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

Teruko Danjo[a], Kenji Yoshimi[b], Kazuo Funabiki[a], Satoshi Yawata[a], and Shigetada Nakanishi[a,1]

[a]Department of Systems Biology, Osaka Bioscience Institute, Suita, Osaka 565-0874, Japan; and [b]Department of Neurophysiology, Juntendo University School of Medicine, Bunkyo-ku, Tokyo 113-0821, Japan

Contributed by Shigetada Nakanishi, March 23, 2014 (sent for review January 24, 2014)

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 predicted 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. S1 *A* and *C*). Two weeks after surgery,

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.

Author contributions: T.D. and S.N. designed research; T.D. performed research; T.D., K.Y., K.F., and S.Y. contributed new reagents/analytic tools; and T.D., S.Y., and S.N. wrote the paper.

The authors declare no conflict of interest.

[1]To whom correspondence should be addressed. E-mail: snakanis@obi.or.jp.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1404323111/-/DCSupplemental.

Arch-eGFP was restrictedly detected in the VTA (Fig. S1*B*). 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. 1*A*). After habituation, the WT mice stayed preferentially in the dark room either with or without optical stimulation in the dark room (Fig. S1*D*), 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. S1*E*). 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. 1*B*). The TH-Cre mice did not reverse their avoidance against the dark room even though they received no optical stimulation at the posttest (Fig. 1*C*). 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

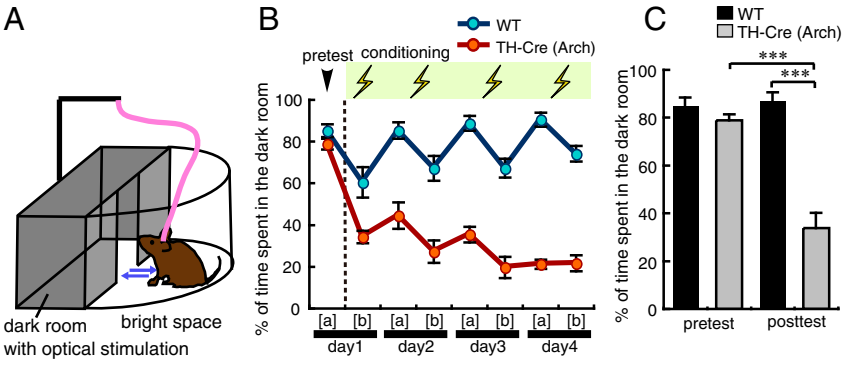

Fig. 1. Optogenetic inactivation of DA neurons blocks dark-room preference of freely behaving mice. (*A*) An illustration of the apparatus used in the dark-room preference test. Mice were allowed to freely move around the dark room and bright space. (*B*) Time course of the dark-room preference test. *a* and *b* in the graph denote the data from the first 5 min and the last 5 min of each day, respectively. TH-Cre mice showed significantly different behavioral performance during conditioning compared with WT ($F_{9,90} = 9.77$, $P < 0.0001$, $n = 6$). (*C*) Time spent in the dark room at pretest and posttest. TH-Cre animals showed a significant reduction in staying time in the dark room at the posttest ($F_{3,20} = 35.84$, $P < 0.0001$; post hoc test, ***$P < 0.001$, $n = 6$).

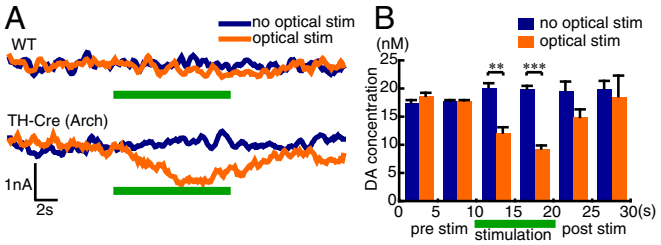

Fig. 2. Optical inactivation of DA neurons in the VTA reduces DA level in the NAc. (*A*) Averaged DA responses to optical stimulation in the NAc as measured by FSCV. The green line indicates the duration of optical stimulation ($n = 7$–11 traces). (*B*) Averaged DA levels determined every 5 s. The DA level was significantly decreased during optical stimulation ($t_4 = 7.03$, $^{**}P < 0.01$ during 10–15 s; $t_4 = 14.80$, $^{***}P < 0.001$ during 15–20s; *t* test, $n = 3$ mice, 45 traces in total).

MSNs, and D2R is predominantly expressed in the enkephalin (coded by Penk gene)-expressing MSNs; each type of MSNs constitutes the direct and indirect pathways, respectively, in the NAc (3). As the affinity for DA is much higher for the D2R (nM order) than for the D1R (μM order) (22, 23), a reduction in DA levels is thought to result in the inactivation of G_i-coupled D2R but to have no appreciable effect on the D1R (3, 24), thereby up-regulating the neural activity specifically in the indirect pathway. Moreover, the Fos activation was more prominently observed in Penk- or Drd2 (D2R)-expressing cells than in the Tac1- or Drd1a (D1R)-expressing cells (Fig. S5). Based on these observations, we hypothesized that DA signaling through D2R could play a major role in the observed aversive conditioning.

To test this hypothesis, we performed the three-chamber conditioned place aversion (CPA) test (Fig. S6). We prepared a behavioral apparatus containing two chambers with virtually identical circumstances and one small corridor. This unbiased environmental condition in the CPA test enabled us to further examine whether the inactivation of VTA DA neurons is capable of inducing aversive reaction and learning, in addition to blocking the dark room preference. When animals were allowed to freely move around the entire apparatus, most of them stayed in two chambers without any typical behavioral difference at pretest. The optical conditioning was then performed by pairing optical stimulation with one fixed chamber. Even when either of the chambers was used for the conditioning, the TH-Cre mice persistently and significantly avoided staying in the optically conditioned chamber during the conditioning and at the posttest (Fig. S6 *B–E*). Statistical analysis validated a significant reduction in staying time of the TH-Cre mice in the optically conditioned chamber at the posttest compared with the staying time for the WT mice (Fig. S6*F*).

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. 4*B*). The effective knockdown of mRNA expression of each receptor was confirmed by quantitative real-time PCR analysis (Fig. S7*A*). 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. 4*C* 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. 4*C*). 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 VTA (Fig. 4*A*). When the three-chamber CPA test was conducted at three weeks after surgery, the TH-Cre mice injected with lenti:shD1R-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 lenti:shD2R-mCherry failed to show obvious CPA during conditioning (Fig. 4*D*). The exclusive learning deficit of the TH-Cre mice injected with lenti:shD2R-mCherry was further substantiated by analysis of aversive learning at the posttest (Fig. 4*E*). 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 G_s-coupled D1R facilitates its firing, whereas activation of the G_i-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

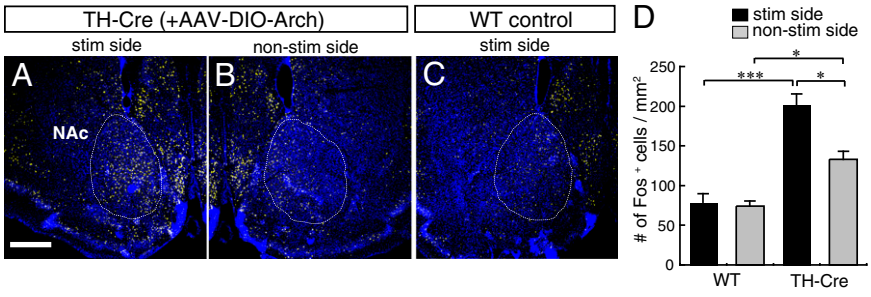

Fig. 3. Activity-related expression of Fos gene induced by optogenetic DA neuron inactivation. (*A–C*) Representative photographs for Fos expression (yellow) in the NAc. Pictures were taken of the stimulated side of a TH-Cre mouse (*A*), of the nonstimulated side of it (*B*), and of the stimulated side of a WT control (*C*). (*D*) Number of Fos^+ cells per square millimeter in the NAc ($F_{3,8} = 31.19$, $P < 0.0001$; post hoc test, $^{*}P < 0.05$, $^{***}P < 0.001$, $n = 3$). (Scale bar, 500 μm.)

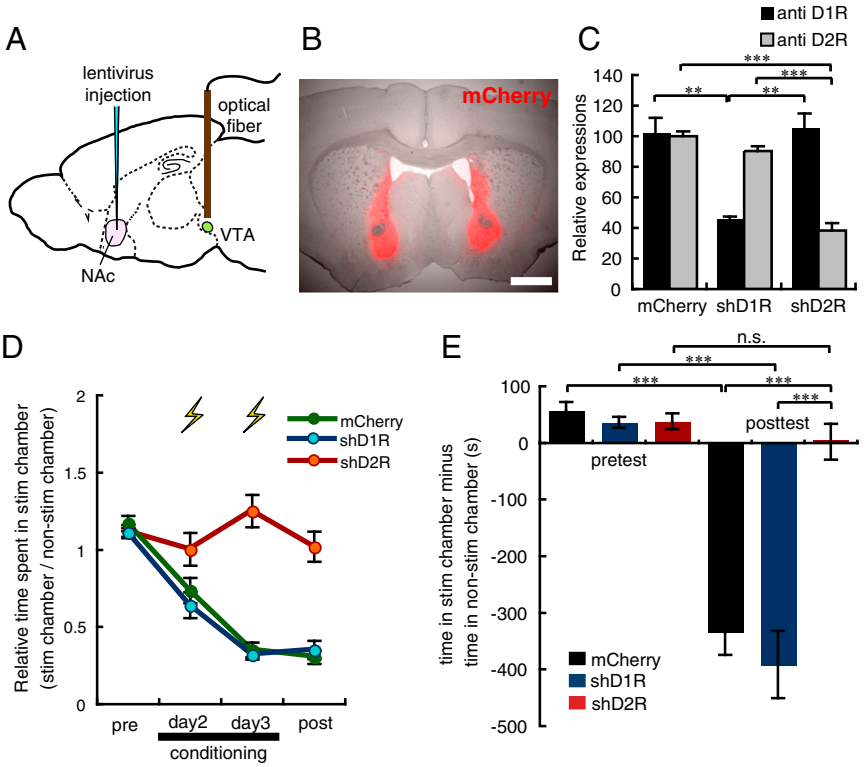

Fig. 4. DA signaling through D2R is critical for optogenetically induced CPA. (*A*) An illustration showing the surgical procedure. The shRNA-encoding lentivirus for D1R or D2R was injected bilaterally into the NAc. AAV-DIO-Arch was injected unilaterally into the VTA. (*B*) Lentivirus-induced mCherry expression (red) in the NAc. (Scale bar, 1 mm.) (*C*) Relative level of D1R and D2R proteins quantified by densitometry of Western blots. D1R and D2R protein levels were significantly decreased by the respective shD1R ($F_{2,7} = 15.84$, $P < 0.01$; post hoc test, $^{**}P < 0.01$, $n = 3$–4) and shD2R ($F_{2,7} = 68.17$, $P < 0.0001$; post hoc test, $^{***}P < 0.001$, $n = 3$–4). mCherry implies samples with Lenti:mCherry injection, and shD1R and shD2R denote samples with Lenti:shD1R-mCherry and Lenti:shD2R-mCherry injection, respectively. (*D*) Time course of time spent in the stimulated chamber relative to that in the nonstimulated chamber in the CPA test. TH-Cre mice injected with shD2R-containing lentivirus showed significantly different behavioral performance ($F_{6,66} = 13.96$, $P < 0.0001$, $n = 8$–9). (*E*) Time spent in the stimulated chamber was subtracted from that in the nonstimulated chamber. Lenti:mCherry- or Lenti:shD1R-injected TH-Cre mice showed significantly reduced staying in the stimulated chamber at the posttest, whereas Lenti:shD2R-injected TH-Cre mice showed a significant difference in behavior compared with Lenti:mCherry- or Lenti:shD1R-injected TH-Cre mice ($F_{5,44} = 39.30$, $P < 0.0001$; post hoc test, $^{***}P < 0.001$, n.s., not significant, $n = 8$–9).

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 optogenic 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::IRES-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 a standard 12-h light/12-h dark cycle and given food and water ad libitum. Cre^+ and Cre^- mice from the same litters (3–6 mo of age) were used for the experiments. All animal experiments were approved by the animal committee of Osaka Bioscience Institute under the guidelines of animal experiments.

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 was tested without optical stimulation (posttest: 5 min; Fig. S1*E*).

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. Mice that stayed 1.5 times 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. S6*A*).

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 stereotaxic 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. 1*B*, 4*D*, and Fig. S6 *D* and *E*) or one-way ANOVA (Figs. 1*C*, 3*D*, 4 *C* and *E*, and Figs. S4 *K–M*, S6*F*, and S7*A*), and post hoc analyses were done by using the Bonferroni test. All marks/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*.

ACKNOWLEDGMENTS. We thank E. Boyden for the Arch construct, R. Matsui for technical advice in lentivirus production and purification, and Y. Hayashi for technical advice in the programming of data analysis. This work was supported by Research Grants-in-Aid 22220005 (to S.N.), 23120011 (to S.Y. and S.N.), 24700339 (to T.D.), and 25871080 (to S.Y.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and a grant from Takeda Science Foundation (to S.N.).

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