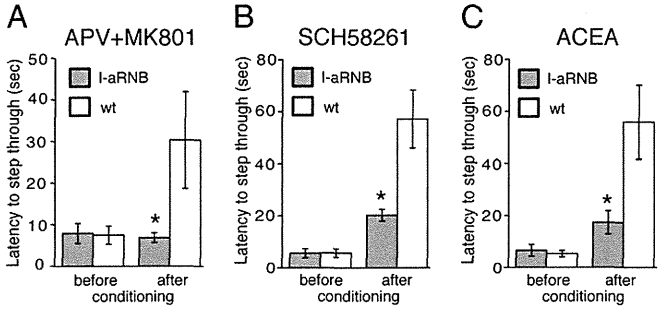

Fig. 5. Aversive learning by activation of NMDA and A2a receptors and inhibition of CB1 receptors in the indirect pathway. Twenty minutes after injection of NMDA receptor antagonists APV and MK801 (*A*), A2a receptor antagonist SCH58261 (*B*), or a CB1 receptor agonist ACEA (*C*), latencies to step through from the lighted chamber to the preferred dark chamber were measured in mice before conditioning with electric shocks. They were then subjected to electric shocks at the dark chamber; latencies for the mice to enter the dark chamber were measured 24 h after shocking ($n = 5$ each). Columns and bars represent the mean ± SEM, respectively. I-aRNB vs. WT, $*P < 0.05$.

inactivation of D2 receptors and selectively disrupted aversive learning (Fig. 6*B*). D2 receptors are located both presynaptically and postsynaptically, but the D2L receptors are located postsynaptically (23, 33). The D2L deficiency has been shown to abolish postsynaptic responses (21, 22) but keep presynaptic functions intact, including transmission by GABA, glutamate, and DA in the NAc (23, 33–36). In the present investigation, the D2L knockout mice exhibited impaired aversive learning. Thus, taking into account impairments of the D2 agonist-treated I-aRNB mice, the results indicated that the postsynaptic D2 receptors in the NAc play a pivotal role for induction of aversive learning. Furthermore, consistent with our proposed model, previous studies have indicated that D2L knockout mice retain the normal ability for CPP after repeated cocaine administration (37, 38). Importantly, previous electrophysiological studies have indicated that several key neurotransmitter receptors play a critical role in inducing LTP in glutamatergic transmission in the indirect pathway (10, 11). Our behavioral study explicitly demonstrated that these LTP-evoking receptors, when pharmacologically manipulated, all prevented aversive learning in the I-aRNB mice. Our model thus holds that aversive learning is induced by inactivation of the postsynaptic D2 receptors and then the sequential mechanistic events mediated by the key neurotransmitter receptors at the NAc circuit of the indirect pathway.

Interestingly, pharmacological manipulation by DA receptor agonist or antagonist before and after learning conditioning impaired cocaine sensitization and aversive learning, but once appetitive reward learning had been developed, such manipulation was no more effective in blocking this learning. Thus, dopaminergic synaptic modulation is required for both acquisition and expression of cocaine sensitization and aversive learning, but this modulation is specifically involved in acquisition but not expression of appetitive reward learning. The corticostriatal circuitry is functionally segregated in parallel loops conveying limbic, associative, and sensory motor information (6). Because transmission blockade was restricted to the NAc in this investigation, appetitive rewarding signals may shift from the NAc loop to other striatal loops and additional neural circuits. The difference in encoding information about abusive drugs versus natural rewards would be important for a better understanding of abnormal responses to abusive drugs. In conclusion, reward and aversive learning is controlled by the pathway-specific, segregated synaptic modulation in the NAc via selective transmitter receptors; this finding should open additional perspectives on the development of specific and efficient treatments for drug addiction and psychiatric disorders.

Materials and Methods

Animals. All animal handling procedures were approved by the animal research committees of Osaka Bioscience Institute, Kyoto University Graduate School of Medicine, and Kitasato University. The RNB and aRNB mice were generated by the respective bilateral and unilateral injection of the recombinant AAV viruses into four sites of each NAc by stereotaxic techniques (14, 17). Virus-injected WT littermates were used as controls. The heterozygous $D2L^{+/-}$ mice (23) were backcrossed to a C57BL/6 genetic background for more than six generations; homozygous $D2L^{-/-}$, heterozygous $D2L^{+/-}$, and WT mice were generated by mating the heterozygous $D2L^{+/-}$ mice.

Drug Infusion in aRNB Mice. After the recombinant virus had been unilaterally injected into the NAc of anesthetized mice, the other side of the NAc was implanted with a cannula aimed toward the NAc for drug infusion as described previously (17). The concentrations of infused drugs were 100 μM SCH23390, 300 μM SKF81297, 1 mM quinpirole, 400 μM eticlopride (all from SIGMA), 100 μM aripiprazole (Toronto Research Chemicals), 25 mM APV, 20 mM MK801 (both from Wako), 100 μM SCH58261 (Tocris), and 300 μM ACEA (abcam). After behavioral analysis, injection sites of drugs were confirmed by direct visualization of a series of slice sections of the NAc region. When the injection site was found to be outside the NAc, these data were discarded (about 2% of animals analyzed).

Behavior Tests. Behavioral analysis was started 2–3 wk after viral injection together with surgery for drug infusion. The CPP test and the one-trial inhibitory avoidance test were performed as described previously (14). Cocaine was obtained from Shionogi. Fear responses were analyzed by scoring percentages of positive freezing responses at 2-s intervals in a 1-min period following electric shocks (14). All tests of animal behaviors were conducted in a blind fashion.

Statistical Analysis. Statistical analysis was conducted by using StatView 5.0. Data were analyzed by two-way ANOVA or repeated-measured ANOVA.

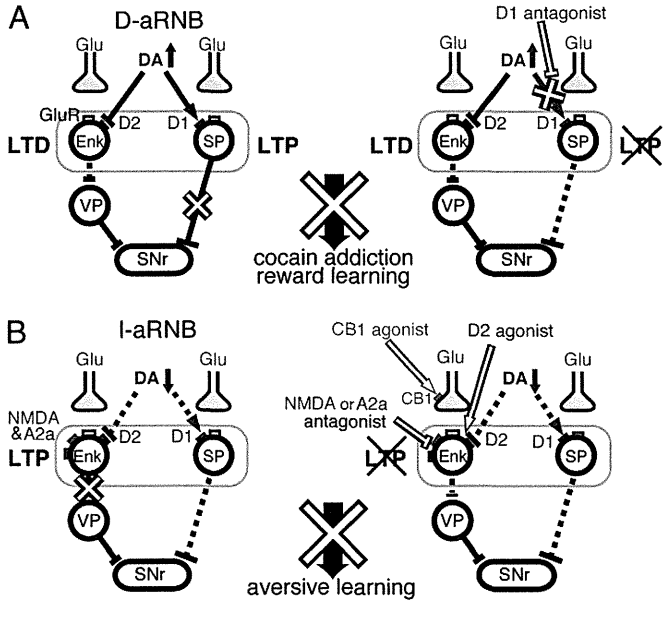

Fig. 6. A mechanistic model for reward-based and aversive learning by neural plasticity in the direct and indirect pathways of the NAc. For explanation of a mechanistic model of pathway-specific regulation of reward learning/cocaine addiction (*A*) and aversive learning (*B*) via selective transmitter receptors, see *Discussion*. The solid and dashed lines indicate the active and inactive states of input transmission, respectively. Arrowed and blocked lines note excitatory and inhibitory transmissions, respectively. A2a, A2a receptors; CB1, CB1 receptors; D1, D1 receptors; D2, D2 receptors; GluR, glutamate receptors; NMDA, NMDA receptors.

ACKNOWLEDGMENTS. We thank A. Uto for preparing figures. This work was supported by Research Grants-in-Aid 2222005 (to S.N.), 23120011 (to S.N., T.H., and S.Y.), and 23680034 (to T.H.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and by grants from Japan Science and Technology Agency, Precursory Research for Embryonic Science and Technology (to T.H.); TK Project of Medical Innovation Center of Kyoto University (to T.H.); and the Ministry of Health, Labor and Welfare of Japan (to T.H.); the Takeda Science Foundation (to S.N.); the Naito Foundation (to T.H.); the Uehara Memorial Foundation (to T.H.); and the Senri Life Science Foundation (to T.H.).

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Pathway-specific control of reward learning and its flexibility via selective dopamine receptors in the nucleus accumbens

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Contributed by Shigetada Nakanishi, June 26, 2012 (sent for review June 6, 2012)

In the basal ganglia, inputs from the nucleus accumbens (NAc) are transmitted through both direct and indirect pathways and control reward-based learning. In the NAc, dopamine (DA) serves as a key neurotransmitter, modulating these two parallel pathways. This study explored how reward learning and its flexibility are controlled in a pathway-specific and DA receptor-dependent manner. We used two techniques (*i*) reversible neurotransmission blocking (RNB), in which transmission of the direct (D-RNB) or the indirect pathway (I-RNB) in the NAc on both sides of the hemispheres was selectively blocked by transmission-blocking tetanus toxin; and (*ii*) asymmetric RNB, in which transmission of the direct (D-aRNB) or the indirect pathway (I-aRNB) was unilaterally blocked by RNB techniques and the intact side of the NAc was infused with DA agonists or antagonists. Reward-based learning was assessed by measuring goal-directed learning ability based on visual cue tasks (VCTs) or response-direction tasks (RDTs). Learning flexibility was then tested by switching from a previously learned VCT to a new VCT or RDT. D-RNB mice and D1 receptor antagonist-treated D-aRNB mice showed severe impairments in learning acquisition but normal flexibility to switch from a previously learned strategy. In contrast, I-RNB mice and D2 receptor agonist-treated I-aRNB mice showed normal learning acquisition but severe impairments not only in the flexibility to the learning switch but also in the subsequent acquisition of learning a new strategy. D1 and D2 receptors thus play distinct but cooperative roles in reward learning and its flexibility in a pathway-specific manner.

goal-directed behavior | perseveration | transmission modulation | neurotransmission blockade | striatal neurons

The basal ganglia are the key neural substrates that control reward-based learning and its flexibility to effectively acquire rewards under changing environmental circumstances (1–3). Dysfunction of the basal ganglia leads to severe cognitive and learning impairments as exemplified in Parkinson's disease, schizophrenia, and drug addiction (4–6). In the basal ganglia circuitry, the projection neurons in the striatum and the nucleus accumbens (NAc), which is the ventral part of the striatum, are divided into two subpopulations, i.e., striatonigral neurons of the direct pathway and striatopallidal neurons of the indirect pathway (1, 7, 8). The outputs of these two parallel pathways converge at the substantia nigra pars reticulata/ventral tegmental area (VTA) and control the dynamic balance of the basal ganglia–thalamocortical circuitry (1, 9). The two types of the striatal projection neurons are morphologically indistinguishable, but the striatonigral and striatopallidal neurons selectively express D1 and D2 dopamine (DA) receptors (1, 10, 11). This difference in expression profile as well as the distinct ligand affinities of these two DA receptors is thought to be critical for modulating transmission of the pathways involved in rewarding behaviors (3, 12). However, whether and how different types of DA receptors in the two parallel pathways control reward learning and its flexibility are questions largely remaining to be answered.

In our previous study, we developed a gene-manipulating technique referred to as reversible neurotransmission blocking (RNB), in which neurotransmission in a specific neural pathway is reversibly blocked in a doxycycline-regulated manner (13–15). In this technique, the transmission-blocking tetanus toxin is expressed in a pathway-specific and doxycycline-regulated manner, thus allowing separate and reversible blockade of neurotransmission in the direct pathway (D-RNB mice) or the indirect pathway (I-RNB mice) in vivo (15, 16). The function of the basal ganglia circuitry becomes defective only when both sides of the basal ganglia circuit are simultaneously impaired in the brain hemispheres (17, 18). We thus extended the RNB technique to an asymmetric RNB (aRNB) technique, in which one side of the basal ganglia circuit is blocked by the RNB technique, and the other intact side is treated with an agonist or antagonist specific for D1 or D2 receptors. This aRNB technique allowed us to disclose what type of DA receptors is responsible for pathway-specific modulation of rewarding behaviors. Here, we report that D1 and D2 receptors play a distinct and critical role in reward learning and its flexibility in a pathway-specific manner.

Results

Function of the Two Pathways in Visual Cue-Based Reward Learning. In the RNB mice, the expression of transmission-blocking tetanus toxin (TN) is driven by interaction of the tetracycline-responsive element (TRE) and the tetracycline-repressive transcription factor (tTA) (13–16) (Fig. 1*A*). The separate expression of tTA in either pathway is achieved by using the adeno-associated virus (AAV)–mediated gene-expression system, in which the expression of tTA is restricted in the direct and indirect pathways by the substance P promoter and the enkephalin promoter, respectively (15). Recombinant AAVs were bilaterally injected into the NAc (15), and 2–3 wk after the viral injection, we tested animal behaviors to investigate how the direct and indirect pathways were involved in reward-based learning and learning flexibility.

We first examined the learning ability of D-RNB and I-RNB mice to gain a reward in a visual cue task (VCT) (Fig. 1*B*). In this task, mice were randomly placed in one of two arms of a plus maze. The mice had to learn to make a correct left or right turn on the basis of visual cues to gain a reward placed at the terminal of the fixed arm. The control mice (wt mice) and I-RNB mice both progressively learned the correct choice by repeated training and reached more than 90% correct choices by the fifth session in the VCT (Fig. 1*C*). In contrast, D-RNB mice were impaired in correct choices throughout repeated sessions of the training. The wt mice and I-RNB mice reached the criterion for learning acquisition in 4.4 ± 0.2 and 5.4 ± 0.4 sessions, respectively (Fig. 1*D*). There was no statistical difference in acquisition criterion between these two groups. In contrast, the D-RNB mice reached the criterion in 8.0 ± 0.5 sessions, and

Author contributions: S.Y., T.H., and S.N. designed research; S.Y., T.D., and T.H. performed research; S.Y., T.Y., and T.H. analyzed data; and S.Y. and S.N. wrote the paper.

The authors declare no conflict of interest.

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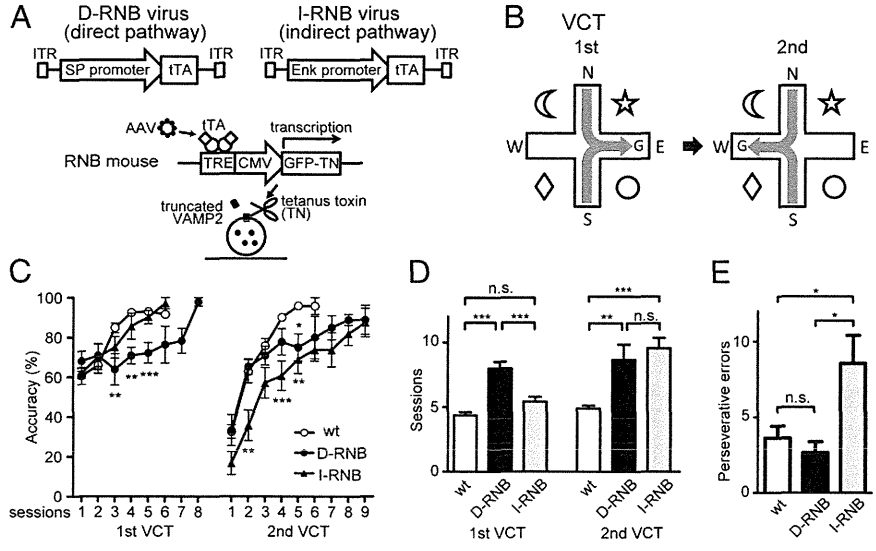

Fig. 1. Reward-based learning and its flexibility of D- and I-RNB mice in the VCT. (*A*) Schema showing preparation of D- and I-RNB mice. The D- and I-RNB viruses contained the *tTA* gene following the substance P (SP) and enkephalin (Enk) promoters, respectively. When the NAc of RNB mice was transfected with the recombinant virus, the GFP-TN fusion protein was selectively expressed in the neurons of the direct or the indirect pathway by the interaction of the virus-driven tTA with the TRE and separately blocked transmission in the respective pathway. CMV is cytomegalovirus promoter and ITR is inverted terminal repeat of the viral DNA. (*B*) Schema of learning analysis with the VCT. In the first VCT, mice were started from the north or south arm and on the basis of visual cues, they learned the east arm to receive a reward. After learning sufficiently in the first VCT, the goal (G) position was changed to the opposite, west arm in the second VCT. (*C*) Accuracy represents percentages of trials in which the mice succeeded in turning correctly to receive a reward at each session. (*D*) Number of sessions that animals required to reach the criterion in the VCT. (*E*) Number of perseverative errors on the shift of the reward position in the second task; $n = 11$ (wt), 6 (D-RNB), and 7 (I-RNB). Marks/columns and bars represent the mean and ± SEM, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, n.s., not significant.

a significant difference in reaching the criterion was noted between the wt/I-RNB mice and D-RNB mice ($P < 0.001$).

Next we addressed how blockade of each pathway would affect the ability to learn the shift of a reward position in the VCT. In this test, animals were trained to reach the acquisition criterion in the first VCT. The reward was then placed at the end of the opposite arm so that the animal needed to make a reverse turn to receive a reward in the second task (Fig. 1*B*). In this test, the wt and D-RNB mice showed the comparable ability to learn this shift at the early sessions of training, but the learning ability of the D-RNB mice was significantly reduced at the late sessions of training (Fig. 1*C*). The I-RNB mice were impaired not only at the early sessions but also at the late sessions of training. In the second test, the wt mice achieved the learning criterion in 4.9 ± 0.2 sessions, but the D-RNB and I-RNB mice needed more training to reach it, requiring 8.7 ± 1.2 and 9.6 ± 0.8 sessions, respectively ($P < 0.01$, D-RNB vs. wt; $P < 0.001$, I-RNB vs. wt) (Fig. 1*D*).

We then addressed whether blockade of each pathway would affect perseveration after switching the VCT (Fig. 1*E*). In this analysis, perseverative errors were assessed by analyzing the trial numbers required for making a first correct turn when the reward position had been switched on the second VCT. Upon this analysis, the wt and D-RNB mice turned to the error side repeatedly at 3.6 ± 0.8 and 2.7 ± 0.7 trials, respectively; and these perseverative errors were not significantly different between these two groups. In contrast, perseverative errors by the I-RNB mice increased to 8.6 ± 1.8 trials, and this increase was significant ($P < 0.05$) compared with the trial numbers for the wt and D-RNB mice. Thus, the transmission blockade of the indirect pathway, but not that of the direct pathway, impaired learning on the switch due to perseveration.

Pathway-Dependent Function in Response-Direction Learning and Its Flexibility. The role of each pathway in reward learning was further examined by performing a response-direction task (RDT). In this task, a mouse was randomly started from two of the four arms and had to make a 90° turn in the same direction to receive a reward (Fig. 2*A*). The wt and I-RNB mice comparably learned to make a correct turn and reached the acquisition criterion in 5.3 ± 0.5 and 5.3 ± 0.8 sessions, respectively, with no statistical difference between them (Fig. 2*B*). In contrast, the D-RNB mice showed reduced learning ability, as the number of sessions to reach the criterion significantly increased to 11.8 ± 0.9 sessions ($P < 0.001$, compared with the wt/I-RNB mice) (Fig. 2*B*). Thus, the transmission blockade of the direct pathway, as noted in the VCT test, selectively impaired acquisition of reward-based learning in the RDT test as well.

We next analyzed the ability of animals to switch to a different type of reward-based learning. When animals reached the criterion in the VCT, the task was changed to the RDT in the second test (Fig. 2*C*). Similar to the learning shift in the VCT, the D-RNB mice could learn the shift comparably as the wt mice at the early sessions of the RDT and then appeared to be partially impaired at the late sessions. It is important to note that the I-RNB mice were defective at both the early and late sessions of the learning switch. The I-RNB mice thus required a larger number of sessions (11.3 ± 2.1 sessions) to reach the criterion than did the wt mice (4.7 ± 0.6 sessions, $P < 0.01$) (Fig. 2*D*). This session number of the D-RNB mice also tended to increase (7.4 ± 0.9 sessions) although this number was not statistically significant compared with that for the wt mice ($P = 0.14$). We then analyzed perseverative errors in the VCT–RDT switching task. These errors significantly increased in the I-RNB mice (6.5 ± 1.2 trials) ($P < 0.05$), but not in the D-RNB mice (3.9 ± 0.6 trials) compared with the number for the wt mice (2.7 ± 0.6 trials) (Fig. 2*E*). Thus, blockade of the indirect pathway increased perseveration and impaired a different type of reward-based learning shift.

D1-Receptor Regulation of the Direct Pathway in Acquisition of Reward-Based Learning. To assess how DA could regulate reward-based learning and its flexibility, we generated asymmetric RNB mice, in which transmission of either the direct or the

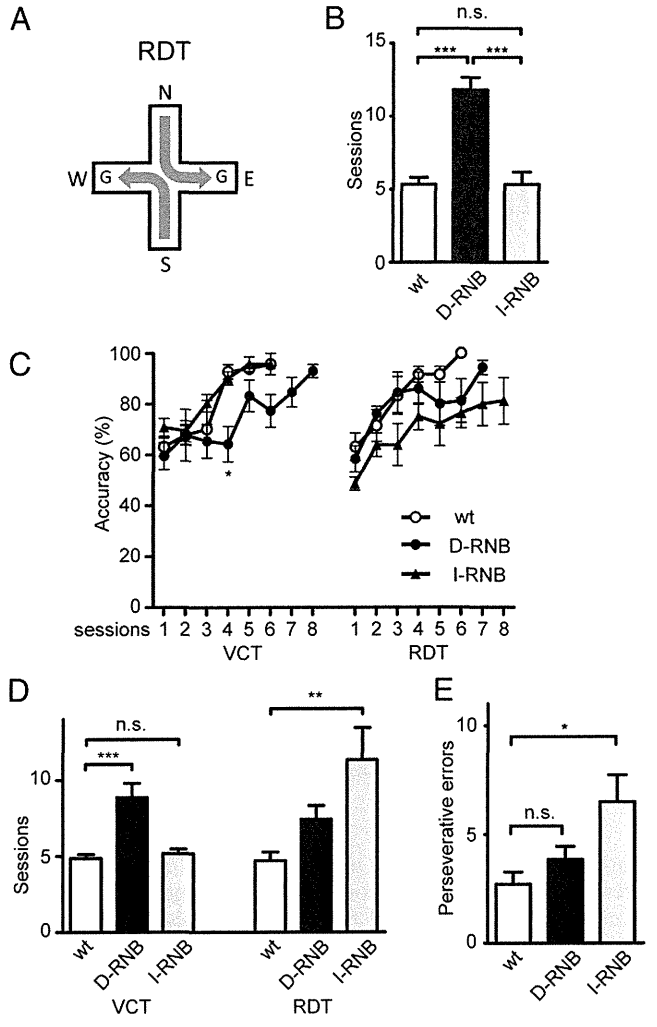

Fig. 2. Learning acquisition in the RDT and learning flexibility on the shift from the VCT to the RDT. (*A*) Schema of learning analysis with the RDT. Mice were started from either the north or south arm and learned a fixed turning direction to receive a reward. (*B*) Number of sessions that the animals required to reach the criterion in the RDT; $n = 9$ (wt), 5 (D-RNB), and 6 (I-RNB). (*C–E*) Learning ability was tested by the VCT in the first test. After sufficiently learning in the first test, the mice were then examined for learning ability in the second test using RDT. Accuracy (*C*), session numbers to reach the criterion (*D*), and perseverative errors (*E*) were determined as described in Fig. 1; $n = 7$ (wt), 7 (D-RNB), and 6 (I-RNB). Marks/columns and bars represent the mean and ± SEM, respectively. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, n.s., not significant.

indirect pathway in the NAc was unilaterally blocked by the D- or I-RNB virus injection (D-aRNB and I-aRNB mice), respectively (Fig. 3). Two to three weeks after the viral injection, a DA agonist or antagonist was infused into the intact side of the NAc through an implanted cannula (Fig. 3*A*). Animal behavior was analyzed 15–30 min after drug infusion in each session, and the location of the implanted cannula was confirmed once the behavioral analysis had been completed. SKF81297 (SKF) and SCH23390 (SCH) were used as a D1 agonist and a D1 antagonist, respectively; whereas quinpirole and eticlopride were used as a D2 agonist and a D2 antagonist, respectively (19–22).

The D1 receptor is predominantly expressed in the striatonigral neurons of the direct pathway (11, 23, 24). We first addressed whether and how D1 receptors could be involved in reward-based learning and its flexibility. In this analysis, we performed the VCT test to examine the effects of treatment of D-aRNB mice with either saline, SCH, or SKF (Fig. 3*A*). The saline-injected D-aRNB mice showed normal learning acquisition in the first VCT test and normal learning switch in the second VCT test, as the accuracies were comparable to those of the saline-injected wt mice (Fig. 3*B*). This finding verified that unilateral blockade of transmission had no effect on the reward-based learning ability. Then, when D1 receptors were inhibited by contralateral injection of SCH into the intact NAc, these mice were severely impaired in their learning ability throughout training in the first test. Furthermore, they normally learned the shift of a reward at the early sessions but became defective at the late sessions of the second test (Fig. 3*C*). The SCH–D-aRNB mice thus exhibited a defective profile identical to that of the bilaterally blocked D-RNB mice in terms of both learning acquisition and learning switch. In contrast, stimulation of D1 receptors with SKF had no obvious effects on either learning acquisition or learning switch (Fig. 3*D*). Also, the virus-transfected wt mice never showed any defect by contralateral injection of either SCH or SKF (Fig. 3 *C* and *D*). As a result, only the SCH–D-aRNB mice showed a significant increase in the session number required to reach the learning criterion in both the first and second tests. The session numbers needed to reach the criterion in the first and second tests were 5.3 ± 0.2 and 4.5 ± 0.3 for saline–wt; 5.5 ± 0.4 and 6.2 ± 0.7 for saline–D-aRNB; and 9.4 ± 1.2 and 9.4 ± 1.3 for SCH–D-aRNB ($P < 0.05$–0.001, SCH–D-aRNB vs. other groups) (Fig. 3*E*). In addition, when the D-aRNB mice were treated with either saline, SCH, or SKF, none of these mice showed an increase in perseverative errors in response to switching of the reward position (Fig. 3*F*). The results thus indicate that the activation of D1 receptors in the direct pathway plays a key role in learning acquisition but not in learning switch.

D2-Receptor Regulation of the Indirect Pathway in Flexibility of Reward-Based Learning. The D2 receptor is predominantly expressed in the striatopallidal neurons of the indirect pathway (11, 23, 24). We next assessed the role of D2 receptors in the indirect pathway by examining the effects of a D2 agonist or antagonist on the learning ability of I-aRNB mice (Fig. 4). Infusion of saline into I-aRNB mice had no effect on either learning acquisition in the VCT of the first test or on the VCT–VCT learning switch in the second test (Fig. 4*A*). The injection of the D2 agonist quinpirole into I-aRNB mice tended to rather elevate learning acquisition in the first test and then, like the bilateral blockade in I-RNB mice, markedly impaired both the early and late sessions of learning switch in the second test (Fig. 4*B*). In contrast, the D2 antagonist eticlopride had no inhibitory effect on the learning ability in either the first or second test (Fig. 4*C*). Thus, the number of sessions to reach the criterion increased in learning switch only when the D2 receptor was activated in the I-aRNB mice: the session numbers required to reach the criterion in the first and second tests were 5.0 and 5.2 ± 0.7 for saline–wt; 5.3 ± 0.4 and 5.6 ± 0.6 for saline–I-aRNB; and 4.3 ± 0.4 and 9.0 ± 1.2 for quinpirole–I-aRNB ($P < 0.01$–0.001; quinpirole–I-aRNB vs. other groups in the second test) (Fig. 4*D*). Furthermore, the perseverative errors significantly increased in the VCT–VCT switching when quinpirole (8.0 ± 1.8 trials), but not eticlopride (3.7 ± 1.1 trials), was administered to the I-aRNB mice ($P < 0.01$, quinpirole–I-aRNB vs. saline–I-aRNB or quinpirole–wt) (Fig. 4*E*). Thus, the profile of the quinpirole–I-aRNB mice in terms of defective reward learning and its flexibility was identical to that of the bilaterally blocked I-RNB mice. These results indicate that inactivation of the D2 receptor in the indirect pathway is necessary to flexibly adapt learning switching and promote the subsequent new learning.

In this investigation, we focused on the functional role of the pathway-specific DA receptors in the NAc. Both D-aRNB and I-aRNB mice showed no alteration in locomotor activity in the plus maze regardless of treatment with DA agonists or antagonists

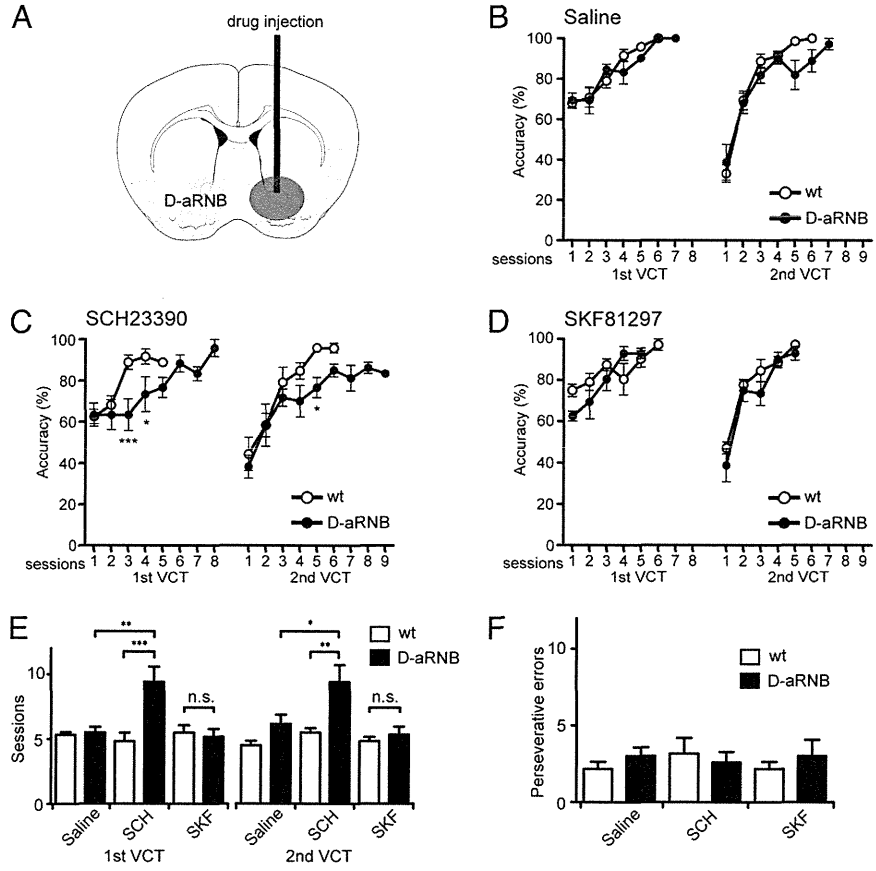

Fig. 3. Learning acquisition with D1 receptor in the direct pathway. (*A*) Schema of the aRNB technique combined with pharmacological analysis. One side of transmission of the direct pathway in the NAc was blocked by the RNB technique, and the other intact side of the NAc was infused with saline (*B*), SCH23390 (*C*), or SKF81297 (*D*). Learning ability was tested by the VCT in the first test. Then after the mice had sufficiently learned in the first test, a reward was placed at the end of the opposite arm; and the learning ability was then tested by the VCT in the second test. Accuracy (*B–D*), session numbers to reach the criterion (*E*), and perseverative errors (*F*) were determined as described in Fig. 1; n = 5–6. Marks/columns and bars represent the mean and ± SEM, respectively. $*P < 0.1$, $**P < 0.01$, $***P < 0.001$, n.s., not significant.

(8.4–10.5 cm/s). Our previous study showed that when the direct or the indirect pathway was unilaterally blocked in the whole striatal region and then these mice were forced to rotate on a hemispherical container, such blockade induced abnormal ipsilateral or contralateral rotations, respectively (15). However, none of the drugs infused into the NAc elicited such abnormal turning in either the D-aRNB or I-aRNB mice. Thus, the observed impairments of learning ability in the drug-infused aRNB mice were not due to imbalance of motor movement and indeed reflected deficits in reward-based learning and its flexibility.

Discussion

This study has established a technique that allowed us to define the role of pathway-specific DA receptors in reward-based learning and its flexibility. The results explicitly indicate that learning deficits in both D1 antagonist-treated D-aRNB mice and D2 agonist-treated I-aRNB mice reflected those in bilaterally blocked D- and I-RNB mice, respectively. These results indicate that the activation of D1 receptor in the direct pathway is essential for the animals to acquire reward-based learning but is not necessary for them to flexibly switch the previously learned reward-seeking behavior. In contrast, the modulation of D2 receptors in the indirect pathway is not necessary for acquisition of reward-based learning; but their inactivation is indispensable for learning flexibility to switch from the previously learned behavior. Thus, the D1 and D2 receptors play a key role in learning acquisition and learning flexibility, respectively, in a pathway-specific manner. The results further indicate that the indirect pathway-defective naive mice normally learned a reward-gaining strategy but that these mice, once having learned it, showed difficulty in learning the switch to a new strategy even after repeated reward presentation. Furthermore, the observed functional deficits were caused by restricted blockade of the NAc circuit, indicating that input convergence at the NAc is critical for both reward learning and its flexibility.

D1 and D2 receptors are almost exclusively expressed in the direct and indirect pathways, respectively, the former exhibiting low-affinity binding of DA and the latter, high-affinity binding (11, 23, 24). In addition, DA neurons in the VTA exhibit two different patterns of firings, a phasic firing and a tonic firing, which differentially modulate D1 and D2 receptors in the NAc (12, 17, 25). On the basis of these characteristic features of DA transmission, Frank proposed a neurocomputational model to explain "Go" and "No Go" signals in reward-based learning (26). Our study has provided explicit experimental evidence that is in good agreement with their proposal and has further extended our understanding of reward-based learning mechanisms, as depicted in Fig. 5. When naive animals encounter unexpected rewards or sensory signals predicting such rewards, DA neurons evoke a burst of phasic firings and increase synaptic concentrations of DA in the NAc (27, 28). This increase effectively activates the low-affinity D1 receptor and enhances the response of the NAc neurons to reward-related input, thereby triggering reward-directed learning (Fig. 5*A*). Thus, the defect of the direct

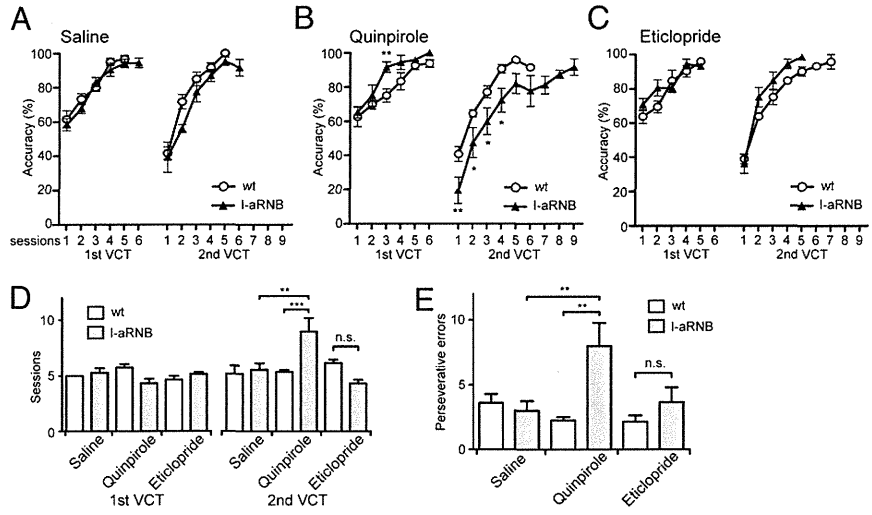

Fig. 4. Learning acquisition and flexibility with D2 receptor in the indirect pathway. One side of transmission of the indirect pathway in the NAc was blocked by the RNB technique, and the other intact side of the NAc was infused with saline (*A*), quinpirole (*B*), or eticlopride (*C*). Learning ability was tested as described in Fig. 3. Accuracy (*A–C*), session numbers to reach the criterion (*D*), and perseverative errors (*E*) were determined as described in Fig. 1; $n = 5–8$. Marks/columns and bars represent the mean and ± SEM, respectively. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, n.s., not significant.

pathway in both D-RNB mice and D1 antagonist-treated D-aRNB mice resulted in impairments of reward-based learning. The functional deficit of the D1 receptor in the direct pathway also impaired the learning ability at the late sessions of the second test. The activation of the D1 receptor is thus required for learning a new strategy when the learning strategy was switched in the second test. By contrast, absence of an expected reward suppresses tonic firings of DA neurons and lowers DA concentrations in the NAc (12). This reduction in DA relieves the D2-receptor-mediated inhibition of the indirect pathway but has no effect on the low-affinity D1 receptor in the direct pathway. The selective disinhibition of the indirect pathway then precludes the previously learned actions in response to reward omission (Fig. 5*B*). Thus, both I-RNB mice and D2-receptor-activated I-aRNB mice showed normal learning ability to initial reward presentation but were severely impaired in their flexibility of the learning switch. The important finding of this investigation is that when the learning strategy was shifted, the defective indirect pathway significantly slowed down the ability to learn a new reward-gaining strategy in both the VCT–VCT and VCT–RDT tests. When the rewarding system is changed, it is necessary to prevent recalling a previously learned strategy in addition to promoting a new reward-based learning (Fig. 5*C*). Our finding thus strongly suggests that the indirect pathway is indispensable not only for rapid suppression of perseveration toward a previously learned strategy but also to persistently preclude the reward-negative outcome (Fig. 5*C*).

The integrative processes in the NAc circuit that regulate reward learning and its flexibility have important implications for disease states where DA signaling is abnormal. In both hyperdopaminergic states, such as drug abuse and schizophrenia, and hypodopaminergic states, as seen in Parkinson's disease, the modulatory mechanisms of the direct and indirect pathways are disrupted (4–6, 29). This is reflected in the abnormal behaviors of animal models and human patients. Cocaine-sensitized animals are not impaired in acquiring reward learning but show significant perseveration if the goal is switched (30). A similar type of abnormality is observed in probabilistic reversal learning after repeated L-DOPA administration in Parkinson's patients and is proposed to be due to the lack of D2-receptor inactivation necessary for learning flexibility (29, 31). Our findings that indicate pathway-specific and receptor-dependent dopaminergic modulation in reward learning and flexibility will provide more informative approaches for the treatment of Parkinson's disease and psychiatric disorders.

Materials and Methods

RNB Mice. All animal-handling procedures were performed according to the guidelines of Osaka Bioscience Institute. The RNB mice were generated as described previously (15), and the schema of the RNB technique is presented in Fig. 1*A*. The recombinant AAV was unilaterally or bilaterally injected into four sites of the NAc by a stereotaxic technique (15). The RNB and aRNB mice and their wt littermates were used for all experiments.

Drug Infusion in aRNB Mice. After anesthesia and retraction of the scalp, the recombinant virus was injected unilaterally into the NAc (15). Then, the

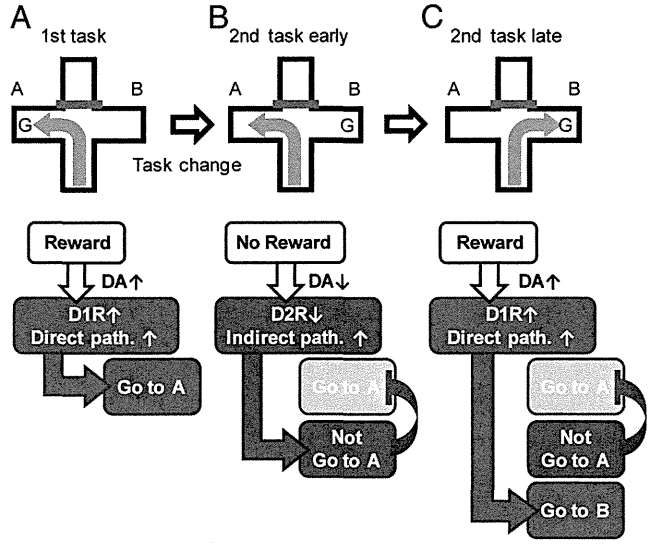

Fig. 5. Schematic model of pathway-specific, DA receptor-dependent modulation of the NAc in reward learning and flexibility. D1R is D1 receptor and D2R is D2 receptor. Note that this model holds that the D2 receptor in the indirect pathway is involved in not only the flexibility to learning switch but also the subsequent suppression of learning conflicts between a previously learned strategy and a new one.

contralateral NAc was implanted with a 5-mm guide cannula (26-gauge) possessing a dummy cannula (33-gauge) aimed toward the NAc. The guide cannula was secured in place with dental acrylic. The stereotaxic coordinates for drug infusion were 1.2 mm anterior to bregma, 1.2 mm lateral to the midline, and 3.5 mm ventral to dura according to the atlas of Franklin and Paxinos (32). Drug infusion into the NAc was made through an inner cannula (33-gauge) attached to a Hamilton syringe. The syringe was driven in a volume of 1 μL per side for 2 min by a microinfusion pump. The concentrations of infused drugs were 100 μM SCH23390, 300 μM SKF81297, 1 mM quinpirole, and 400 μM eticlopride, all purchased from Sigma. After behavioral analysis, injection sites of drugs were confirmed by direct visualization of a series of slice sections of the NAc region. When the injection site was found to be outside the NAc, these data were discarded (about 3% of drug-injected aRNB mice).

Behavioral Analysis. A four-arm cross maze was made of a clear plastic wall and a gray floor and placed 90 cm above the floor. Each arm was 25 cm long and 5 cm wide, and the center platform was 5 × 5 cm. Visual cues such as balls and shopping baskets were hung outside the maze, and one side of the room and the other side of it were surrounded by a black and a yellow curtain, respectively. The position of a mouse was detected by video camera suspended over the maze and was analyzed by use of Labview software. Behavioral analysis was started 2–3 wk after manipulation with either bilateral viral injection or unilateral viral injection together with surgery for drug infusion. Animals were food-restricted to reach approximately 80% of their original ad libitum weight by the beginning of behavioral analysis, which was started after 3 d of habituation. On each day of habituation, three pieces of chocolate were placed in the food well of each arm. A mouse was allowed to freely navigate and consume the chocolate within 15 min. During the habituation period, the mouse was handled for 10 min per day. After the habituation procedure, a possible bias to turn to a preferred arm or to a preferred direction was assessed in the absence of chocolate by the use of a T maze, in which one arm was closed by a clear acrylic wall. Then, to avoid the possible turning bias, a reward was placed on the opposite arm as its turn bias during testing (17, 33). In the VCT, a mouse was started from either the north or south arm and had to make a 90° turn to the left or to the right on the basis of visual cues. In the RDT, a mouse had to make a 90° turn in the same direction. Each start arm was used with an equal number of trials in a pseudorandom fashion. Two sessions, each consisting 12 trials, were carried out per day. Between trials, the mouse was placed back in the holding cage. The maze arms were wiped down with a sponge moisturized with ammonium chloride solution. The intertrial interval was ~10 s. Accuracy was calculated as percentages of correct choices per session. The acquisition criterion was defined as more than 11 correct choices in two consecutive sessions. Regardless of reaching this criterion, at least five sessions were performed in each test. Perseverative errors were calculated as number of repeated incorrect choices in the beginning of the second test in which the first VCT was switched to either the second VCT or the RDT. Locomotor activity and forced rotation were measured as described previously (15). All tests of animal behaviors were conducted in a blind fashion.

Statistical Analysis. Statistical analysis was conducted by using GraphPad PRISM 5.0 (GraphPad Software) and StatView 5.0. Data were analyzed by one-way ANOVA or repeated measure ANOVA and post hoc comparisons were made by using the Bonferroni test.

ACKNOWLEDGMENTS. This work was supported by Research Grants-in-Aid 2222005 (to S.N.), 23120011 (to T.H., S.Y., and S.N.), and 23680034 (to T.H.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and the Japan Science and Technology Agency Precursory Research for Embryonic Science and Technology Program (to T.H.), the Takeda Science Foundation (to S.N.), the Naito Foundation, and the Senri Life Science Foundation (to T.H.).

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Pathway-specific engagement of ephrinA5-EphA4/EphA5 system of the substantia nigra pars reticulata in cocaine-induced responses

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Contributed by Shigetada Nakanishi, May 12, 2011 (sent for review April 28, 2011)

The nucleus accumbens (NAc) serves as a key neural substrate that controls acute and adaptive behavioral responses to cocaine administration. In this circuit, inputs from the NAc are transmitted through two parallel pathways, named the direct and indirect pathways, and converge at the substantia nigra pars reticulata (SNr). Our previous study using reversible neurotransmission blocking (RNB) of each pathway revealed that the dual stimulation of the SNr by both pathways is necessary for the acute response, but that the direct pathway predominantly controls the adaptive response to repeated cocaine administration. This study aimed at exploring the pathway-specific mechanism of cocaine actions at the convergent SNr. We examined a genome-wide expression profile of the SNr of three types of experimental mice: the direct pathway-blocked D-RNB mice, the indirect pathway-blocked I-RNB mice, and wild-type mice. We identified the up-regulation of ephrinA5, EphA4, and EphA5 specific to D-RNB mice during both acute and adaptive responses to cocaine administration. The activation by EphA4 and EphA5 in the SNr of wild-type mice by use of the immunoadhesin technique suppressed the adaptive response to repeated cocaine administration. Furthermore, cocaine exposure stimulated the phosphorylation of Erk1/2 in ephrinA5-expressing SNr cells in a direct pathway-dependent manner. The results have demonstrated that the ephrinA5-EphA4/EphA5 system plays an important role in the direct pathway-dependent regulation of the SNr in both acute and adaptive cocaine responses and would provide valuable therapeutic targets of cocaine addiction.

basal ganglia | drug addiction | Eph-ephrin signaling | gene regulation | transmission blocking

The basal ganglia are the key neural substrates that control motor balance and reward-based and aversive learning (1, 2). Dysfunction of the basal ganglia leads to devastating neurological disorders, such as Parkinson disease and drug addiction (3–5). The projection neurons in the striatum and the nucleus accumbens (NAc), the ventral part of the striatum, are GABA-containing medium-sized spiny neurons, which are divided into two subpopulations: striatonigral neurons in the direct pathway and striatopallidal neurons in the indirect pathway (1, 3, 6). The inputs of these two pathways converge at the substantia nigra pars reticulata (SNr) and control the dynamic balance of the basal ganglia-thalamocortical circuitry (1, 7). Cocaine and other psychostimulants massively increase dopamine levels in the NAc and the striatum and induce abnormal behavioral responses both acutely and chronically (8). We previously developed a gene-manipulating technique that allows separate and reversible neurotransmission blocking (RNB) of the direct pathway (D-RNB mice) and the indirect pathway (I-RNB mice) in vivo (9). The use of this technique revealed the distinct regulatory function of the two pathways in acute and chronic responses to cocaine exposure (9). Blockade of the direct pathway abrogates the acute response and then markedly attenuates the chronic response to cocaine administration. In contrast, blockade of the indirect pathway abolishes the acute response as well; but the ability to induce normal levels of the chronic response after repeated cocaine administration is retained. The two pathways are thus necessary for the acute cocaine response but the direct pathway plays a predominant role in the adaptive response to repeated cocaine administration (9). However, the molecular and signaling mechanisms that underlie these different adaptive reactions by the two pathways remain to be clarified.

The SNr is composed mostly of GABAergic projection neurons and serves as a main target nucleus that receives GABAergic inputs from the direct pathway and both GABAergic and glutamatergic inputs from the indirect pathway (1, 7). In this study, we investigated what signaling molecules are involved in the pathway-dependent regulation of the SNr after cocaine administration. To address this question, we examined a genome-wide expression profile of the SNr of the D-RNB, I-RNB, and WT mice by using microarray and quantitative RT-PCR techniques. We identified the specific up-regulation of ephrinA5, EphA4, and EphA5 in the D-RNB mice after cocaine administration. We also revealed the inhibitory role and downstream signaling of the ephrinA5-EphA4/EphA5 system in cocaine-induced behaviors. This study has thus disclosed an important mechanism of the pathway-specific regulation of cocaine actions in the basal ganglia circuitry and would provide valuable therapeutic targets of drug addiction.

Results

Profiling of Gene Expression of the SNr in the D-RNB Mice After Cocaine Administration. In this study, we used previously developed RNB transgenic mice, in which the tetanus toxin light chain (TN) is restrictedly expressed in cells of either the direct or the indirect pathway (9). TN is a bacterial toxin that cleaves the synaptic vesicle-associated membrane protein-2 and thus blocks transmitter release from the synaptic vesicles. In RNB mice, the expression of TN is controlled by the tetracycline-responsive element (TRE) and thus driven by its interaction with the tetracycline-repressive transcription factor (tTA) in a tetracycline-derivative doxycycline-regulated manner. The restricted expression of tTA in either pathway is achieved by using the adeno-associated virus (AAV)-mediated gene-expression system, in which the expression of tTA is directed by the substance P promoter or the enkephalin promoter. Recombinant AAVs were bilaterally injected into the NAc, and 2 wk after the viral injection, locomotor activity was measured for 10 min immediately after cocaine (10 mg/kg) or saline administration. Both D-RNB

Author contributions: K.K., T.H., and S.N. designed research; K.K., T.H., S.Y., and T.Y. performed research; K.K. and T.H. analyzed data; and K.K., T.H., and S.N. wrote the paper.

The authors declare no conflict of interest.

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and I-RNB mice failed to show acute hyperlocomotion after cocaine administration (9).

The SNr, which is a main target nucleus of the direct and indirect pathways, is rich in glutamic acid decarboxylase67 (GAD67)-immunoreactive cells and is located adjacent to the substantia nigra pars compacta (SNc), which is characterized by a high density of tyrosine hydroxylase (TH) immunoreactivity (Fig. 1*A*) (10, 11). The SNr and the SNc could thus be easily separated and dissected by the characteristic architecture and cell shapes of these nuclei. We performed quantitative RT-PCR of dissected SNrs and confirmed that the SNrs used exhibited a high level of the GAD67 mRNA and a minimal contamination of the TH mRNA from the SNc (Fig. 1*B*). Furthermore, there was no difference in expression levels of the GAD67 mRNA among the D-RNB, I-RNB, and WT mice, regardless of whether the animals were treated or not with cocaine (Fig. 1*B*).

After confirmation of the lack of cocaine-induced hyperlocomotion in individual D-RNB and I-RNB mice, SNrs were mechanically isolated from D-RNB, I-RNB, and WT mice 1 h after cocaine or saline administration. Total RNA was extracted from microdissected SNrs and subjected to microarray analysis. As criteria for the selection of candidate genes, we used hybridization signals of >150 at least in one of the three types of the experimental animals and more than 1.4-fold changes between cocaine and saline treatments in either D-RNB or I-RNB mice, but not in the WT mice. Candidate genes thus selected were further confirmed by quantitative RT-PCR analysis. Among a few candidate genes, we focused on and analyzed in detail the ephrinA5, EphA4, and EphA5 mRNAs, all of which were up-regulated in the SNr of only the D-RNB mice (Fig. 1*C*) (one-way ANOVA analysis for ephrinA5, $P < 0.001$–0.01; for EphA4, $P < 0.01$–0.05; for EphA5, $P < 0.01$–0.05). The up-regulation of these mRNAs was not only specific to D-RNB mice after cocaine administration but in addition, the expression of these mRNAs was not altered in saline-treated RNB or WT mice (Fig. 1 *C* and *D*), indicating that the up-regulation of these mRNAs depended on both blockade of the direct pathway and cocaine administration.

Because the previous blockade study indicated a key role of the direct pathway in the adaptive response to repeated cocaine administration (9), we next addressed whether the ephrinA5, EphA4, and EphA5 mRNAs remained up-regulated in the SNr of D-RNB mice after repeated administration of cocaine. The SNr was isolated and microdissected after repeated cocaine administration for 5 d. Quantitative RT-PCR showed that the ephrinA5 and EphA5 mRNAs remained up-regulated in the SNr of only the D-RNB mice (Fig. 1*D*) (one-way ANOVA analysis for ephrinA5, $P < 0.05$; for EphA5, $P < 0.05$). The EphA4 mRNA, although not being statistically significant, tended to be up-regulated in the D-RNB mice (Fig. 1*D*). These results indicate that ephrin-Eph receptor signaling molecules are specifically up-regulated in the D-RNB mice not only at the acute phase but also at the adaptive phase of cocaine administration.

Localization of EphrinA5, EphA4, and EphA5 in the SNr. GABAergic neurons represent a major cell population, amounting to more than 90% of the neurons in the SNr (12). We investigated the cellular expression patterns of ephrinA5, EphA4, and EphA5 in the SNr by double immunostaining for each of these molecules and either gephyrin, a postsynaptic marker of GABAergic neurons (13), or glial fibrillary acidic protein (GFAP), a marker of astrocytes (14). This analysis showed that gephyrin-immunoreactive GABAergic neurons expressed both the ephrinA5 ligand and the EphA4 and EphA5 receptors (Fig. 2 *A*, *C*, and *E*). In contrast, the GFAP-positive astrocytes expressed the EphA4 and EphA5 receptors, but not the ephrinA5 ligand (Fig. 2 *B*, *D*, and *F*). In gephyrin-immunoreactive neurons, EphA4 and EphA5 were mostly localized in the soma and proximal dendrites, whereas the ephrinA5 localization extended from the soma to the distal dendrites. Furthermore, double immunostaining among ephrinA5, EphA4, and EphA5 indicated that all three molecules were ubiquitously colocalized in more than 80% of the SNr neurons (Fig. 3).

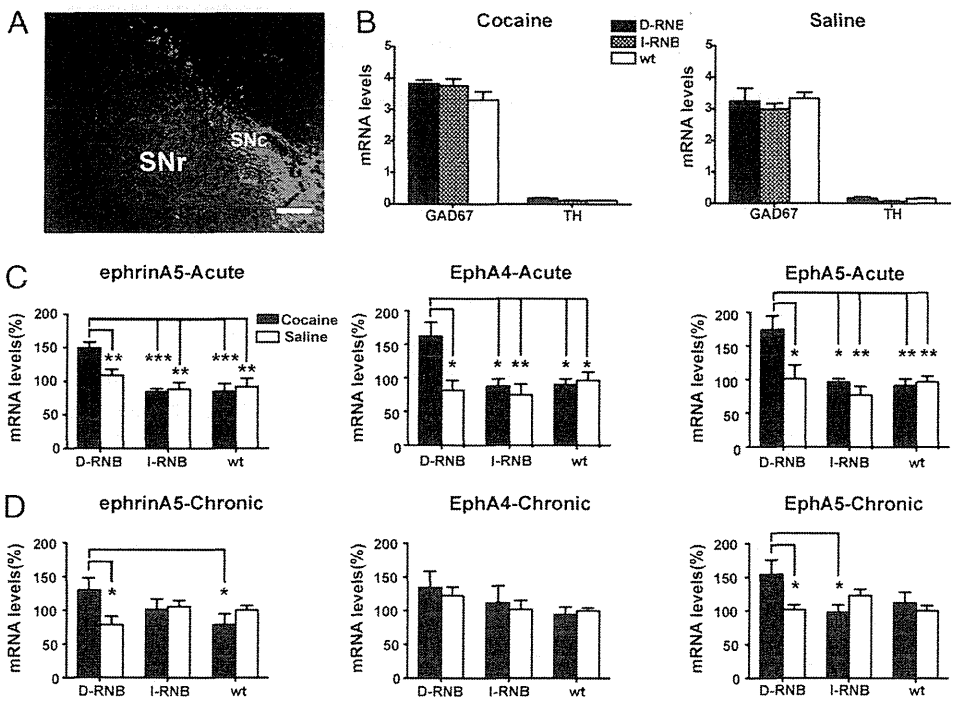

Fig. 1. Direct pathway-specific regulation of expression of ephrinA5, EphA4, and EphA5 mRNAs in the SNr by cocaine administration. (*A*) Double immunostaining of coronal sections of the SNr and SNc of WT mice with the GAD67 antibody and the TH antibody. A merged view showed that the GAD67-immunoreactive SNr (red) is located adjacent to the TH-immunoreactive SNc (green). (Scale bar, 100 μm.) (*B*) D-RNB, I-RNB, and WT mice were prepared by bilateral injection of the AAVs into the NAc. Two weeks after the viral injection, the animals received a single intraperitoneal injection of either cocaine (10 mg/kg) or saline and were killed 1 h later. The SNr was then isolated, and the levels of the GAD67 and TH mRNAs were quantified by RT-PCR. mRNA levels were normalized by referring to that level of the β-actin mRNA as 1 ($n = 6$ for D-RNB and I-RNB; $n = 12$ for WT). (*C*) The SNr was isolated as in *B*, and mRNA levels were quantified by PCR ($n = 6$ for D-RNB and I-RNB; $n = 12$ for WT). (*D*) Experiments were performed as in *B*, except that the animals daily received a single intraperitoneal injection of cocaine (10 mg/kg) or saline for 5 d and the SNr was then isolated 1 h after the last intraperitoneal injection ($n = 6$ each). In *C* and *D*, levels of each mRNA were expressed by referring to those of the corresponding mRNA in saline-injected WT mice. Columns and bars represent the mean ± SEM; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

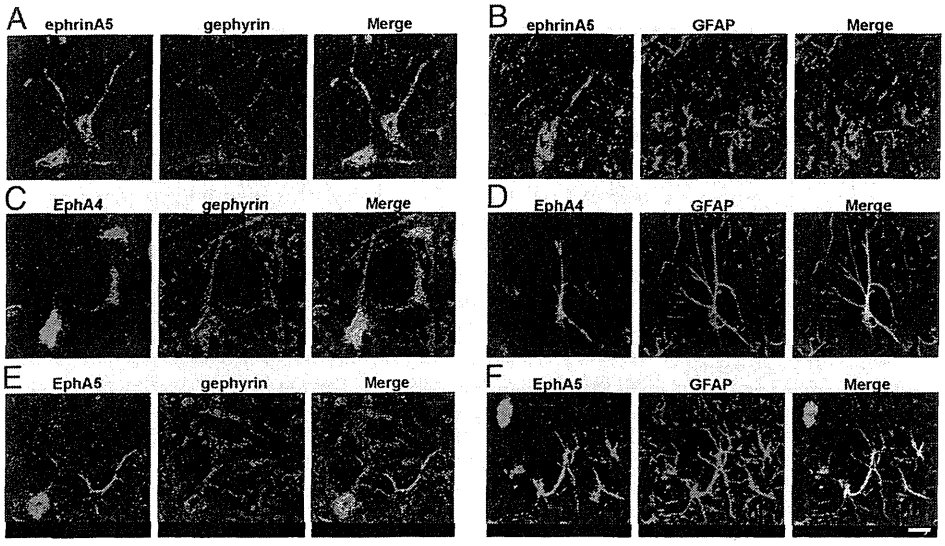

Fig. 2. Immunohistological analysis of ephrinA5, EphA4, and EphA5 in the SNr cells. Coronal sections of the SNr of WT mice were double-immunostained with the following antibodies and visualized by confocal microscopic analysis: (*A* and *B*) green, ephrinA5; (*C* and *D*) green, EphA4; (*E* and *F*) green, EphA5; (*A*, *C*, and *E*) red, gephyrin; (*B*, *D*, and *F*) red, GFAP. (Scale bar, 10 μm.)

Effects of EphrinA5, EphA4, and EphA5 on Cocaine Sensitization. Because the above results indicate that the deficit of the direct-pathway transmission selectively up-regulated the ephrin-Eph signaling molecules in the SNr of the cocaine-treated mice, we next addressed whether the activation of this signaling could suppress the adaptive response induced by repeated cocaine administration. To address this question, we used specific immunoadhesin chimeras, which were the fusion proteins consisting of the Fc domain of human IgG and the respective extracellular binding domain of the ephrin or the Eph receptors (15, 16). These immunoadhesins were the dimerized forms, which activate the corresponding receptors or ligands (17, 18). The immunoadhesin or the control Fc was attached to fluorescent microspheres to prevent diffusion into other brain regions (19).

The immunoadhesin- or the control Fc-attached microspheres were bilaterally injected into the SNr of WT mice, and restricted injection into the SNr was confirmed by visualization of the microsphere fluorescence in brain slices of individual mice killed after behavioral analysis. Four days after injection of the immunoadhesins, cocaine (10 mg/kg) was daily administered for 4 d and locomotor activity was measured immediately after each cocaine administration. Repeated cocaine administration induced a progressive increase in locomotor activity, called locomotor sensitization (9). Both EphA4-Fc and EphA5-Fc significantly suppressed cocaine-induced locomotor sensitization compared with that of the control Fc-injected mice (Fig. 4 *B* and *C*) (analyzed by repeated-measure ANOVA: between EphA4-Fc (n = 10) and control Fc (n = 6), for immunoadhesin, $P < 0.005$; for day, $P < 0.005$; for interaction immunoadhesin × day, $P < 0.01$; between EphA5-Fc (n = 8) and control Fc(n = 6), for immunoadhesin, $P < 0.005$; for day, $P < 0.005$; for interaction immunoadhesin × day, $P < 0.05$). EphrinA5-Fc showed no statistically significant suppression of cocaine-induced hyperlocomotion, as analyzed by repeated-measure ANOVA but tended to reduce locomotor sensitization on days 3 and 4 (Fig. 4*A*). These results indicate that the EphA4 and EphA5 receptors in the SNr play an important role in controlling adaptive responses to repeated administration of cocaine.

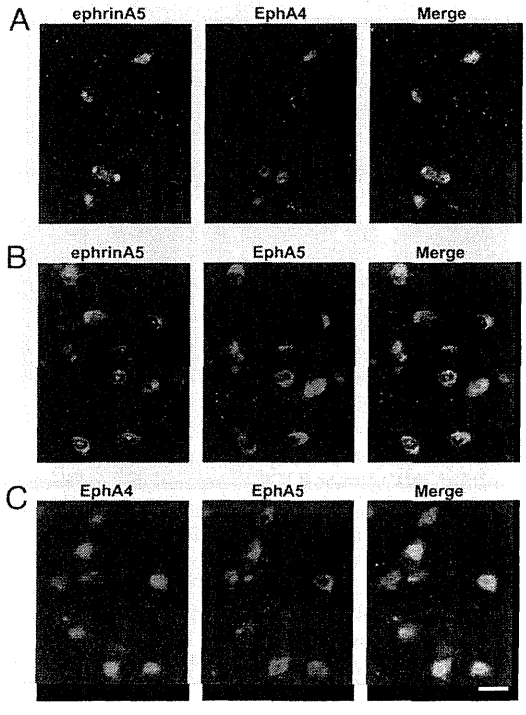

Fig. 3. Coexistence of ephrinA5, EphA4, and EphA5 in the SNr neurons. Coronal sections of the SNr were double-immunostained with the following antibodies and visualized by light microscopic analysis: (*A*) ephrinA5 (green) and EphA4 (red); (*B*) ephrinA5 (green) and EphA5 (red); (*C*) EphA4 (green) and EphA5 (red). (Scale bar, 50 μm.)

Erk Phosphorylation in EphrinA5-Positive Cells Specific to the Cocaine-Treated D-RNB Mice. Both EphA4 and EphA5 bind to ephrinA5, and this binding reversely stimulates the phosphorylation of the MAP kinases, Erk1 and Erk2, in ephrinA5-bearing cells (20). Therefore, we addressed whether cocaine could enhance phosphorylation of Erk1/2 in ephrinA5-bearing neurons specific to the SNr of D-RNB mice. The SNr of D-RNB, I-RNB, or WT mice was analyzed by double immunostaining with antibodies against ephrinA5 and phospho-Erk1/2 (pErk1/2) after acute or chronic cocaine administration (Fig. 5 *A–I*). The numbers of cells positive for ephrinA5 or pErk1/2 were counted, and the ratio of pErk1/2-ephrinA5 double-positive cells to ephrinA5-positive cells was calculated. This ratio markedly increased in D-RNB mice in the acute phase of cocaine administration (D-RNB, 69.0 ± 3.8%; I-RNB, 37.0 ± 1.4%; WT, 33.2 ± 2.3%; $P < 0.001$, D-RNB vs. I-RNB or WT) (Fig. 5*J*). Similarly, this ratio significantly increased in D-RNB mice at the chronic phase of cocaine

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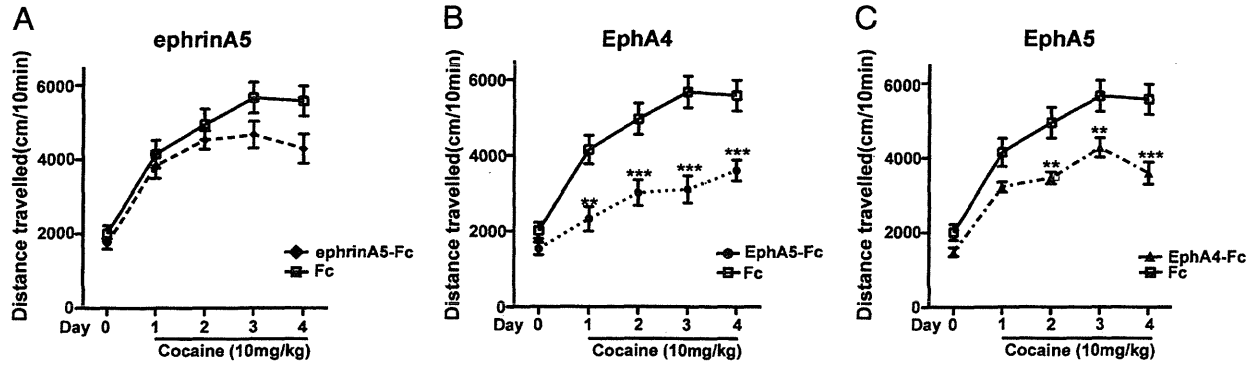

Fig. 4. Suppression of cocaine-induced hyperlocomotion by EphA4 and EphA5 in the SNr. Fluorescent microspheres with attached ephrinA5-Fc (*A*), EphA4-Fc (*B*), EphA5-Fc (*C*), or control Fc (*A–C*) were bilaterally injected into the SNr of WT mice. One day after immunoadhesin injection,. animals received intraperitoneal saline once a day and were habituated for 3 d. Cocaine (10 mg/kg) was then intraperitoneally injected once a day from day 1 to day 4; and immediately after each cocaine injection, locomotor activity was counted for a 10-min period. Symbols and bars represent the mean ± SEM (ephrinA5-Fc, n = 14; EphA4-Fc, n = 10; EphA5-Fc, n = 8; control Fc, n = 6). Statistical significance was analyzed by repeated-measure ANOVA; $^{**}P < 0.01$, $^{***}P < 0.001$ (EphA4-Fc or EphA5-Fc vs. control Fc).

administration (D-RNB, 70.4 ± 7.8%; I-RNB, 37.8 ± 2.9%; WT, 31.8 ± 7.8%; $P < 0.001$–0.01, D-RNB vs. I-RNB or WT) (Fig. 5*K*). Importantly, there was no difference in the relative ratio of two types of cells in three groups of saline-treated mice (Fig. 5 *J* and *K*). Upon double immunostaining for NeuN, a marker of mature neurons (21), ephrinA5-immunoreactive cells amounted to 94% to 98% of the NeuN-positive cells in all three groups of mice, regardless of treatment or not with cocaine. Thus, there

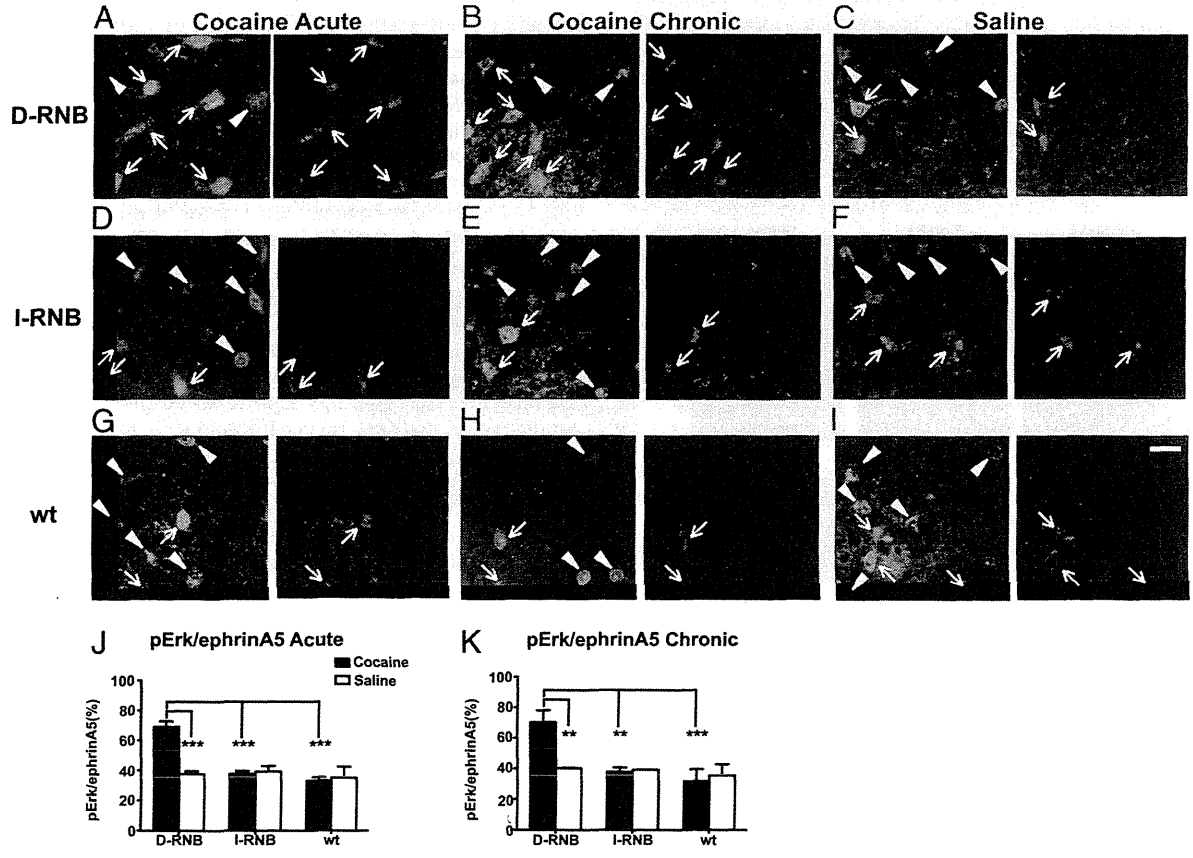

Fig. 5. Activation of Erk1/2 in ephrinA5-expressing SNr neurons specific to D-RNB mice. For Cocaine Acute (*A*, *D*, and *G*) and Saline (*C*, *F*, and *I*), D-RNB, I-RNB, and WT mice received a single intraperitoneal injection of cocaine (10 mg/kg) and saline, respectively, and the SNr was isolated 6 h after cocaine or saline injection. For Cocaine Chronic (*B*, *E*, and *H*), three groups of mice daily received a single intraperitoneal injection of cocaine (10 mg/kg) for 5 d and the SNr was isolated 1 h after cocaine injection. Coronal sections were double-immunostained with antibodies against ephrinA5 (green) and pErk1/2 (red) and visualized by light microscopic analysis. Arrows and arrowheads indicate the pErk1/2-positive and pErk1/2-negative cells, respectively, that were also immunopositive for ephrinA5. (Scale bar, 50 μm.) (*J* and *K*) The numbers of Erk1/2-immunopositive and Erk1/2-immunonegative cells among the ephrinA5-immunoreactive cells were counted, and the ratios of pErk1/2-ephrinA5 double-immunopositive cells to ephrinA5-immunoreactive cells are indicated in *J* and *K*. Columns and error bars represent the mean ± SEM (n = 4 each). The statistical significance was analyzed by one-way ANOVA. $^{**}P < 0.01$, $^{***}P < 0.001$.

was a good correlation in the pathway specificity between the regulation of the EphA4/EphA5-ephrinA5 system and phosphorylation of Erk1/2 in the SNr neurons. This correlation suggests that the Erk1/2 signaling downstream of ephrin actions in the SNr is important for the cocaine-induced behavioral responses.

Discussion

The principal striatal neurons receive inputs from the cerebral cortex and thalamus and send their inputs to the SNr through two parallel pathways (1, 7). In the basal ganglia circuit, cocaine inhibits the dopamine transporter and massively increases dopamine levels in the striatum and the NAc (8). This rapid increase in dopamine activates both the low-affinity D1 receptor in the direct pathway and the high-affinity D2 receptor in the indirect pathway (22). The chronic cocaine exposure then persistently activates the D1 and D2 receptors and differentially induces long-term potentiation at striatonigral neurons of the direct pathway and long-term depression at striatopallidal neurons of the indirect pathway (23). The long-term potentiation of the direct pathway is thought to be critical for inducing the adaptive response to chronic cocaine exposure (9, 23). However, the pathway-specific regulatory mechanisms of cocaine actions at the convergent SNr remained to be clarified. This investigation has revealed an important mechanism, in which the ephrinA5 ligand-EphA4/EphA5 receptors are regulated in the SNr via a direct pathway-specific mechanism in both the acute and chronic phases of cocaine responses. These ephrin-Eph molecules were up-regulated specifically by blocking inputs of the direct pathway after cocaine administration. Conversely, the EphA4 and EphA5 receptors in the SNr suppressed adaptive response to repeated cocaine administration. Furthermore, cocaine exposure activated the Erk1/2 signaling cascade in ephrinA5-expressing SNr cells in a direct pathway-dependent manner. These results indicate that the ephrinA5-EphA4/EphA5 signaling molecules are specifically regulated by inputs of the direct pathway and play an important role in the acute and adaptive responses to cocaine exposure.

The ephrin-Eph system consists of the large family of both ephrins and Eph receptors and controls a large variety of cellular responses, including contact-mediated attraction or repulsion, synapse formation, spine morphogenesis, and neural plasticity (24, 25). One of the characteristic features of the ephrin-Eph system is its bidirectional signaling cascade, in which the interaction of ephrin with the Eph receptor induces a forward signaling in the Eph-bearing cells and simultaneously elicits a reverse signaling in the ephrin-bearing cells (20, 26). Although ephrinA5, EphA4, and EphA5 were all up-regulated by blocking the direct pathway at least at the acute phase of cocaine administration, activation by EphA4 and EphA5 was more effective than activation by ephrinA5 in suppressing the cocaine sensitization. This reverse signaling could thus play a predominant role in the transmission regulation of the direct pathway in the SNr. This regulation may occur by interaction of the presynaptic EphA4/EphA5 of striatal cells (27, 28) and the postsynaptic ephrinA5 of the SNr neurons. Astrocytes also highly express EphA4 and EphA5, which may stimulate ephrinA5 in neurons (26). Recently, the ephrin-Eph *cis* interaction within the same cellular membrane has been shown to transduce a key signaling in the ephrin-Eph system (26, 29, 30). Because both ephrinA5 and EphA4/EphA5 are commonly distributed in most of the SNr neurons, the *cis* interaction of this system could be involved in the direct pathway-specific regulation of cocaine responses. Whatever the mechanisms of the ephrinA5-EphA4/EphA5 system in the SNr, our finding that the Erk1/2 signaling is pathway-specifically regulated in ephrinA5-expressing cells strongly suggests that the ephrinA5-EphA4/EphA5–expressing SNr neurons play an important role in cocaine-induced input transmission of the direct pathway.

No alteration of the ephrinA5-EphA4/EphA5 system was observed in saline-treated D-RNB mice, indicating that the observed changes in this ephrin-Eph system were linked to the action of cocaine and not a consequence of impaired transmission per se of the direct pathway. Our previous study using the RNB technique revealed that blockade of either the direct or the indirect pathway abolished the acute cocaine response (9). The dual stimulation of the two pathways is thus necessary for the rapid response to cocaine administration (9). In the chronic response to repeated cocaine administration, blockade of the direct pathway—but not that of the indirect pathway—severely impaired cocaine-induced adaptive responses, indicating that the direct pathway plays a predominant role in input transmission for the adaptive response to cocaine (9). However, despite the defectiveness of the acute response by blockade of either of the two pathways (9), up-regulation of the ephrinA5-EphA4/EphA5 system as well as activation of Erk1/2 was observed in a direct pathway-selective manner at both acute and chronic phases of cocaine administration. The ephrinA5-EphA4/EphA5 system is thus most likely to contribute to triggering the acute response and then inducing the adaptive response to cocaine actions. The cellular response to ephrin-Eph engagement is often repulsive between the two cells, although a repulsive or attractive response depends on the cellular context (26). This ephrin-Eph system is also important for cell-cell communication by controlling spine morphogenesis and neural plasticity (18, 24, 26, 31). Present findings of the pathway-selective ephrin-Eph engagement in cocaine-induced responses thus shed light on the action of cocaine and would provide valuable therapeutic targets for the treatment of drug addiction.

Materials and Methods

Animals and Behavioral Analysis. All animal handling procedures were performed according to the guidelines of the Osaka Bioscience Institute. The RNB mice, in which transmission of either the direct or the indirect pathway was selectively blocked, was generated as described previously (9). Briefly, the expression of TN was driven in the TN mice by the TRE and induced by interaction with the tTA (32). The expression of tTA was restricted to the direct or indirect pathway by injecting one of two types of the recombinant AAVs into the NAc, in which tTA was exclusively expressed in either the direct or the indirect pathway under the control of the substance P promoter or the enkephalin promoter, respectively (9). The recombinant AAV was bilaterally injected into four sites of the NAc by stereotaxic techniques (9). The RNB mice and their WT littermates were used for all experiments.

Locomotor activity was measured with an infrared activity monitor (MED Associates). For measurement of cocaine-induced hyperlocomotion, animals received intraperitoneal saline once a day and were habituated to a novel chamber for 3 d. Cocaine (10 mg/kg) or saline was then intraperitoneally injected once a day from day 1 to day 4, and immediately thereafter the locomotor activity was counted for a 10-min period.

Microdissection of the SNr. One hour after cocaine or saline administration, mice were killed, and frozen coronal sections (40 μm) were obtained from the brain embedded in OCT compound. Microdissection was performed by using a Micro Dissector PPMD (Eppendorf), consisting of a 1-mm diameter stainless-steel needle (Eppendorf) set at a 45° angle to the surface of the microscope table. A micropipette was mounted on a 3-axis–controlled, motorized micromanipulator (Eppendorf) attached to the microscope. After cryosections were covered with a pool of 15 μL of xylene for visualization, the SNr was dissected as an ultrasonically oscillating needle was moved along a selected tissue area.

Microarray Analysis. Total RNA of dissected SNrs was extracted with the reagents of an RNeasy Mini Kit (Qiagen) after evaporation of xylene in a vacuum concentrator. Approximately 5 ng of total RNA was labeled by using GeneChip Two-Cycle Target Labeling and Control Reagents (Affymetrix). Hybridization signals were calculated by analyzing raw data with Microarray Suite 5.0 (Affymetrix) and further analyzed with GeneSpring GX 11.0 software (Agilent Technologies) and Ingenuity Pathway Analysis 6.0 software (Ingenuity Systems). The data were normalized to the 75th percentile for per-chip normalization.

Quantitative RT-PCR. Reverse transcription was carried out by using the SuperScript First-Strand Synthesis System (Affymetrix) with the T7-oligo(dT) primer. cDNAs thus synthesized were amplified by a cycle of T7 amplification by using the MEGAscript High Yield Transcription Kit (Applied Biosystems). Specific primers were designed to generate 60- to 150-bp PCR products corresponding to the 3′ region of each mRNA. All reactions were performed in duplicate, and β-actin mRNA was used as an internal control for mRNA quantification.

Immunohistological Analysis. Immunohistochemistry of frozen coronal sections (20 μm) of the adult mouse brain was performed as described by Schneider Gasser et al. (33) by using the primary antibodies against ephrinA5 (Abcam), EphA4, EphA5 (for both, Abcam or Santa Cruz), pErk1/2 (Santa Cruz), GAD67, TH (Millipore), gephyrin (Synaptic Systems), and GFAP (Sigma Aldrich). The secondary antibodies used were Alexa488- or Alexa594-conjugated goat IgG (Molecular Probes), and specific immunoreactivity was confirmed by performing immunohistochemical analysis without addition of the primary antibody.

Immunoadhesin Analysis. Three different immunoadhesins were used; that is, fusion proteins consisting of the binding domain of either ephrinA5, EphA4, or EphA5 attached to the Fc domain of human IgG (R&D Systems). To prevent diffusion of immunoadhesins into other brain regions, we attached fluorescent microspheres (Lumafluor) to each immunoadhesin, as described by Riddle et al. (19). Immunoadhesin was injected stereotaxically at four sites in the SNr of WT mice (3.4-mm and 3.6-mm posterior to the bregma, ± 1.5-mm lateral from the midline, 4.0-mm depth from the dura). Four days after immunoadhesin injection, locomotor activity was measured immediately after daily administration of cocaine (10 mg/kg). After the behavioral analysis, injection sites of immunoadhesins were confirmed by visualization of immunoadhesin-attached fluorescent microspheres in the SNr of brain-slice preparations.

Statistical Analysis. Statistical analysis was conducted by using Graph Pad PRISM 5.0 (GraphPad Software). Data were analyzed by one-way ANOVA or repeated-measure ANOVA and were presented as the mean ± SEM.

ACKNOWLEDGMENTS. This work was supported by Research Grants KAKENHI 22220005 (to S.N.), 22659069, and 23110522 (to T.H.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, Grant on Regulatory Science of Pharmaceuticals and Medical Devices from the Ministry of Health and Labour and Welfare (to T.H.), a grant from the Japan Science and Technology Agency Precursory Research for Embryonic Science and Technology Program (T.H.), and grants from the Takeda Science Foundation (to S.N.) and Daiichi-Sankyo Foundation of Life Science (to T.H.)

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Neuron
Article

Distinct Roles of Synaptic Transmission in Direct and Indirect Striatal Pathways to Reward and Aversive Behavior

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DOI 10.1016/j.neuron.2010.05.011

SUMMARY

In the basal ganglia, convergent input and dopaminergic modulation of the direct striatonigral and the indirect striatopallidal pathways are critical in rewarding and aversive learning and drug addiction. To explore how the basal ganglia information is processed and integrated through these two pathways, we developed a reversible neurotransmission blocking technique, in which transmission of each pathway was selectively blocked by specific expression of transmission-blocking tetanus toxin in a doxycycline-dependent manner. The results indicated that the coordinated modulation of these two pathways was necessary for dopamine-mediated acute psychostimulant actions. This modulation, however, shifted to the predominant roles of the direct pathway in reward learning and cocaine sensitization and the indirect pathway in aversive behavior. These two pathways thus have distinct roles: the direct pathway critical for distinguishing associative rewarding stimuli from nonassociative ones and the indirect pathway for rapid memory formation to avoid aversive stimuli.

INTRODUCTION

The basal ganglia are the key neural substrates that control motor balance and reward-based learning (Graybiel, 2000; Wickens et al., 2003). Dysfunction of the basal ganglia leads to devastating neurological disorders such as Parkinson's disease, Huntington's disease, and drug addiction (Albin et al., 1989; Chesselet and Delfs, 1996; Hyman et al., 2006). The striatal projection neurons are GABA-containing medium-sized spiny neurons (MSNs), which are divided into two subpopulations, i.e., striatonigral neurons in the direct pathway and striatopallidal neurons in the indirect pathway (Albin et al., 1989; Alexander and Crutcher, 1990; Graybiel, 2000). The inputs of these two pathways converge at the substantia nigra pars reticulata (SNr) and control the dynamic balance of the basal ganglia-thalamocortical circuitry (Deniau et al., 2007; Graybiel, 2000). Accumulated evidence has indicated vast differences in the expression profiles of functional proteins and intracellular signaling molecules between these two subpopulations of MSNs (Gerfen et al., 1990; Heiman et al., 2008; Surmeier et al., 2007; Valjent et al., 2009). The striatonigral neurons selectively express substance P (SP) and D1 dopamine receptor, in marked contrast to the confined expression of enkephalin (Enk) and D2 dopamine receptor in the striatopallidal neurons (Gerfen et al., 1990; Graybiel, 2000; Surmeier et al., 2007). Gene targeting and pharmacological studies have shown that the D1 receptor-expressing direct pathway acts more predominantly than the D2 receptor-expressing indirect pathway in adaptive responses to cocaine and other dopamine agonists (Baker et al., 1996, 1998; Caine et al., 2007; Smith et al., 2002; Welter et al., 2007; Xu et al., 2000, but see also Miner et al., 1995). However, these techniques would have global effects on many other brain regions as well. Furthermore, two types of MSNs are morphologically indistinguishable and exist in a similar number (Surmeier et al., 2007). This similarity has made it extremely difficult to determine how the neural information is processed and integrated through these two pathways.

To explore the regulatory mechanisms of the basal ganglia function, we adopted a gene-manipulating technique termed reversible neurotransmission blocking (RNB) (Wada et al., 2007; Yamamoto et al., 2003). For this technique, we used previously developed transgenic mice (TN mice), in which the expression of tetanus toxin light chain (TN) was controlled by the tetracycline-responsive element (TRE) in a tetracycline-derivative doxycycline (DOX)-dependent manner (Wada et al., 2007; Yamamoto et al., 2003). TN is a bacterial toxin that cleaves the synaptic-vesicle-associated VAMP2 protein (Schiavo et al., 1992) and abolishes neurotransmitter release from synaptic vesicles (Wada et al., 2007). SP and Enk are specifically expressed in striatonigral and striatopallidal neurons, respectively, but are also distributed in other brain regions (Cuello and Kanazawa, 1978; Miller and Pickel, 1980). To restrict the expression of the tetracycline-repressive transcription factor (tTA) to striatonigral and striatopallidal neurons, we combined with the adeno-associated virus- (AAV-) mediated gene expression system, which has been reported to effectively express incorporated transgenes in MSNs and modify properties of these cells in a transgene-dependent manner (Lerchner et al., 2007; Pulipparacharuvil et al., 2008). We constructed two types of AAVs, termed V-S-tTA and V-E-tTA, in which expression of tTA was

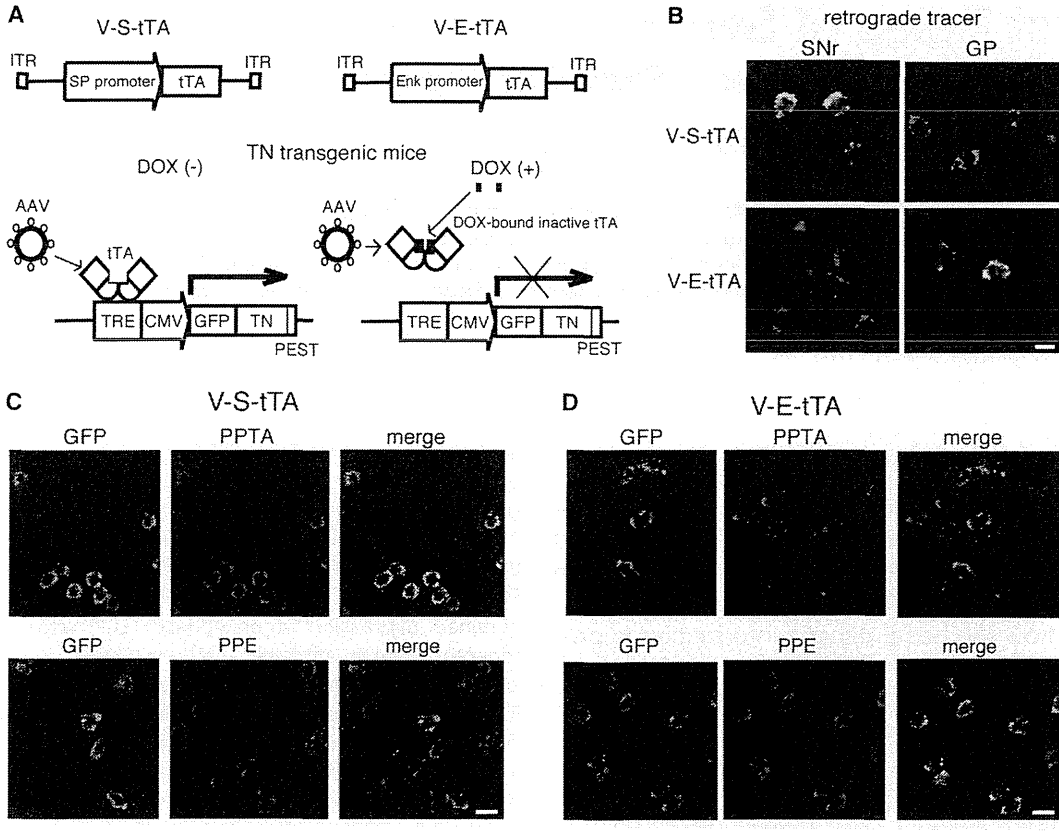

Figure 1. RNB Technique and Selective Expression of TN in Striatonigral or Striatopallidal Neurons

(A) Schema of cell-type-specific reversible expression of TN. The V-S-tTA and V-E-tTA viruses incorporated the *flag*-tagged *tTA* gene following the SP and Enk promoters, respectively. The TN transgenic mice contained the *GFP-TN* fusion gene. When the striatum was transfected with the recombinant virus, the expression of the GFP-TN was driven by the interaction of tTA with the TRE under the DOX-free condition, but this expression was abolished by DOX treatment. The expression of TN was confined to the striatonigral and the striatopallidal neurons by transfection with V-S-tTA and V-E-tTA, respectively. ITR, inverted terminal repeat; CMV, cytomegalovirus promoter; PEST, the degradation-facilitating PEST sequence.

(B) Cell-type-specific expression of flag-tTA. The CTB-Alexa 594 retrograde tracer was injected into the SNr or GP of the V-S-tTA-transfected and V-E-tTA-transfected wild-type mice. Two weeks after the injection, coronal sections of the striatum were immunostained with anti-flag antibody, and the retrograde tracer (red) and the flag-tagged tTA (green) were visualized. Scale bar, 10 μm.

(C and D) Cell-type-specific expression of TN. Coronal sections of the V-S-tTA-transfected (C) and the V-E-tTA-transfected TN mice (D) were double-immunostained with the GFP and the indicated antibodies. Scale bar, 10 μm.

directed by the SP or the Enk promoter, respectively. When these recombinant viruses were injected into the striatum of TN mice, TN was specifically expressed and in turn separately blocked neurotransmission in striatonigral and striatopallidal neurons. This investigation has indicated that transmission blockade of either of the two pathways abolished the psychostimulant-induced acute responses but that the blockade of the direct and indirect pathways caused selective impairments of the reward-based and aversive behavior, respectively. Synaptic transmission of the two pathways is thus coordinated, but each plays a distinct role in controlling adaptive mechanisms involved in the basal ganglia function.

RESULTS

Cell-Type-Specific Expression of TN

The selective and reversible blockade of synaptic transmission of the two types of MSNs in vivo was achieved by combining the TN transgenic mice (Wada et al., 2007; Yamamoto et al., 2003) with the recombinant AAV transfection technique (Figures 1A and S1, available online). In the TN mice, the expression of the fusion protein of TN and green fluorescent protein (GFP) was driven by the TRE and was induced by interaction with the tTA (Figure 1A). The AAV-mediated gene expression system (V-S-tTA or V-E-tTA) was used to restrict the SP- or Enk-derived expression of tTA within the striatum (Figure 1A). With this strategy, the SP or Enk-promoter-derived tTA would activate expression of the GFP-TN transgene in specific striatal neurons. The administration of DOX would then terminate the expression of TN and allow recovery of synaptic transmission through newly synthesized VAMP2 (Figure 1A).

The success of our strategy relied on accurate and mutually exclusive expression of TN in one of the MSN types. We first examined whether the SP and Enk promoters would faithfully direct the expression of tTA in striatonigral and striatopallidal neurons, respectively, in wild-type mice (Figure 1B). The V-S-tTA or

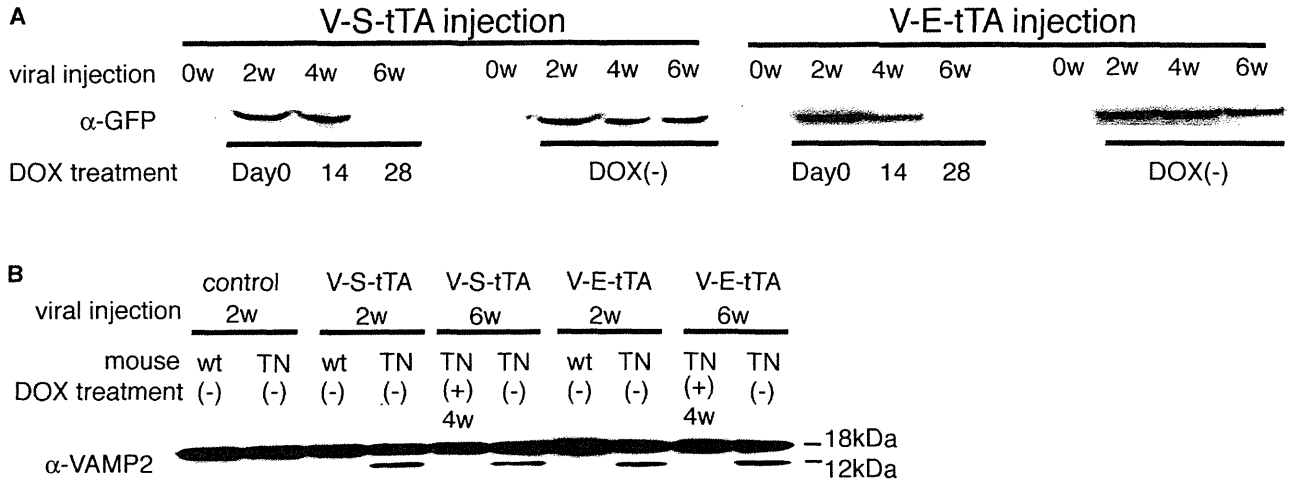

Figure 2. Reversible Expression of TN

(A) Two weeks after the viral injection, TN mice were treated or not with DOX. At the indicated days after the viral injection or DOX treatment, the striatum was isolated, and cell lysates were prepared and then immunoblotted with the GFP antibody.

(B) Animals were treated as in (A), and striatal cell lysates were prepared and then immunoblotted with the N-terminal VAMP2 antibody.

V-E-tTA virus was injected into the striatum. The cholera toxin subunit B (CTB)-Alexa 594 retrograde tracer was then injected into either the globus pallidus (GP) or the SNr of these mice. Two weeks after the injection, the retrograde tracer and the flag-tagged tTA were visualized in serial sections of the striatum by fluorescence and immunostaining with anti-flag antibody, respectively (Figure 1B). Immunoreactivity of the flag-tagged tTA was exclusively seen in striatonigral neurons of the V-S-tTA-transfected mice, which were retrogradely labeled from the SNr; the tTA-positive cells were 68.6% ± 1.0% of retrogradely labeled cells (n = 3). Conversely, the immunoreactivity was restricted to the striatopallidal neurons of the V-E-tTA-transfected mice, which were retrogradely labeled from the GP; the tTA-positive cells were 66.2% ± 3.1% of retrogradely labeled cells (n = 3).

We then examined the tTA-dependent, cell-type-specific expression of TN by injecting the recombinant virus into the striatum of the TN and wild-type mice. Upon double immunostaining of the TN mice 2 weeks after V-S-tTA injection, immunoreactivity of the TN-GFP fusion protein was exclusively seen in SP-immunoreactive but not in Enk-immunoreactive neurons (Figure 1C); the GFP-immunoreactive cells were 74.2% ± 1.9% of SP-immunoreactive striatonigral neurons (n = 4). This immunoreactive colocalization was reversed in the V-E-tTA-transfected TN mice (Figure 1D); the GFP-immunoreactive cells were 71.1% ± 0.9% of Enk-immunoreactive striatopallidal neurons (n = 4). In controls, no GFP immunoreactivity was observed in any other striatal interneurons (Figure S2), nor in the virus-transfected wild-type striatum. Although viral tropism may also contribute to specific expression of transgenes in MSNs, the results indicated that TN was exclusively expressed in striatonigral or striatopallidal neurons, depending on the transfection with the V-S-tTA or V-E-tTA virus, respectively.

Reversibility of TN Expression and VAMP2 Cleavage

We next examined the DOX-mediated reversibility of TN expression and cleavage of VAMP2 in TN mice. The V-S-tTA or V-E-tTA virus or the tTA-free control virus was injected into one side of the striatum of wild-type and TN mice. Two weeks after the viral injection, DOX was continuously administered or the animals were left untreated. At 2, 4, or 6 weeks after the viral injection, the striatum was dissected from DOX-treated and DOX-untreated animals. No anatomical alterations with Nissl staining nor changes in patterns and intensities of immunoreactivity of dopamine transporter and hybridization signals of *SP* and *Enk* mRNAs were observed at the striatum in virus-transfected TN mice regardless of treatment or not with DOX (Figure S3). Immunoblot analysis showed intense GFP immunoreactivity in both the V-S-tTA-injected and the V-E-tTA-injected TN mice at least up to 6 weeks after the viral injection (Figure 2A). This immunoreactivity completely disappeared 4 weeks after DOX treatment in both cases (Figure 2A). No GFP immunoreactivity was seen in striatal sections transfected with the control AAV or in those of wild-type mice transfected with either the V-S-tTA or V-E-tTA (data not shown). The expression of TN was thus reversibly controlled in a DOX-dependent manner.

The reversibility of the TN-mediated cleavage of VAMP2 was examined by immunoblot analysis of striatal lysates with antibody against N-terminal VAMP2 (Figure 2B). The intact 18 kDa VAMP2 was significantly cleaved to the 12 kDa N-terminal fragment in TN mice with either V-S-tTA or V-E-tTA transfection. This cleavage disappeared 4 weeks after DOX treatment (Figure 2B). Upon densitometric measurement, cleavage of the 18 kDa VAMP2 was calculated to be about 30% in both virus-transfected TN mice (n = 2). If striatonigral and striatopallidal neurons are assumed to exist in a 1:1 ratio within the striatum, the densitometric calculation indicated that about two-thirds of the VAMP2 was cleaved in targeted cells in both viral transfections. This value was consistent with more than 70% of GFP-positive target cells in the virus-transfected striatum (Figures 1C and 1D). No VAMP2 cleavage was detected in the virus-transfected wild-type mice nor in the control virus-transfected TN mice (Figure 2B).

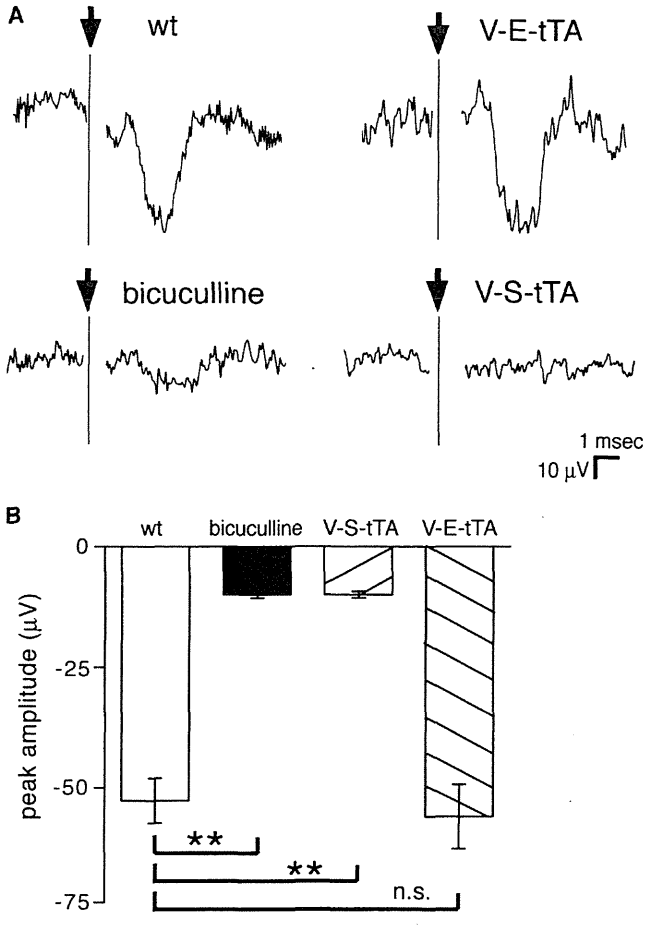

Figure 3. Selective Blockade of Striatonigral Transmission in the V-S-tTA-Transfected TN Mice

(A) Field potentials recorded in the SNr of wild-type and virus-transfected TN mice after striatal stimulation. For bicuculline treatment, 5 mM bicuculline methochloride was perfused into the SNr 15 min before striatal stimulation. The arrow denotes time of striatal stimulation.

(B) Peak amplitudes of bicuculline-sensitive, short-latency responses (<10 ms). Columns and error bars represent the mean ± SEM (n = 4–5). **p < 0.01; n.s., not significant.

Electrophysiological Characterization of the Virus-Transfected TN Mice

We tested transmission blockade of the virus-transfected TN mice by extracellular recordings of the GP or the SNr in anesthetized animals after electrical stimulation of the striatum. The striatal stimulation evoked a rapid and monophasic response in the SNr of wild-type mice (Figure 3A). This response was inhibited by perfusion with the GABA receptor antagonist bicuculline methochloride into the SNr (Figure 3A), confirming GABA-mediated transmission from the striatum to the SNr in wild-type mice. Importantly, the striatal stimulation failed to evoke an electrophysiological response in the V-S-tTA-transfected TN mice but induced a normal level of response in the V-E-tTA-transfected TN mice (Figures 3A and 3B). These results indicate that neurotransmission of the direct pathway was selectively blocked in the V-S-tTA-transfected TN mice. Extracellular recordings of the GP after striatal stimulation of anesthetized wild-type mice were also conducted, but this attempt failed to identify GABA-mediated transmission from the striatum to the GP. This failure was due to close localization of the GP from the striatum, so that electrical excitation within the striatum hampered identification of the striatum-stimulated response in the GP. However, the blockade of striatopallidal transmission was confirmed by a different approach that examined the striatopallidal transmission-mediated upregulation of c-*fos* mRNA in the ventral pallidum (VP) (see Figure 5E). These results demonstrate that neurotransmission of the direct and indirect pathways was selectively blocked in the V-S-tTA-transfected and the V-E-tTA-transfected TN mice, respectively.

Abnormal Turning Behavior by Blocking Either the Striatonigral or Striatopallidal Transmission

The two types of MSNs exert opposing effects on the SNr neurons (Deniau et al., 2007; Graybiel, 2000). Consequently, deficit of the striatonigral transmission on one side of the striatum induces abnormal turning ipsilateral to the impaired side, whereas deprivation of one side of the striatopallidal transmission elicits contralateral turning (Kaneko et al., 2000; Pycock, 1980) (Figure S4). To examine the DOX-dependent, reversible motor imbalance in the virus-transfected TN mice, we injected the recombinant virus unilaterally into the left side of the striatum. Two weeks after the injection, DOX was continuously administered or the animals were left untreated. The abnormal turning behavior was then analyzed by forcing the animals to rotate on a hemispherical container. In the absence of DOX treatment, unilaterally V-S-tTA-injected TN mice all showed rotation ipsilateral to the side of the viral injection (Figure 4A). Conversely, contralateral rotation was induced in unilaterally V-E-tTA-injected TN mice (Figure 4B). Consistent with the TN expression ontogeny (Figure 2A), the abnormal turning behavior remained up to 2 weeks and then disappeared 4 weeks after DOX treatment in both the V-S-tTA and V-E-tTA-injected TN mice (Figures 4A and 4B). In the virus-injected wild-type mice, the abnormal turning never occurred, irrespective of DOX treatment (Figures 4A and 4B). The abnormal turning was thus not only induced in a TN-expression-dependent manner but also was consistent with the coordinated role of the striatonigral and striatopallidal transmission in motor balance (Figure S4). The results described above all support the conclusion that synaptic transmission of the direct and indirect pathways was selectively and reversibly blocked in our model animals. We hereafter refer to the direct-pathway-blocked and the indirect-pathway-blocked TN mice as the D-RNB and I-RNB mice, respectively.

Critical Role of Both Striatonigral and Striatopallidal Transmission on Psychostimulant-Induced Acute Responses

Psychostimulants methamphetamine and cocaine both stimulate the dopamine system in the striatum and enhance acute responses of locomotor activity (Hyman et al., 2006). So we examined whether the methamphetamine-induced acute hyperlocomotion would be influenced in the D-RNB and I-RNB mice by bilateral injection of the V-S-tTA or V-E-tTA virus, respectively, into the striatum including the nucleus accumbens (NAc)

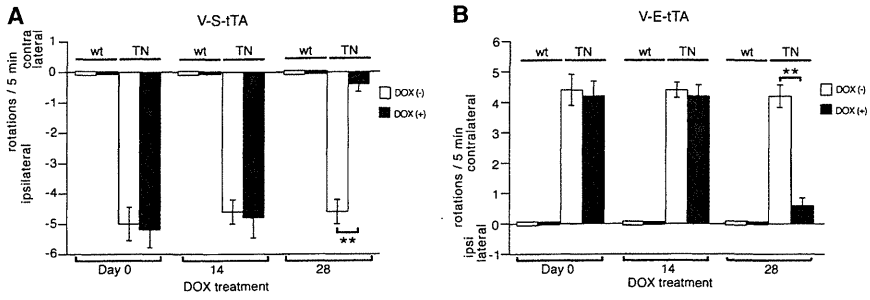

Figure 4. Abnormal Rotation of Unilaterally Virus-Transfected TN Mice

The V-S-tTA virus (A) or the V-E-tTA virus (B) was injected into the left striatum of the TN and wild-type mice. Two weeks after the viral injection, the animals were treated or not with DOX. Numbers of rotations were counted for a 5 min period at days 0, 14, and 28 after the start of the DOX treatment. Columns and error bars represent the mean ± SEM (n = 5 each). **p < 0.01.

(Figures 5A and 5B). The D-RNB and I-RNB mice showed no abnormal locomotor activity under the ordinary condition. The wild-type mice showed a significant methamphetamine-induced acute hyperlocomotion regardless of the viral transfection and DOX treatment. Similarly, hyperlocomotion was induced in the TN-transgenic mice before the viral injection. Remarkably, blockade of either the direct or the indirect pathway abrogated the methamphetamine-induced acute hyperlocomotion. Importantly, when DOX was administered for 4 weeks, the methamphetamine-induced hyperlocomotion recovered in both the D-RNB and I-RNB mice to levels comparable to those of the wild-type mice. In controls, the injection of saline alone had no effect on locomotor activity in any experimental stages of the D-RNB or I-RNB mice.

The cocaine-induced acute hyperlocomotion was also examined by bilateral viral injection into the NAc, the ventral part of the striatum that is the central neural substrate for cocaine actions (Di Chiara and Imperato, 1988) (Figures 5C and 5D). In remarkable contrast to the wild-type mice, neither the D-RNB mice nor the I-RNB mice showed the cocaine-induced acute hyperlocomotion immediately after a single cocaine administration. Synaptic transmission of both pathways is thus required for psychostimulant-enhanced acute responses.

The dopamine stimulation induced by cocaine administration excites the pallidal neurons of the indirect pathway and suppresses the SNr neurons, which receive convergent inputs from the direct and indirect pathways (Albin et al., 1989; Graybiel, 2000). This modulatory effect on the VP and SNr neurons was examined by quantitative in situ hybridization analysis of the activity-dependent up- and downregulation of c-*fos* mRNA at the corresponding brain regions 30 min after cocaine administration (Marshall et al., 1998). This analysis showed that c-*fos* mRNA was upregulated in the VP of both the wild-type and the D-RNB mice but not in the VP of the I-RNB mice (Figure 5E). Furthermore, the cocaine treatment downregulated c-*fos* mRNA in the SNr of the wild-type mice but failed to show such downregulation in both the D-RNB and I-RNB mice (Figure 5F). This finding further confirmed the conclusion that the input transmission from the striatum/NAc to the SNr was impaired by selective blockade of the direct and indirect pathways in the D-RNB and I-RNB mice, respectively.

Differential Control of Cocaine-Induced Adaptation by Striatonigral and Striatopallidal Transmission

Repeated cocaine administration induces neural adaptation of the dopamine system and causes a progressive increase in locomotor activity, called locomotor sensitization (Hikida et al., 2001, 2003; Kalivas and Stewart, 1991). When the recombinant virus was bilaterally injected into the NAc, the I-RNB mice showed delayed cocaine-induced sensitization but reached hyperlocomotor levels comparable to those of the wild-type mice on days 3–5 (Figure 6B). In contrast, the D-RNB mice showed not only reduced locomotor activity in the early stage but also markedly attenuated locomotor sensitization by repeated cocaine administration (Figure 6A). The partial reduction in locomotor sensitization could have resulted from insufficient transfection of the NAc with the V-S-tTA virus; but this interpretation seems to be unlikely, because a large number of the SP-immunoreactive striatonigral cells were GFP positive (Figure 1C), and acute cocaine-induced hyperlocomotion was abolished in the D-RNB mice (Figure 5C).

The adaptive response to chronic cocaine administration persists even after omission of cocaine administration (Grimm et al., 2001; Hikida et al., 2003). To examine this long-lasting adaptive response, we treated animals with cocaine for 5 days and then treated them with DOX from day 6 to day 33 in the absence of cocaine administration (Figures 6A and 6B). They were then challenged with cocaine administration on day 33. In both the wild-type and I-RNB mice, the locomotion was significantly higher on day 33 than the initial cocaine-induced locomotion on day 1 (Figure 6B). Importantly, the cocaine-induced locomotion of the D-RNB mice was strikingly higher on day 33 than the locomotion on day 5 and became comparable to that of the wild-type mice (Figure 6A). These results indicate that the adaptive mechanism remained effective even when synaptic transmission was blocked in the striatonigral and striatopallidal neurons.

The distinct influences of the two pathways on the adaptive responses were further analyzed by conditioned place preference (CPP), which is NAc-mediated associative learning (Tzschentke, 2007). Animals were conditioned by repeated cocaine administration in one of two chambers that differed visually and textually (Figures 6C and 6D). Before conditioning, all animals showed no preference in visiting the two chambers. After conditioning with cocaine for 3 days, blockade of the direct pathway significantly reduced the cocaine-induced CPP (Figure 6C). In contrast, the same manipulation of the indirect pathway showed the ability to induce CPP, which was comparable to that of the wild-type mice (Figure 6D). Both hyperlocomotion and CPP analyses indicate that the direct pathway plays a predominant role in adaptive behaviors of cocaine sensitization.

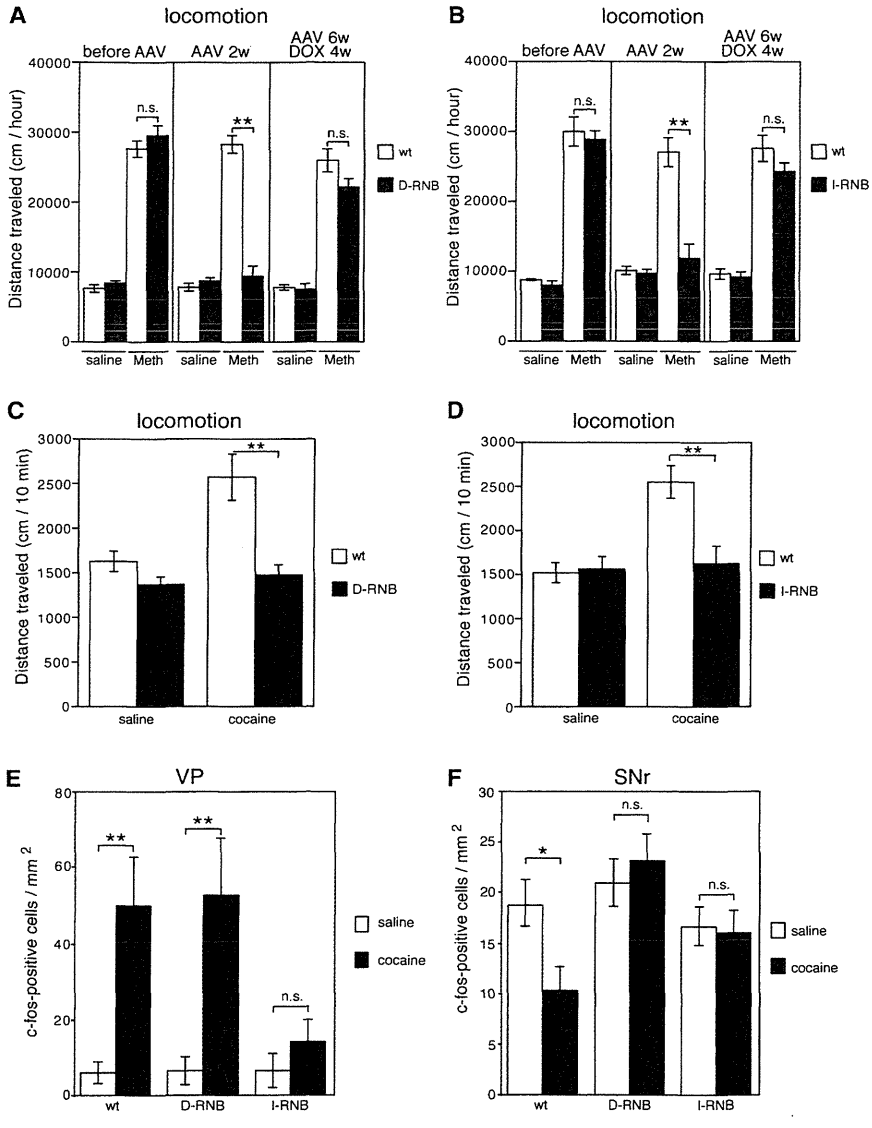

Figure 5. Effects of Blockade of Transmission on Methamphetamine-Induced and Cocaine-Induced Acute Responses

(A and B) Two weeks after bilateral injection of the V-S-tTA virus (A) or the V-E-tTA virus (B) into the striatum, the animals were treated or not with DOX for 4 weeks (n = 5 or 6). Locomotion was measured for 60 min immediately after i.p. injection of methamphetamine (Meth, 2 mg/kg) or saline before and 2 and 6 weeks after the viral transfection.

(C and D) Two weeks after the viral injection into the NAc, locomotion was measured for 10 min immediately after i.p. injection of cocaine (10 mg/kg) or saline (n = 8 each).

(E and F) Mice were treated with cocaine as in (C) and (D), and coronal sections of the brain were prepared 30 min after cocaine or saline treatment and subjected to in situ hybridization with [^{35}S]-labeled c-*fos* cRNA. c-*fos* mRNA-positive cells were counted in the VP (E) and the SNr (F) from four to six brain sections of two to four animals and averaged. Columns and error bars represent the mean ± SEM. *p < 0.05, **p < 0.01; n.s., not significant.

Distinct Roles of Striatonigral and Striatopallidal Transmission in Reward-Based and Aversive Behavior

Dopamine neurons recorded in vivo exhibit two different patterns of firings, i.e., a burst of phasic firing and a slow single-spike or tonic firing (Grace et al., 2007; Schultz, 2007). The phasic firing is responsible for stimulating the low-affinity D1 receptor and is believed to be the functionally relevant signal involved in reward-related behavior (Grace et al., 2007; Mirenowicz and Schultz, 1994). The tonic firing is crucial for modulating the high-affinity D2 receptor and has been reported to be suppressed by aversive stimuli (Grace et al., 2007; Mirenowicz and Schultz, 1996; Ungless et al., 2004). The D-RNB and I-RNB mice showed a normal preference for chocolate over a standard food, when they were freely given access to the two foods (Figure S5). So we extended the CPP test to the naturally occurring appetitive reward learning (Figures 7A and 7B). Animals were trained by pairing a standard food with one chamber and a palatable chocolate food with the other chamber. After conditioning for 3 days, the wild-type and the I-RNB mice learned to visit the chocolate-paired chamber with no statistical difference between the two groups (Figure 7B). In contrast, this learning ability was markedly impaired in the D-RNB mice (Figure 7A). The direct pathway is thus critical in the adaptive mechanism of the naturally occurring reward learning.

The effect of transmission blockade on aversive learning was then examined by the one-trial inhibitory avoidance task. In this task, mice received electric shocks, when they entered from a light chamber to a preferred dark chamber. Aversive behavior was then tested 24 hr later by measuring latencies, in which the mouse avoided entering an electrically shocked dark chamber. The wild-type mice avoided entering the dark chamber after experiencing electric shocks. This aversive behavior was not impaired in the D-RNB mice (Figure 7C). In contrast, the I-RNB mice failed to show aversive behavior and entered the dark chamber with no significant difference before and after electric shocks (Figure 7D). The indirect pathway is thus critical for evoking aversive behavior.

To exclude the possibility that the deficit in aversive behavior was due to an impaired fear system, we examined the freezing response after applying electric footshocks (Figure 7E). During the three 1 min intervals after electric footshocks, all three animals showed immediate postshock freezing that increased by repeated presentation of footshocks. There was no statistical difference in extent or pattern of freezing among wild-type, I-RNB, and D-RNB mice.