Aversive behavior induced by optogenetic inactivation of ventral tegmental area dopamine neurons is mediated by dopamine D2 receptors in the nucleus accumbens

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Dopamine (DA) transmission from the ventral tegmental area (VTA) is critical for controlling both rewarding and aversive behaviors. The transient silencing of DA neurons is one of the responses to aversive stimuli, but its consequences and neural mechanisms regarding aversive responses and learning have largely remained elusive. Here, we report that optogenetic inactivation of VTA DA neurons promptly down-regulated DA levels and induced up-regulation of the neural activity in the nucleus accumbens (NAc) as evaluated by Fos expression. This optogenetic suppression of DA neuron firing immediately evoked aversive responses to the previously preferred dark room and led to aversive learning toward the optogenetically conditioned place. Importantly, this place aversion was abolished by knockdown of dopamine D2 receptors but not by that of D1 receptors in the NAc. Silencing of DA neurons in the VTA was thus indispensable for inducing aversive responses and learning through dopamine D2 receptors in the NAc.

The mesolimbic dopaminergic system not only plays a pivotal role in a wide range of motivation and learning (1–3), but its dysfunction has also been implicated in severe neuropsychiatric disorders as exemplified in Parkinson disease, schizophrenia, and drug addiction. Dopamine (DA) neurons in the ventral tegmental area (VTA) react to rewarding stimuli by phasic firing, and the main function of this firing is theorized to encode “the reward prediction error,” the difference in the value between the expected reward and the actual reward (4). In contrast to the response to rewarding stimuli, their reactions to aversive stimuli are far from homologous; i.e., some DA neurons are activated in response to aversive stimuli, whereas most others react by transiently suppressing their firings (5–9). In fact, recent studies have revealed that optogenetic activation of GABAergic neurons and resultant inactivation of DA neurons suppress reward consumption and induce an aversive response (10, 11). However, it has largely remained elusive as to which mechanisms in the neural circuits are essential for the acquisition of aversive learning following the inactivation of DA neurons in the VTA and as to how behavioral responses are controlled toward suppressing reward consumption and inducing aversive behaviors.

Accumulated evidence has revealed that the motivational and cognitive learning in response to positive and negative stimuli is largely regulated by the neural circuits including the basal ganglia (12), which receive a large amount of the dopaminergic projection from the midbrain. In the striatum, two fundamental neural circuits are constituted by specified medium-sized spiny neurons (MSNs), each expressing a distinct type of DA receptor (13). One circuit is the direct pathway, consisting of the MSNs directly projecting to the output nuclei of the basal ganglia, substantia nigra pars reticulata (SNr), and predominantly expressing dopamine D1 receptors (D1Rs). The other is the indirect pathway, consisting of the MSNs that project indirectly through the globus pallidus to the SNr and primarily express dopamine D2 receptors (D2Rs). DA signals from the midbrain dynamically modulate these two parallel pathways in the opposite manner via D1Rs and D2Rs, and this modulation is supposed to facilitate motivational learning (3, 14). As for the rewarding stimuli, up-regulated DA levels induced by rewarding signals are considered to activate the D1Rs and thus predominantly facilitate the direct pathway in the nucleus accumbens (NAc). On the other hand, the suppression of DA neuron firings in response to aversive stimuli decreases DA levels in the NAc, and this reaction is supposed to specifically promote the signal transmission in the indirect pathway through activated D2Rs.

Although studies using the pharmacological strategies and reversible neurotransmission blocking (RNB) method have supported this mechanism of regulation in the NAc (15, 16), it has remained unknown whether the suppression of DA neuron firing is sufficient to promote the activity of the indirect pathway and subsequently induce the avoidance behavior. In this present study, we addressed this issue by selectively inactivating DA neurons in the VTA by optogenetically manipulating membrane-hyperpolarizing Arch protein (17) and explicitly demonstrated that the suppression of DA neurons in the VTA subsequently decreased DA levels in the NAc and induced aversive reaction and learning. Furthermore, we investigated the mechanisms of the regulation of this reaction and disclosed that this aversive reaction was specifically controlled by D2Rs in the NAc.

**Results**

**Optogenetic Inactivation of DA Neurons Blocks Dark-Room Preference.** To selectively inactivate firings of DA neurons, we injected a Cre-inducible adeno-associated viral construct encoding Arch-eGFP [AAV-double-floxed inverted open reading frame (DIO)-Arch] (17) unilaterally into the VTA of adult tyrosine hydroxylase (TH)-Cre mice (18) and wild-type (WT) littermates and placed an optical fiber above the VTA (Fig. S1A and C). Two weeks after surgery, optogenetically controlling DA neurons in the VTA and found that the inactivation of DA neurons resulted in aversive response and learning. The nucleus accumbens (NAc), the major output nuclei of VTA DA neurons, was considered to be responsible for this response, so we examined which of the fundamental pathways in the NAc was critical to this behavior by using knockdown of D1 or D2 receptor, and found that the D2 receptor-specific pathway was crucial for this behavior.

**Significance**

Dopamine (DA) neurons in the ventral tegmental area (VTA) react to aversive stimuli mostly by transient silencing. It remains unclear whether this reaction directly induces aversive responses in behaving mice. We examined this question by optogenetically controlling DA neurons in the VTA and found that the inactivation of DA neurons resulted in aversive response and learning. The nucleus accumbens (NAc), the major output nuclei of VTA DA neurons, was considered to be responsible for this response, so we examined which of the fundamental pathways in the NAc was critical to this behavior by using knockdown of D1 or D2 receptor, and found that the D2 receptor-specific pathway was crucial for this behavior.

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Arch-eGFP was restrictedly detected in the VTA (Fig. S1B). We tested the hyperpolarizing effect of the Arch protein by electrophysiological recording and measured the effect of optical stimulation of the VTA of TH-Cre mice injected with AAV-DIO-Arch. In vivo electrophysiological recordings from the VTA of anesthetized TH-Cre mice revealed that optical stimulation of putative DA neurons inhibited their firings (Fig. S2), indicating that the optical stimulation sufficiently hyperpolarized the membrane potential of Arch-expressing DA cells and thus inhibited their spontaneous firing.

By using these mice, we next examined whether the optical inactivation of DA neurons in the VTA could serve as an aversive signal for behavioral learning. Mice possess an innate tendency to prefer a dark environment (19). We designed a behavioral apparatus in which mice could freely explore the dark room and open bright space (Fig. 1A). After habituation, the WT mice stayed preferentially in the dark room either with or without optical stimulation in the dark room (Fig. S1D), ensuring that optical stimulation itself had no influence on their dark-room–preferring behavior. We scheduled the behavioral experiment of animals to test the effect of optical inactivation of DA neurons on their behavior (Fig. S1E). After habituation and pretest, mice were conditioned by optically stimulating the DA neurons in the VTA when they stayed in the dark room. Even during the first 5 min of conditioning, the TH-Cre mice stayed out of the previously preferred dark room and successively avoided the dark room throughout the conditioning (Fig. 1B). The TH-Cre mice did not reverse their avoidance against the dark room even though they received no optical stimulation at the posttest (Fig. 1C). These data indicate that hyperpolarization of DA neurons not only induced transient aversive behavior but also served as a signal for aversive learning against the dark room and also demonstrate that the inactivation of DA neurons played a causal role in both transient aversive behavior and prolonged aversive learning.

**Optogenetic Down-Regulation of DA Levels in the NAc.** We next investigated whether the inactivation of DA neurons in the VTA actually modified the concentration of DA in its major targeting region, the NAc. We measured DA levels in the NAc by fast-scan cyclic voltammetry (FSCV) in anesthetized TH-Cre mice that had been injected with AAV-DIO-Arch into their VTA. DA levels in the NAc were promptly elevated by electrical stimulation of the VTA, and the evoked DA release was significantly reduced by simultaneous optical stimulation of the VTA (Fig. S3). We then tested whether optical stimulation of VTA could reduce the tonic DA level in the NAc. In the same experimental settings, we observed that the DA level in the NAc was transiently decreased by 20 s of optical stimulation of the VTA (Fig. 2), which is consistent with the reported FSCV reaction against the aversive stimuli (20). These data demonstrate that optical stimulation of the VTA was effective enough to inactivate the VTA DA neurons and to diminish the DA level in the NAc during the behavioral experiment.

**Up-Regulation of Fos Gene Expression by Optical Inactivation of DA Neurons in the VTA.** The behavioral change caused by conditioned inactivation of DA neurons in the VTA suggested that optical stimulation directly altered neural activity and resulted in the shift of behavioral performance. So we next investigated the regions in which neural activity was elevated by the conditioned inactivation of DA neurons by examining the expression of Fos, an immediate early gene. Soon after the conditioning was performed in the dark-room test, mice were quickly processed to determine the amount of Fos expression by quantitative in situ hybridization analysis (Fig. 3 and Fig. S4). The NAc, the region that receives a large amount of dopaminergic projections from the VTA, showed a significantly increased amount of Fos expression in the TH-Cre mice (Fig. 3). This up-regulation was also detected in the contralateral side of optical stimulation, which was supposedly caused by a small amount of virus infection into that side. However, the up-regulation was much higher at the ipsilateral side than at the contralateral side of optical stimulation, suggesting that optical inactivation of DA neurons directly up-regulated the neural activity of the NAc. The increased Fos expression was also observed in other brain regions including the septum, periventricular regions of the striatum, basolateral amygdala (BLA), and lateral hypothalamus, but not in the lateral habenula or medial prefrontal cortex (mPFC; Fig. S4). These results indicate that the regions activated by optical inactivation of DA neurons were not restricted to the direct target regions of VTA DA neurons, but rather included the regions that could be indirectly activated in a neural circuit-dependent manner. This observation suggests that optical inactivation of DA neurons modified circuit-wide neuronal activity and could not only evoke an aversive reaction but also trigger several other brain functions such as anxiety, fear, and stress responses (21).

**DA Signaling Through D2R Is Critical for Optogenetically Induced Conditioned Place Aversion.** The majority of dopaminergic signals from the VTA are transmitted to the MSNs in the NAc through DA receptors, D1R and D2R. D1R is almost exclusively expressed in the substance P (coded by Tac1 gene)-expressing...
We then attempted to specify DA receptor subtypes involved in this aversive behavior by specifically suppressing each of the DA receptors in the NAc (Fig. 4 and Fig. S7). We designed and validated lentiviral vectors containing short hairpin RNA (shRNA) specific for each DA receptor with constitutive expression of mCherry. Three weeks after injecting the lentivirus into the NAc, robust expression of mCherry was localized in the NAc (Fig. 4B). The effective knockdown of mRNA expression of each receptor was confirmed by quantitative real-time PCR analysis (Fig. S7A). Measuring protein levels through Western blotting also revealed that injection of each of the lentiviruses selectively reduced its target protein product without affecting the expression of the other subtype of DA receptor (Fig. 4C and Fig. S7 B–G). The shD1R- and shD2R-expressing lentiviruses decreased their target protein level to 46.2 ± 1.1% and 38.4 ± 4.9%, respectively, compared with the level for the control virus (Fig. 4C). These results verified that the lentiviral vectors expressing shRNA specific for D1R and D2R selectively and sufficiently suppressed their target RNAs and down-regulated the amount of the respective protein products. We also confirmed that the virus-mediated expression of mCherry was not detected in the VTA, excluding the possibility that the lentivirus-mediated shRNA directly affected the VTA.

Using these lentiviruses containing shRNA, we tested which type of DA receptor was responsible for the aversive behavior induced by optogenetic inactivation of DA neurons. We injected shRNA-containing lentivirus or control lentivirus into the bilateral NAc together with AAV-DIO-Arch into the left VTA of the TH-Cre mice. The optical fiber was also inserted above the lateral NAc together with AAV-DIO-Arch into the right VTA of the TH-Cre mice. The optical fiber was also inserted above the VTA (Fig. 4A). When the three-chamber CPA test was conducted at three weeks after surgery, the TH-Cre mice injected with lentishD1R-mCherry still showed explicit CPA against the optical stimulation-paired chamber comparable to that of the TH-Cre mice injected with the control lentivirus (lenti:mCherry). In contrast, the TH-Cre mice injected with lentishD2R-mCherry failed to show obvious CPA during conditioning (Fig. 4D). The exclusive learning deficit of the TH-Cre mice injected with lentishD2R-mCherry was further substantiated by analysis of aversive learning at the posttest (Fig. 4E). These results demonstrate that the aversive behavior to the place conditioned by the DA neuron inactivation was specifically evoked through D2R, and not through D1R, in the NAc.

**Discussion**

In the striatum, studies have revealed that activation of the Gs-coupled D1R facilitates its firing, whereas activation of the Gi-coupled D2R results in suppressed firing efficiency (25). According to the specificity of DA receptor expression, phasic firings of DA neurons mainly activate the direct pathway through D1R, whereas a transient decrease in DA neuron firings predominantly promotes the indirect pathway competency through
D2R (3, 26). Based on this mechanism of regulation, it has been proposed that silencing of DA neurons in response to aversive stimuli is mainly processed through the indirect pathway and results in aversive behavior (3). Recent studies have shown that blockade of the synaptic transmission of the indirect pathway impairs the acquisition of aversive behavior elicited by an electric shock (15) and that this impairment is caused by the inhibition of D2R-mediated signal transmission (16). In addition, the optogenetic up-regulation of D2R-expressing MSNs in the indirect pathway evokes behavioral avoidance (27). However, because DA neurons exhibit both enhanced and suppressed firings in response to aversive stimuli and because other shock-related sensory information is simultaneously processed in the brain, it still remains to be clarified whether silencing of DA neurons could directly trigger aversive reaction and learning, and whether this reaction is regulated through D2R-expressing MSNs in the indirect pathway.

In this study, we used optogenetic control of DA neuron firings in the two behavioral tests: the dark-room preference test and three-chamber CPA test. Our optogenetic manipulation showed efficient suppression of DA neuron firings in the VTA and down-regulation of DA levels in the NAc. Our precise optogenetic inactivation of DA neuron firings only during the period that the animals stayed in the conditioned chamber explicitly evoked an aversive reaction and learning, demonstrating that transient DA silencing directly caused passive avoidance behavior. Furthermore, this investigation has elucidated that D2R-mediated signal processing is a key determinant for the induction of this aversive reaction and learning.

Although our data demonstrated that D1R had no effect in the behavioral experiments to evoke the CPA, several studies have documented that phasic firing of DA neurons is required for fear responses and aversive learning (28, 29). This difference is due to the experimental setting: i.e., our optogenetic approach excluded the possibility of the signaling through activated DA neurons to evoke aversive behavior, indicating that inactivating DA neurons was sufficient to induce aversive behavior and learning. The function and signal processing of the activated DA firing evoked by aversive stimuli would have different contributions to aversive behaviors from those studied here and need to be clarified in the future.

DA neurons also project to various other regions including the mPFC, amygdala, and hippocampus. A recent study indicated that optogenetic activation of lateral habenula neurons projecting to DA neurons in the VTA are capable of inducing aversive behavior, and these DA neurons mainly and specifically target to the mPFC (30), although their optogenetic conditioning was different from that in our current study, as their optogenetic stimulation was prolonged for a whole conditioning session. Because the dopaminergic input to the mPFC has been reported to be activated not only by aversive stimuli but also by chronic stress (31, 32), it is possible that their continuous activation of mPFC-projecting DA neurons would be perceived as signals from a highly stressful environment; and, as a result of the
accumulation of stressful conditioning, the animals would show aversive behavior to the conditioned chamber. By contrast, we inhibited firing of DA neurons only while the animals were staying in the conditioned chamber. The results of our behavioral experiments using timing-matched conditioning indicated that a sudden suppression of DA signal would be perceived as a sudden aversive input, which resulted in their quick aversive response. 

DA neurons also project to the amygdala, the region that largely contributes to the fear response. Indeed, the DA signaling to the amygdala has been implicated in the fear response and acquisition of fear memory (33, 34). In our study, labeling DA neurons in the VTA identified a set of DA neurons projecting to the BLA, but the extent of these projections was much lower than that projecting to the NAc. Although we could not exclude a subtle effect of amygdala-projected DA signaling on our observed aversive behavior, the main effect of our optogenetic inactivation of DA neurons should be on the NAc, because our experiments with specific knockdown of the D2R in the NAc dramatically diminished the aversive behavior. Future investigations addressing target-specific DA signaling are required to elucidate the effects of circuit-wide modification of DA neurons on the aversive stimuli and fear conditioning.

Materials and Methods

Subjects. Tyrosine hydroxylase::Cre (TH-Cre) knock-in mice (EM:00254) (18) were obtained from the European Mouse Mutant Archive. All experimental animals had been backcrossed to the C57BL/6J strain for more than 10 generations. Mice were mated with the C57BL/6J WT mice and housed with mental animals had been backcrossed to the C57BL/6J strain for more than 10 months. The position of a mouse was detected by a video camera suspended over the behavioral apparatus and analyzed by a custom-made program using Labview software.

Behavioral Tests. During all behavioral tests, mice were connected with an optical fiber and allowed to move around the entire apparatus. The movement of mice was monitored so that they could move around without any obstacles even when they were connected with an optical fiber on their heads. The position of a mouse was detected by a video camera suspended over the behavioral apparatus and analyzed by a custom-made program using Labview software.

Dark-room preference test. The custom-made behavioral apparatus used in the test was composed of a dark room (15 × 9.5 cm) and a bright open space (15 × 11 cm). The dark room had walls, a floor, and a roof, which were all colored in black and had an entrance (4.5 cm long) to the open bright space. The open bright space was shaped like an ellipse and had a metal grid floor and clear walls without a roof. Before the test, all mice were habituated for 10 min in the apparatus. The test consisted of three sessions: on the early half of day 1 (pretest: 5 min), mice were allowed to explore the entire apparatus. From the late half of day 1 to day 4 (conditioning: 35 min in total), mice received optical stimulation when they stayed in the dark room. On day 5, the dark-room preference test was performed without optical stimulation (posttest: 5 min; Fig. S1D).

Three-chamber CPA test. The custom-made three-chamber conditioned place preference/CPA apparatus used in the test was composed of two chambers (10 × 17 cm) and a connecting corridor. The test consisted of three sessions. Day 1 (pretest: 15 min): Mice were allowed to freely explore the entire apparatus. The mice that stayed 1 s longer in one chamber than in the other were excluded from the test. Days 2 and 3 (conditioning: 15 min each): Mice received optical stimulation when they stayed in the light-paired chamber. The selection of the light-paired chamber was counterbalanced. Day 4 (posttest: 15 min): The test was conducted under the same conditions as in the pretest (Fig. S6A).

In the conditioning session, the optical stimulation was stopped for 30 s when the mice continuously stayed over 30 s in the dark room or the light-paired chamber to avoid overheating. Laser power was controlled to be approximately 5 mW at the tip of the optical fiber in all behavioral tests.

In Vivo Fast-Scan Cyclic Voltammetry. FSCV experiments were conducted by using the method described in previous studies (35–37). Mice were anesthetized with a ketamine/xylazine mixture as described in SI Materials and Methods and placed in a stereotactic frame. An optical fiber used for stimulating Arch-expressing DA neurons was located close to the stimulating electrode. The stimulating optrode was then placed in the VTA (from bregma: anterior−posterior, −3.2 mm; lateral, 0.5 mm; and dorsal−ventral, 3.5 mm) and lowered at 0.25-mm intervals. A carbon−fiber microelectrode (300 µm in length) for voltammetric recording was lowered into the NAc (from bregma: anterior−posterior, −1.0 mm; lateral, 1.0 mm; and dorsal−ventral, 3.5 mm). Voltammetric measurements were made every 100 ms by applying a triangle waveform (−0.4 V to +1.3 V to −0.4 V versus Ag/AgCl, at 400 V/s) to the carbon-fiber microelectrode. A custom-made potentiostat was used for waveform isolation and current amplification. DA release was evoked by electrical stimulation of DA neurons by using 24-pulse stimulation (100 µA, 5 ms duration, 30 Hz). An optical stimulation of DA neurons (532 nm, ~5 mW power at the fiber tip) was applied for 10 s starting 5 s before the onset of an electrical stimulation. Carbon−fiber microelectrodes were calibrated in a solution with known concentrations of DA (0.2 µM, 0.5 µM, and 1.0 µM). All voltammetry data were analyzed by custom-made programs using Labview and Matlab software. Reduction in DA levels by optical stimulation was resolved with principal component analysis, by using the template DA waveforms obtained from electrical VTA stimulations to separate dopamine signals (35, 36).

Statistical Analysis. Statistical analysis was conducted by using GraphPad Prism 5.0 (GraphPad Software). Data were analyzed by repeated measures ANOVA (Figs. 1B, 4D, and Fig. S6 D and E) or one-way ANOVA (Figs. 1C, 3D, 4C and E, and Figs. S4 K−M, S6F, and S7A), and post hoc analyses were done by using the Bonferroni test. All bars/columns and bars represented the mean and ± SEM, respectively.

Other experimental procedures including virus preparation and injection, electrophysiological recording, and immunohistochemical and mRNA analysis are described in detail in SI Materials and Methods.

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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 virus preparation vector carrying the EF1α 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 EF1α promoter and the WPRE. The following shRNA sequences were used: Drd1a, 5′-GCAATGATCTCCATACCTGAGCAAGGTTGATG-GAATCAATGC3′ and Drd2, 5′-GAATGACTAACAGATTTGTCACATCCAGAGGATGACATTCTGGCATGC3′.

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, 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 (LentimCherry, Lentish1DR-RCmCherry or LentshD2R-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 recording electrode by using a double-electrode holder. The optic fiber 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 PBS 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), TacI (GenBank NM_009311, nucleotides 95–995), Penk (GenBank NM_01002927, nucleotides 303–1,109), Drd1a (GenBank NM_0010076, 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 amplification-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% H₂O₂, 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′-CTCCTGTTTTACCTGATCCCTCA-3′ and 5′-GCC-TCTCCCTCTCTCATGTT-3′; Drd2, 5′-TGAAACAGCGCGAGAATGGG-3′ and 5′-CTGGTCTTGAGCACTATCTC-3′; and Gapdh, 5′-TGACCAACAGTTCCATGCTAC-3′ and 5′-GACGG-ACACATTGGGGGTAG-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, 1x Complete Protease Inhibitor mixture (Roche), and 1x 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).


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 (t6 = 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 ($E_{\text{app}}$ 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 $\sim 0.650$ 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. t₄ = 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. t₄ = 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, $F_{3,21} = 3.14, P = 0.0468, n = 4-5$ (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).