± 1.5% of Fos^+ cells were positive for Drd1a and Drd2, respectively. $t_4 = 4.393$, $^*P < 0.05$; *t* test, $n = 3$).

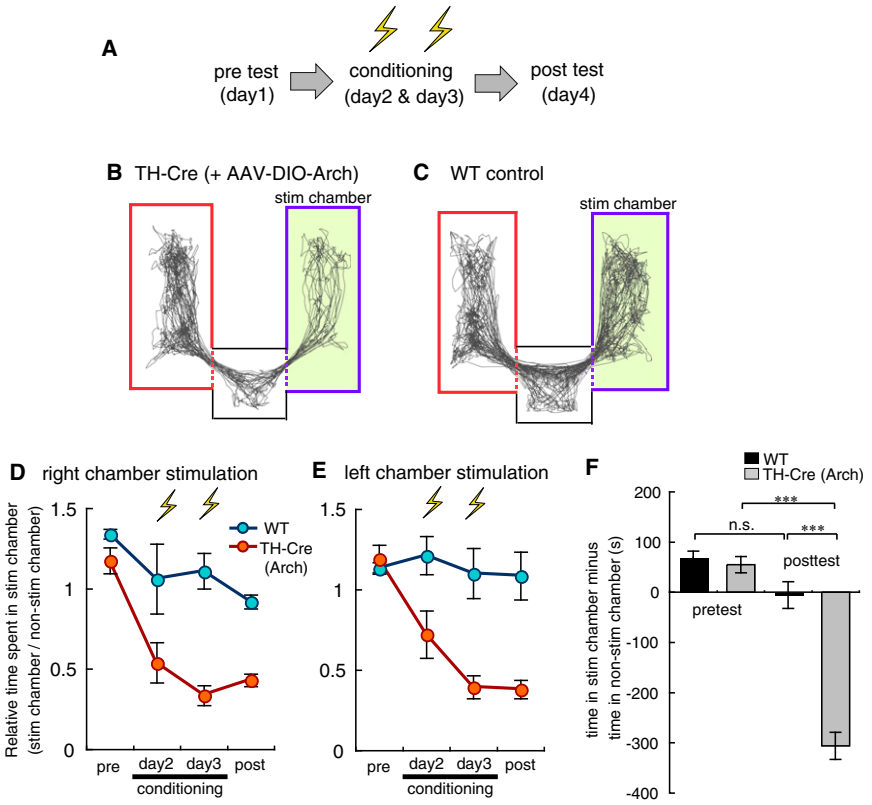

Fig. S6. Optogenetic inactivation of VTA DA neurons evokes conditioned place aversion (CPA) in the three-chamber CPA test. (*A*) Time schedule of the CPA test. During conditioning, mice received optical stimulation when they stayed in a selected chamber. (*B* and *C*) Representative traces of a TH-Cre mouse (*B*) and a WT mouse (*C*) recorded in the posttest. The stimulated chamber is colored in green. (*D* and *E*) Time course of time spent in the stimulated chamber relative to that in the nonstimulated chamber in the CPA test. The right and left chambers were used for optical stimulation in *D* and *E*, respectively. The TH-Cre mice significantly avoided the stimulated chamber in the CPA test [$F_{3,21} = 3.14$, $P = 0.0468$, $n = 4$–5 (*D*) and $F_{3,18} = 7.31$, $P = 0.0021$, $n = 4$ (*E*)]. (*F*) Time spent in the stimulated chamber minus time spent in the nonstimulated chamber. Data from stimulation at the right and left chambers were combined. The TH-Cre mice avoided staying in the stimulated chamber in the posttest ($F_{3,30} = 68.51$, $P < 0.0001$; post hoc test, n.s., not significant, ***$P < 0.001$, $n = 8$–9).

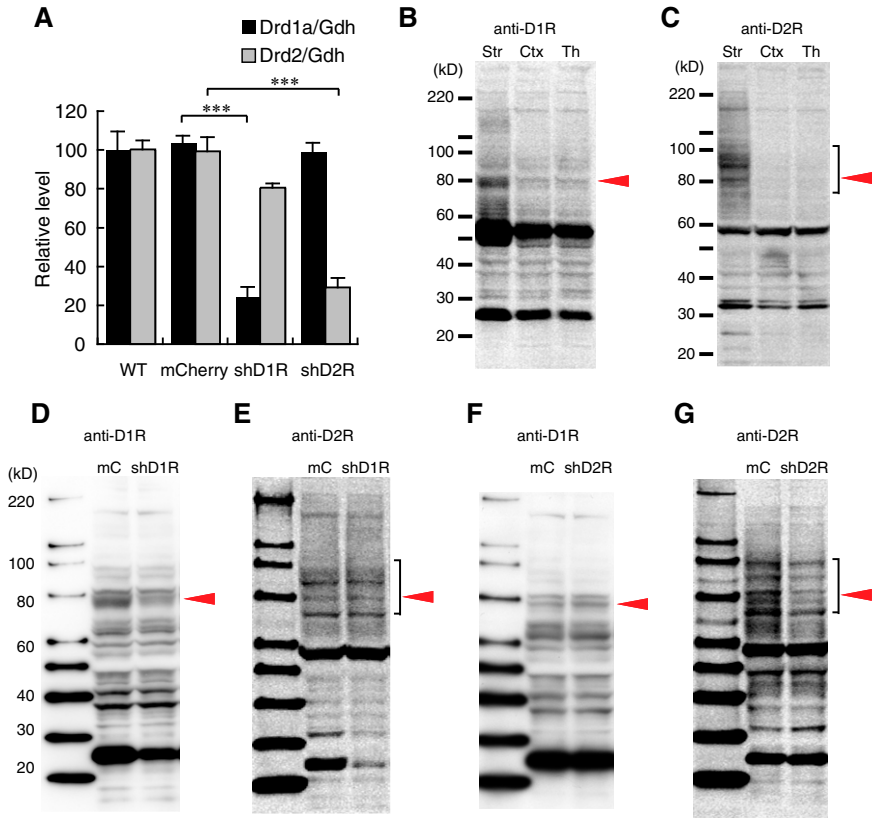

Fig. S7. Efficient knockdown of D1R and D2R with shRNA-coding lentivirus injection in the NAc. (*A*) Quantitative analysis of Drd1a and Drd2 mRNA levels in the NAc after lentivirus injection. Levels were normalized with Gapdh (Gdh) mRNA. The Drd1a level was decreased to 24.4 ± 5.0% in the shD1R-treated mice ($F_{3,8} = 42.54$, $P < 0.0001$; post hoc test, $^{***}P < 0.001$, $n = 3$); and the Drd2 level, to 29.3 ± 4.8% in the shD2R-treated mice ($F_{3,8} = 41.63$, $P < 0.0001$; post hoc test, $^{***}P < 0.001$, $n = 3$). (*B* and *C*) Photographs of full-length immunoblots with anti-D1R (*B*) and anti-D2R (*C*) from a Lenti:mCherry-injected mouse. Str, Ctx, and Th denote samples from the striatum, cortex, and thalamus, respectively. Dense bands around 80 kDa in *B* and 80–100 kDa in *C*, as indicated by arrowheads, were specific for D1R and D2R, respectively. (*D*–*G*) Photographs of full-length immunoblots with anti-D1R (*D* and *F*) and anti-D2R (*E* and *G*) from Lenti:mCherry (mC)- and Lenti:shD1R-injected NAc (*D* and *E*) and from Lenti:mCherry- and Lenti:shD2R-injected NAc (*F* and *G*). Arrowheads indicate bands specific for D1R (*D* and *F*) and D2R (*E* and *G*).