# Cell Type-Specific Alterations in the Nucleus Accumbens by Repeated Exposures to Cocaine

Juhyun Kim, Bong-Hyun Park, Joo Han Lee, Sang Ki Park, and Joung-Hun Kim

**Background:** The nucleus accumbens (NAc) is a brain region critically involved in psychostimulant-induced neuroadaptations. A major proportion of NAc neurons consists of medium spiny neurons (MSNs), commonly divided into two major subsets on the basis of their expression of D1 dopamine receptors (D1R-MSNs) or D2 dopamine receptors (D2R-MSNs). Although NAc MSNs are known to undergo extensive alterations in their characteristics upon exposure to drugs of abuse, the functional and structural changes specific to each type of MSN have yet to be fully resolved.

**Methods:** We repeatedly injected cocaine into transgenic mice expressing enhanced green fluorescent protein under the control of promoters for either D1R or D2R and then analyzed the physiological characteristics of each type of MSN by whole-cell recording. We also analyzed cocaine-induced changes of spine densities of individual MSNs with recombinant lentivirus in a cell type-specific manner and corroborated findings by use of a pathway-specific labeling using recombinant rabies virus.

**Results:** The D1R-MSNs exhibited decreased membrane excitability but increased frequency of miniature excitatory postsynaptic currents after repeated cocaine administration, whereas D2R-MSNs displayed a decrease in miniature excitatory postsynaptic current frequency with no change in excitability. Interestingly, miniature inhibitory postsynaptic currents decreased in D1R-MSNs but were unaffected in D2R-MSNs. Moreover, morphological analyses revealed a selective increase in spine density in D1R-MSNs after chronic cocaine exposure.

**Conclusions:** This study provides the first experimental evidence that NAc MSNs differentially contribute to psychostimulant-induced neuroadaptations by changing their intrinsic, synaptic, and structural characteristics in a cell type-specific fashion.

**Key Words:** Cocaine addiction, dendritic spine, dopamine receptor, intrinsic excitability, nucleus accumbens, synaptic transmission

The nucleus accumbens (NAc) is a primary target of the mesolimbic reward circuitry and has been extensively studied as an important site of action of psychostimulant drugs (1). The γ-aminobutyric acid–ergic medium spiny neurons (MSNs) in the NAc (comprising > 90% of total NAc neurons) receive dopaminergic innervations from the ventral tegmental area (VTA) and glutamatergic inputs from the prefrontal cortex, ventral hippocampus, and basolateral amygdala (2). Repeated exposure to drugs of abuse is known to induce persistent adaptive changes in intrinsic excitability and synaptic plasticity of NAc MSNs, which have been proposed to underlie psychostimulant-specific behaviors (3,4).

The NAc MSNs are commonly classified into two groups: the dopamine D1 receptor (D1R)-expressing MSNs (D1R-MSNs), and the dopamine D2 receptor (D2R)-expressing MSNs (D2R-MSNs) (5). Despite their spatial proximity in the NAc, the two types of MSNs differ in their intracellular responses to dopamine, gene expression profiles, and projection pathways (6–8). Recently, cell type-specific analysis for the D1R- and D2R-MSNs has been tried in the dorsal striatum with transgenic mice expressing enhanced green fluorescent protein (eGFP) under the control of promoters for either D1R or D2R (9). However, physiological changes in the NAc MSNs after repeated exposure to cocaine have not been explored, particularly in a cell type-specific manner.

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Received Jul 16, 2010; revised Dec 23, 2010; accepted Jan 12, 2011.

To investigate how D1R- and D2R-MSNs respond to repeated cocaine exposure, we examined both the intrinsic excitability and synaptic responses of each type of NAc MSNs. We found that both intrinsic membrane excitabilities and basal synaptic transmissions are differentially altered in D1R- versus D2R-MSNs and that spine density increases only in D1R-MSNs after cocaine exposure. Collectively, D1R- and D2R-MSNs in the NAc undergo distinct electrophysiological and structural modifications in response to cocaine exposure as cellular mechanisms that potentially lead to drug addiction and its associated behaviors.

## Methods and Materials

### Animals and Drug Treatment Paradigm

Dopamine $D1_a$ and D2 receptor promoter-dependent eGFP transgenic mice, developed by the GENSAT (Gene Expression Nervous System Atlas) project (9), came from the Mutant Mouse Regional Resource Center. Mice were housed under a 12-hour light/dark cycle and given ad libitum access to food and water. The eGFP-expressing transgenic mice (5–6-week-old, male) were used in electrophysiological studies and structural analyses using lentivirus, and their wild-type littermates were used for morphological analyses using recombinant rabies virus. Either heterozygote transgenic or wild-type mice were used in the locomotion test to confirm cocaine-induced behavioral responses. Mice received five once-daily injections of either saline or cocaine (15 mg/kg, IP) in their home cages. All procedures for animal experiments were performed in accordance with Pohang University of Science and Technology guidelines on animal care and use.

### Electrophysiology

Mice were decapitated, and the brains were quickly removed and chilled in ice-cold sucrose buffer containing (in mmol/L) 20 sodium chloride, 3.5 potassium chloride, 1.4 sodium dihydrogen phosphate, 26 sodium bicarbonate, 11 glucose, 175 sucrose, and 1.3 magnesium dichloride (pH 7.4). Coronal brain slices containing

0006-3223/$36.00
doi:10.1016/j.biopsych.2011.01.013

the NAc (300-μm thickness) were prepared with a vibratome (Leica, Wetzlar, Germany) and incubated in a bath solution containing (in mmol/L) 119 sodium chloride, 2.5 potassium chloride, 2 magnesium sulfate, 1.25 sodium dihydrogen phosphate, 26 sodium bicarbonate, 10 glucose, and 2.5 calcium dichloride (pH 7.3–7.4, equilibrated with 95% oxygen and 5% carbon dioxide). After at least 1 hour of recovery, whole-cell recordings were made from GFP(+)- and GFP(−)-MSNs in the NAc shell under infrared differential interference contrast optics (Leica).

The recording electrodes (5–10 MΩ resistance) were filled with corresponding internal solutions to each specific recording experiment (Supplement 1). Access resistance (20–40 MΩ) was not compensated, and cells were rejected if access resistance changed more than 20% during the experiment. Action potential firing frequency and rheobase were measured by separate applications of depolarizing current (1-sec steps) of 20-pA and 2-pA increments, respectively. Excitatory postsynaptic currents (EPSCs) were evoked by stimulating the prelimbic cortex/NAc shell border area at .05 Hz with a bipolar stimulation electrode. For the measurement of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid type glutamate receptor (AMPAR)/*N*-methyl-D-aspartic acid type glutamate receptor (NMDAR) ratio, AMPAR- and NMDAR-mediated EPSCs were obtained by measuring peak amplitudes at a −70-mV holding potential (AMPAR component) and current amplitudes 50 msec after EPSC onset at a +40-mV holding potential (NMDAR component) in the same cells. Paired-pulse ratios (PPRs) were measured at 25–200-msec interstimulus intervals and calculated as the ratio of the second EPSC amplitude to the first EPSC amplitude. The AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs) (> 100 events/cell) were collected at −70 mV in the presence of tetrodotoxin (1 μmol/L), picrotoxin (100 μmol/L), and 2- amino-5-phosphonovaleric acid (50 μmol/L), whereas miniature inhibitory postsynaptic currents (mIPSCs) were recorded in the presence of tetrodotoxin (1 μmol/L), 2- amino-5-phosphonovaleric acid (50 μmol/L), and 6-cyano-7-nitroquinoxaline-. *2*,3-dione (20 μmol/L). Quantal events with > 5 pA amplitude were selected for analysis and verified by eye. Spike firing and intrinsic membrane properties were analyzed with Clampfit 10.1 software (Molecular Devices, Sunnyvale, California), and the amplitude and frequency of mEPSCs and mIPSCs were analyzed with the Mini Analysis Program (Synaptosoft, Chapel Hill, North Carolina).

### Virus Infection and Spine Analyses

Recombinant lentivirus was produced and concentrated as previously described (10), and deletion-mutant rabies virus encoding eGFP (11) was kindly provided by Edward M. Callaway (Salk Institute for Biological Studies, La Jolla, California). Each mouse was anesthetized with ketamine and rompun, and a total amount of 2 μL/each side of viral solution was infused into either the NAc or the VTA with a stereotaxic frame (Kopf, Tujunga, California) (Supplement 1). After lentivirus and rabies virus infection of 18–20 days and 5–6 days, respectively, mice were subjected to repeated saline or cocaine injection. Twenty-four hours after the last injection, mice brains were collected after transcardiac perfusion of phosphate-buffered saline followed by 4% paraformaldehyde/phosphate-buffered saline, and 40-μm-thick coronal brain sections were prepared with a cryostat. In the experiment with monomeric Cherry red fluorescent variant (mCherry)-expressing lentivirus, NAc-containing brain sections were incubated with rabbit anti-RFP antibody (Medical and Biological Laboratories, Nagoya, Japan) overnight at 4°C and incubated again with Cy3-conjugated antirabbit secondary antibody (Invitrogen, Carlsbad, California) for 2 hours at room temperature.

Three-dimensional images of virus-infected neurons were taken with a Fluoview 1000 confocal microscope (Olympus, Tokyo, Japan). Spines were counted within the 20–40-μm segments on secondary dendrites extending at least 40 μm beyond the soma (Supplement 1). All protrusions, irrespective of their morphological characteristics, were counted as spines if they were in direct continuity with the dendritic shaft.

### Statistical Analysis

Results are denoted as the means ± SEM. Student *t* test was used to determine statistical significance between two datasets. The statistical significance was indicated by $*p < .05$, $**p < .01$, or $***p < .001$.

## Results

### Decreased Excitability of D1R-MSNs but Not of D2R-MSNs Upon Repeated Exposure to Cocaine

We attempted to determine whether chronic exposure to cocaine can induce electrophysiological changes of individual MSNs in the NAc. To this end, we injected cocaine repeatedly in either D1R- or D2R-eGFP transgenic mice (15 mg/kg/day for 5 days) and analyzed the NAc MSNs 24 hours after the last injection, the time point at which alterations of neuronal excitability and structural neuroadaptations were previously demonstrated (12–15). We initially analyzed cocaine-induced behavioral sensitization in the transgenic mice to eliminate the possibility that the genetic modification pertinent to the used mouse models altered the drug-induced neuroadaptations. We found that both transgenic mice exhibit progressively augmented locomotion to repeated cocaine injection over 5 consecutive days, which is not distinguishable from that of wild-type mice (Figure S1 in Supplement 1). This result indicated that the used transgenic mice have no defect in drug-induced behavioral sensitization.

To investigate possible cell type-specific effects of cocaine, acute NAc slices were prepared 24 hours after the final injection (Figure 1A), and whole-cell recordings were made from eGFP-positive MSNs from each corresponding transgenic mouse (Figure 1B). To assess membrane excitability of each MSN, we evoked spikes by injecting a series of depolarizing current steps and found that spike frequency of D1R-MSNs from cocaine-injected mice was significantly reduced compared with that from saline-injected mice (Figures 1C and 1D). Rheobase—the smallest current amplitude for eliciting a single spike—and latency to the first spike were also increased, consistent with a decrease in spike firing, in D1R-MSNs (rheobase, saline, 93.54 ± 7.26 pA vs. cocaine, 124.90 ± 10.01 pA, $p < .05$; latency to the first spike at 160 pA, saline, 124 ± 24.84 msec vs. cocaine, 334.73 ± 92.24 msec, $p < .05$; latency to the first spike at 200 pA, saline, 74.14 ± 12.03 msec vs. cocaine, 163.87 ± 42.84 msec, $p < .05$) (Figures 1E and 1F). We did not detect any difference in the excitability of D2R-MSNs (Figures 1C–1F), unlike D1R-MSNs. To further exclude the possibility that the observed cell type-specific difference in excitability is due to a potential difference between transgenic backgrounds, we performed another set of excitability tests with D2R-eGFP transgenic mice, categorizing cells into GFP(+)-D2R-MSNs or GFP(−)-D1R-MSNs, and corroborated the D1R-MSN-selective decrease of excitability after repeated cocaine treatment (Figure S2 in Supplement 1).

To examine whether repeated administration of cocaine alters other membrane properties, we measured resting membrane potential and spike characteristics, but neither D1R- nor D2R-MSNs showed any significant differences between saline and cocaine groups (Table S1 in Supplement 1). It was previously reported that

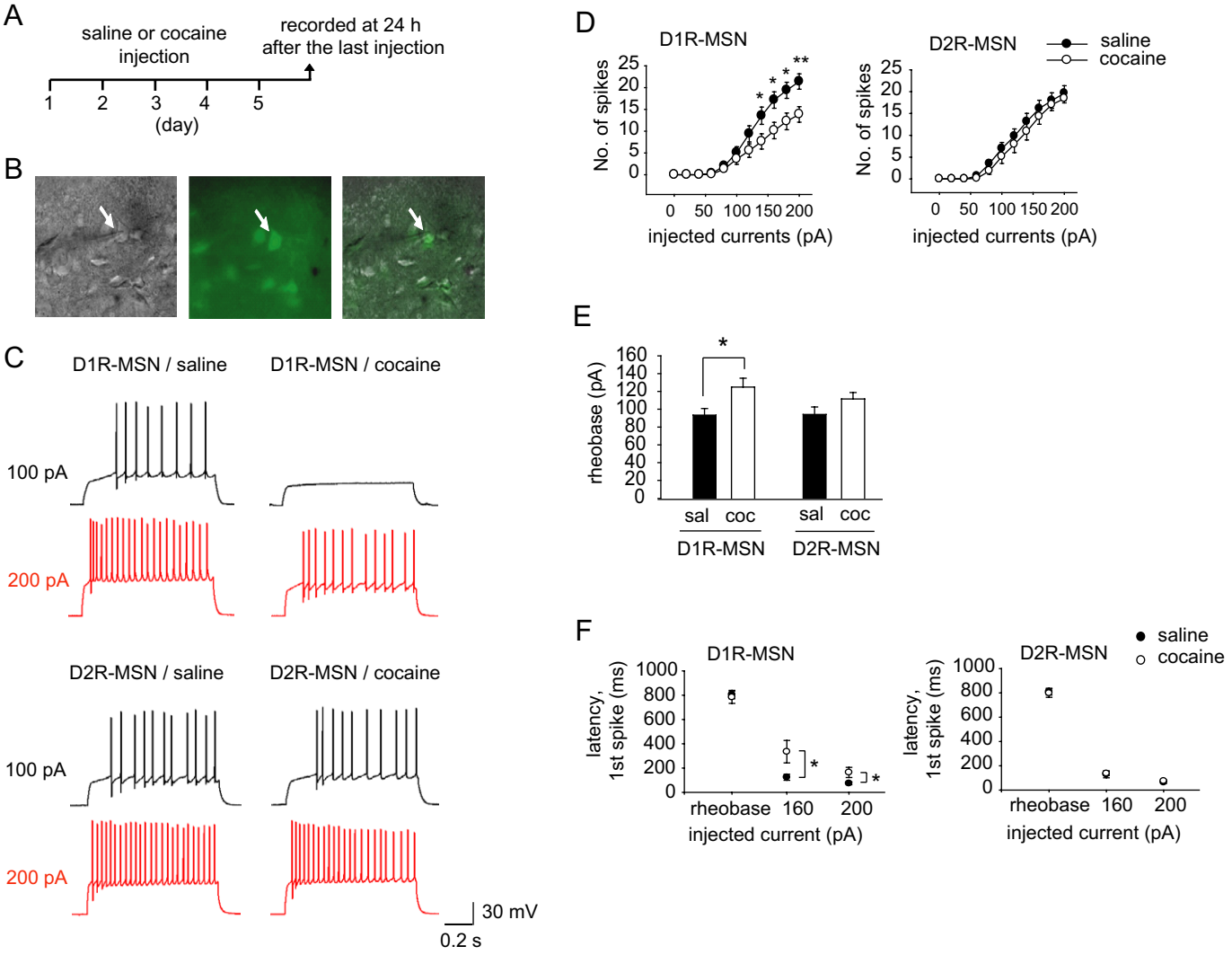


**Figure 1.** Repeated cocaine exposure suppresses the intrinsic excitability of D1 dopamine receptor (D1R)-medium spiny neurons (MSNs) but not of D2 dopamine receptor (D2R)-MSNs. **(A)** Experimental timeline to test the effects of repeated saline or cocaine administration for electrophysiological analyses of nucleus accumbens (NAc) MSNs. **(B)** Representative images of enhanced green fluorescent protein (eGFP)-expressing MSNs in the NAc. Patched MSN (white arrow) images under infrared (left), fluorescence (middle), and merged image (right) are presented. **(C)** Representative voltage traces recorded in D1R- and D2R-MSNs from mice repeatedly injected with saline or cocaine. The voltage responses evoked by 100-pA (black trace) and 200-pA current (red trace) injections are shown. **(D)** Graphs for the numbers of spike firings at the indicated current steps in D1R-MSNs (left) and D2R-MSNs (right). D1R-saline, $n = 12$ cells, seven mice; D1R-cocaine, $n = 18$ cells, four mice; D2R-saline, $n = 21$ cells, six mice; D2R-cocaine, $n = 19$ cells, five mice. $^*p < .05$ at 140–180 pA current injections and $^{**}p < .01$ at 200-pA current injections. **(E)** A histogram of mean rheobases is presented. D1R-saline, $n = 13$ cells, eight mice; D1R-cocaine, $n = 20$ cells, five mice ($^*p < .05$); D2R-saline, $n = 24$ cells, eight mice; D2R-cocaine, $n = 20$ cells, six mice. **(F)** Latency to the first spike in D1R-MSNs (left) and D2R-MSNs (right) at three levels of depolarizing current injections (rheobase, 160, and 200 pA). D1R-saline, $n = 12$ cells, seven mice; D1R-cocaine, $n = 18$ cells, four mice; D2R-saline, $n = 21$ cells, six mice; D2R-cocaine, $n = 19$ cells, five mice. Statistical significances are indicated as $^*p < .05$ between the saline- and cocaine-injected groups.

afterhyperpolarization affects membrane excitability of the NAc MSNs (16), but we failed to see any difference in afterhyperpolarization between groups (Table S1 in Supplement 1). We also measured input resistance and inwardly rectifying potassium channel-mediated currents, one of the negative modulators of membrane excitability in the NAc and striatum (17–19), but found no differences between groups (Figure S3 in Supplement 1). Thus, the decreased excitability of D1R-MSNs by cocaine administration is unlikely due to changes in basal membrane properties. Although the mechanisms involved in the reduction of the intrinsic excitability of D1R-MSNs remain unclear, our cell type-specific recordings indicate that repeated cocaine administration can differently regulate the intrinsic excitability of individual MSN types, decreasing the excitability of D1R-MSNs but leaving that of D2R-MSNs unaffected.

### Evoked Transmission Unaffected by Repeated Administration of Cocaine

Cocaine experience is known to result in the dynamic modification of both the expression and efficacy of glutamatergic transmission to different time points of cocaine exposure. In particular, surface/intracellular expression levels (20–22) or synaptic protein levels (23) of AMPARs were not altered after short-term (1 day) withdrawal from chronic cocaine administration (but see Kourrich *et al.* [24]), whereas long-term (> 1 week) withdrawal increased AMPAR activity (20–26). To explore the possible alteration of

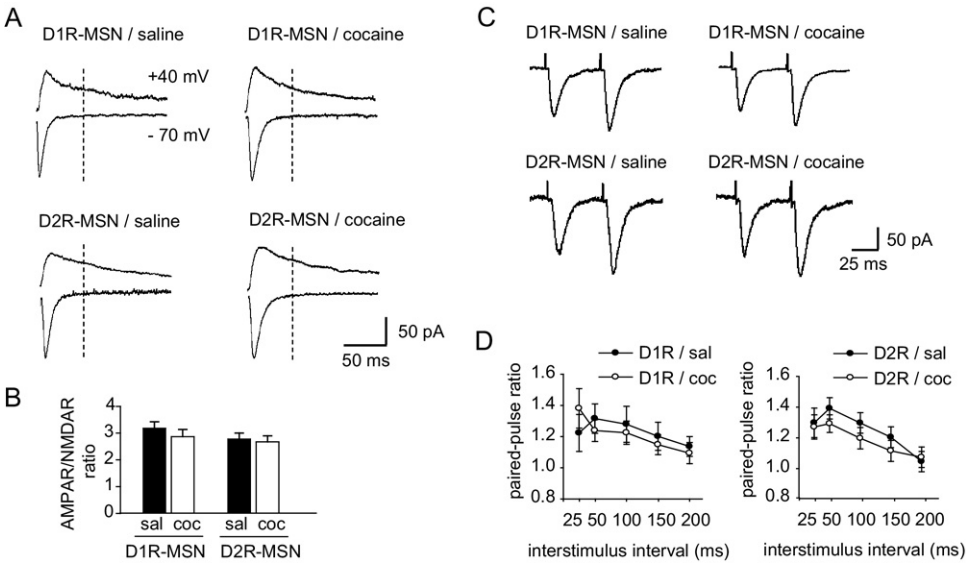


**Figure 2.** Repeated cocaine treatment does not affect evoked excitatory postsynaptic currents (EPSCs) and paired-pulse ratios (PPRs) in NAc synapses. **(A)** Representative traces of *N*-methyl-D-aspartic acid type glutamate receptor (NMDAR)- and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid type glutamate receptor (AMPAR)-mediated EPSCs recorded in the same neurons are presented. Dotted lines indicate the time point, 50 msec after the onset of responses (for measurement of NMDAR component). The NMDAR- and AMPAR-mediated EPSCs were measured at holding potentials of +40 mV and −70 mV, respectively. **(B)** A summary histogram for the AMPAR/NMDAR ratio for each group is depicted. D1R-saline, $n = 17$ cells, five mice; D1R-cocaine, $n = 16$ cells, five mice; D2R-saline, $n = 18$ cells, five mice; D2R-cocaine, $n = 19$ cells, five mice. **(C)** Representative traces in responses to paired-pulse stimulation at a 50-msec interstimulus interval. Stimulus artifacts were truncated for clarity. **(D)** Summary graphs for the PPR of D1R-MSNs (left) and D2R-MSNs (right) are shown for the indicated intervals. D1R-saline, $n = 23$ cells, five mice; D1R-cocaine, $n = 20$ cells, five mice; D2R-saline, $n = 18$ cells, five mice; D2R-cocaine, $n = 18$ cells, five mice. Other abbreviations as in Figure 1.

AMPAR-mediated synaptic transmission in a cell type-specific manner, we measured AMPAR- and NMDAR-mediated EPSCs at holding potentials of −70 mV and +40 mV, respectively, while stimulating the border region between the prelimbic cortex and the NAc (Figure 2A). We compared the ratio of AMPAR-mediated to NMDAR-mediated EPSCs (AMPAR/NMDAR ratio) and found that neither D1R- nor D2R-MSNs undergo any changes after repeated cocaine injections (Figure 2B). This lack of effect of cocaine exposure on the evoked transmission seems to be consistent with previous studies showing the unaltered ratios of surface/intracellular expression levels of AMPARs at time points similar to ours (20,21,25). Interestingly, we found that subject animals exhibited apparent behavioral sensitization and the D1R-MSNs displayed a significant increase in AMPAR/NMDAR ratios in the case of long-term (2 weeks) withdrawal from repeated cocaine administration (Figure S4 in Supplement 1), consistent with previous reports (20–26). This result suggested that the glutamatergic transmission in the NAc is critically subject to withdrawal duration.

We also examined paired-pulse facilitation, a well-characterized form of short-term synaptic plasticity, to test possible alterations in presynaptic transmitter release. We measured PPRs 1 day after repeated cocaine injection, but those obtained from cocaine-treated mice were indistinguishable from those from repeated saline-treated mice in both types of NAc MSNs (Figures 2C and 2D). Therefore, these results show that evoked EPSCs and PPRs from D1R- and D2R-MSNs are not affected significantly by repeated exposure to cocaine, arguing against the possibility for cocaine-induced alterations in glutamatergic transmission or transmitter release probability.

### Bidirectional Changes of mEPSCs but D1R-MSNs-Specific Decreases in mIPSCs

Despite a lack of cocaine effect on AMPAR/NMDAR ratios and PPRs, it is still possible that cocaine exposure might affect basal synaptic transmission. To address this issue, we analyzed mEPSCs in the identified NAc MSNs from either repeated saline- or cocaine-injected mice (Figure 3A). The amplitudes of mEPSCs exhibited no differences between the saline and cocaine groups in either type of MSN (Figures 3B and 3C), in agreement with the unaltered AMPAR/NMDAR ratios (Figure 2B). However, we unexpectedly discovered that the frequencies of mEPSCs displayed bidirectional changes in D1R- versus D2R-MSNs after repeated cocaine treatment: mEPSC frequencies of D1R-MSNs increased after cocaine exposure, whereas those of D2R-MSNs significantly decreased compared with the saline group (D1R-saline, 1.62 ± .27 Hz vs. D1R-cocaine, 2.57 ± .29 Hz, $p < .05$; D2R-saline, 1.70 ± .20 Hz vs. D2R-cocaine, 1.02 ± .12 Hz, $p < .01$) (Figures 3D and DE).

The observed disparate effects of cocaine exposure on the frequency of mEPSCs recorded from D1R- and D2R-MSNs prompted us to test whether cocaine exposure can also regulate inhibitory transmission in the two different types of MSNs. We found that—different from mEPSC results—only D1R-MSNs exhibited decreases in mIPSCs in both their amplitudes (D1R-saline, 33.53 ± 2.90 pA vs. D1R-cocaine, 25.21 ± 1.83 pA, $p < .05$) (Figure 4C) and frequencies (D1R-saline, .56 ± .06 Hz vs. D1R-cocaine, .32 ± .06 Hz, $p < .01$) (Figure 4E) after cocaine exposure, but there was no change in the mIPSCs of D2R-MSNs.

It is not clear whether the marked modifications in excitability, mEPSCs, and mIPSCs are induced only by repeated exposure (5 days) to cocaine or can be induced by a single cocaine injection. To test this possibility, we injected saline or cocaine (15 mg/kg, IP) into mice and examined the properties of NAc MSNs 1 day after the single injection. We found no differences in any parameter between the saline and cocaine groups (Figure S5 in Supplement 1), indicating that a single injection of cocaine is not sufficient to elicit the physiological alterations that we had observed after repeated cocaine injection. Taken together, these results demonstrate that re-

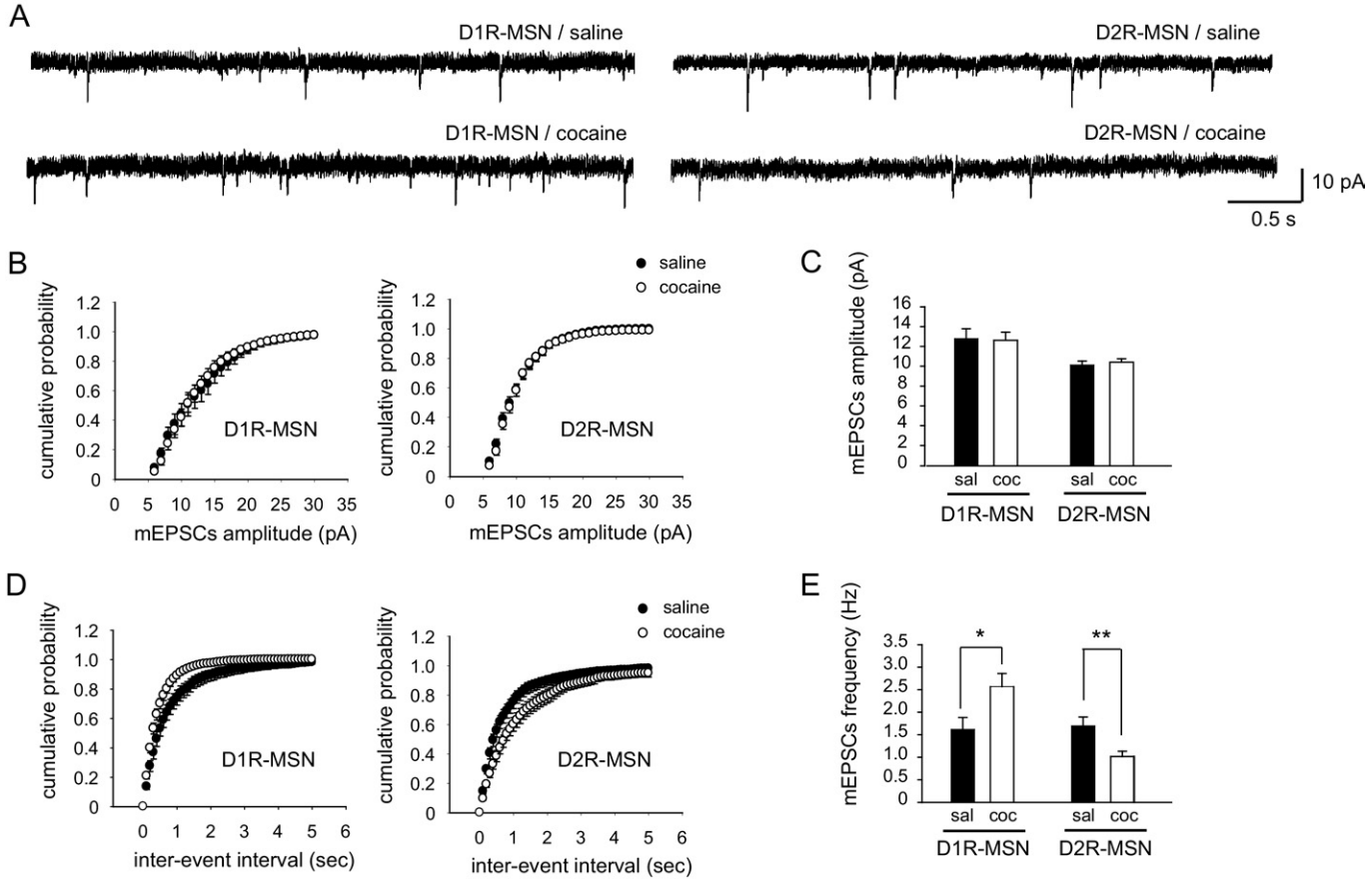


**Figure 3.** The frequencies of miniature excitatory postsynaptic currents (mEPSCs) increase in D1R-MSNs but decrease in D2R-MSNs after repeated cocaine treatment. **(A)** Sample traces of mEPSCs recorded in D1R- and D2R-MSNs from mice repeatedly treated with saline or cocaine. Graphs plotting cumulative probability **(B)** and bar graphs for mean values **(C)** of mEPSC amplitudes are shown. Cumulative probability plot **(D)** and bar graph depicting mean values **(E)** of mEPSC frequencies are presented. D1R-saline, $n = 13$ cells, five mice; D1R-cocaine, $n = 15$ cells, six mice; D2R-saline, $n = 19$ cells, seven mice; D2R-cocaine, $n = 18$ cells, seven mice. In the bar graph for mEPSC frequency **(E)**, statistical significances are indicated as $^{*}p < .05$ in D1R-MSNs and $^{**}p < .01$ in D2R-MSNs between the saline- and cocaine-injected groups. Other abbreviations as in Figure 1.

peated but not a single administration of cocaine induces changes in both intrinsic membrane properties and basal synaptic transmission in a cell type-specific manner in the NAc.

### Increased Spine Density in D1R-MSNs but Not in D2R-MSNs

It has been widely documented that repeated exposure to cocaine causes an increase in spine density of NAc MSNs (13,27–30). The possible morphological changes confined to D1R-MSNs might account for the increased frequency of mEPSCs but the absence of any effect on PPRs that we observed in D1R-MSNs. We attempted to measure the spine density of individual NAc MSNs from mice treated repeatedly with saline or cocaine. Because the fluorescence intensity of eGFP from dendrites and dendritic spines of D1R- or D2R-eGFP transgenic mice is too dim for detailed morphological analysis, we turned to viral gene transfer to unambiguously label neuronal processes (Figure 5A). First, mCherry-expressing lentivirus was infused into the NAc of the transgenic mice. Two to three weeks after viral infusion, mice received either saline or cocaine for 5 consecutive days and were subjected to morphological analyses (Figure S6 in Supplement 1). The expression of mCherry from lentivirus-infected cells enabled us to clearly analyze the dendritic morphology while distinguishing cell types by comparing mCherry signals with eGFP expression levels from the cell bodies (Figures 5B and 5C). Quantitative analyses for dendritic morphology revealed that the spine densities of D1R-MSNs significantly increased after repeated cocaine administration (D1R-saline, 6.13 ± .27 vs. D1R-cocaine, 7.97 ± .24, $p < .001$) (Figure 5D), whereas those of D2R-MSNs were not altered. These results indicate that repeated cocaine administration selectively increases the spine density in D1R-MSNs. Two previous studies suggested that both D1R- and D2R-MSNs undergo a similar increase in spine density after chronic cocaine administration (28,29). Their results are partly inconsistent with our results in D2R-MSNs, but the cocaine administration paradigms and analytic methods used in the previous studies—which apparently differ from ours—make a direct comparison difficult.

Despite the apparent increase in spine density of D1R-MSNs elucidated by viral infusion into the NAc, we cannot completely exclude the possibility that MSNs coexpressing both D1R and D2R—which were previously estimated to comprise up to 17% of the total MSNs in the NAc shell (31)—might have confounded our analyses. To rule out this possibility, we performed another set of morphological analyses, a pathway-specific labeling with rabies virus. The recombinant eGFP-expressing rabies virus has a unique characteristic of retrograde neuronal tracing (11). Previous neuronal tracing studies using fluorescence dyes indicated that most dopamine receptor-containing NAc neurons innervating the VTA express D1R (approximately 99% MSNs), whereas in the accumbens-pallidal projection, D1R- and D2R-expressing NAc neurons

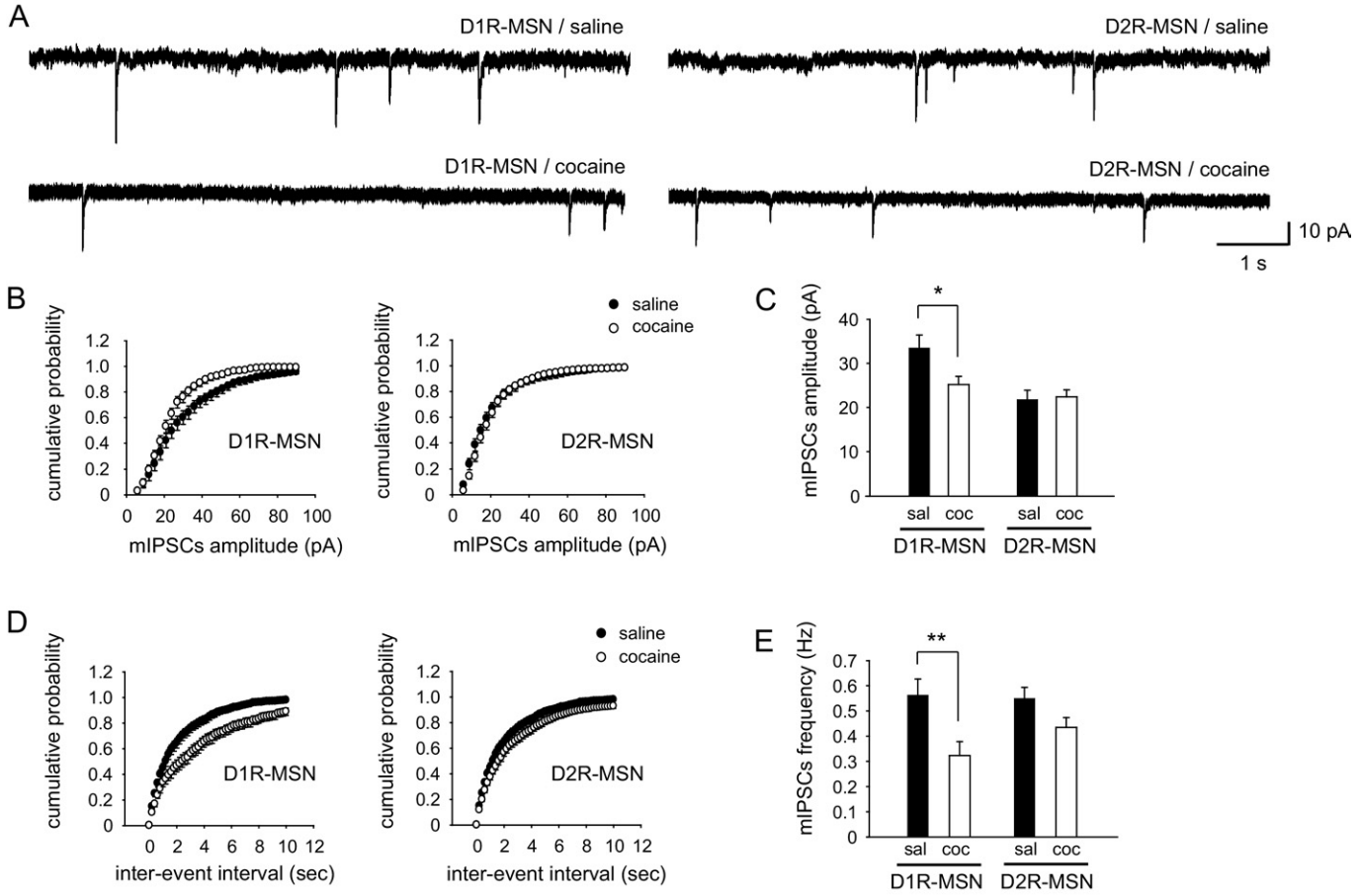


**Figure 4.** Repeated cocaine administration decreases the amplitudes and frequencies of miniature inhibitory postsynaptic currents (mIPSCs) in D1R-MSNs but not in D2R-MSNs. **(A)** Sample traces of mIPSCs recorded in D1R- and D2R-MSNs from mice repeatedly treated with saline or cocaine. Graphs plotting cumulative probability **(B)** and bar graphs for mean values **(C)** of mIPSC amplitudes are shown. Cumulative probability plot **(D)** and bar graph depicting mean values **(E)** of mIPSC frequencies are presented. D1R-saline, $n = 20$ cells, five mice; D1R-cocaine, $n = 18$ cells, five mice; D2R-saline, $n = 27$ cells, five mice; D2R-cocaine, $n = 40$ cells, six mice. Both amplitudes and frequencies of mIPSCs recorded in D1R-MSNs are significantly reduced after repeated cocaine treatment (*$p < .05$ and **$p < .01$ in mIPSC amplitude and frequency, respectively). Other abbreviations as in Figure 1.

innervate the ventral pallidum at similar proportions (7,8,32). Thus, we infused eGFP-expressing rabies virus into the VTA of wild-type mice for the retrograde tracing of D1R-MSNs that project to the VTA (Figure 5A). Entire neuronal processes of virus-infected NAc MSNs, including dendritic spines, were labeled by the rabies virus infusion (Figure 5F). Quantitative determination for dendritic spines revealed that the spine densities of NAc MSNs that were labeled via viral infusion into the VTA considerably increased after repeated cocaine administration (saline, 7.31 ± .23 vs. cocaine, 10.11 ± .36, $p < .001$) (Figure 5H). Therefore, both the cell-type specific staining with mCherry lentivirus and the pathway-specific labeling with eGFP rabies virus provided identical evidence that repeated cocaine exposure increases spine densities of D1R-MSNs almost exclusively. Along with our electrophysiological results (i.e., increased mEPSCs frequency in D1R-MSNs), this D1R-MSN-selective increase of spine density represents cocaine-induced morphological plasticity associated with the functional modification of dendritic spines on these neurons.

## Discussion

The D1R and D2R are principally expressed in separate neurons of the dorsal and ventral striatum (5). Recent studies using dopamine receptor promoter-derived fluorescent protein-expressing transgenic mice have confirmed a high degree of segregation (28,31,33). Previous studies, performed with either dopamine receptor agonist or antagonist (34–37), have implicated the importance of D1R rather than D2R in cocaine addiction. However, physiological responses of the NAc MSNs after repeated exposure to cocaine have not been explored, particularly in a cell type-specific manner, because of the issue of cell-type identification. Here, we were able to distinguish individual MSN types with transgenic mice and simultaneously characterize in detail how the D1R- and D2R-MSNs differently respond to the chronic administration of cocaine at the single-cell level.

Repeated cocaine administration has been reported to decrease membrane excitability of the NAc MSNs (14,15). However, we found that the intrinsic excitability of D2R-MSNs was not affected by cocaine exposure. This might be ascribed to the distinct dopamine receptor-mediated intracellular signaling pathways between the two types of MSNs. For example, repeated exposure to cocaine causes an accumulation of ΔFosB via D1R/protein kinase A activation, which in turn increases the expression of cyclin-dependent kinase 5 (CDK5) (38,39), a negative modulator of intrinsic membrane excitability (40). Therefore, the intrinsic excitability of NAc MSNs would be modulated by the activity of CDK5 that is enhanced after repeated cocaine

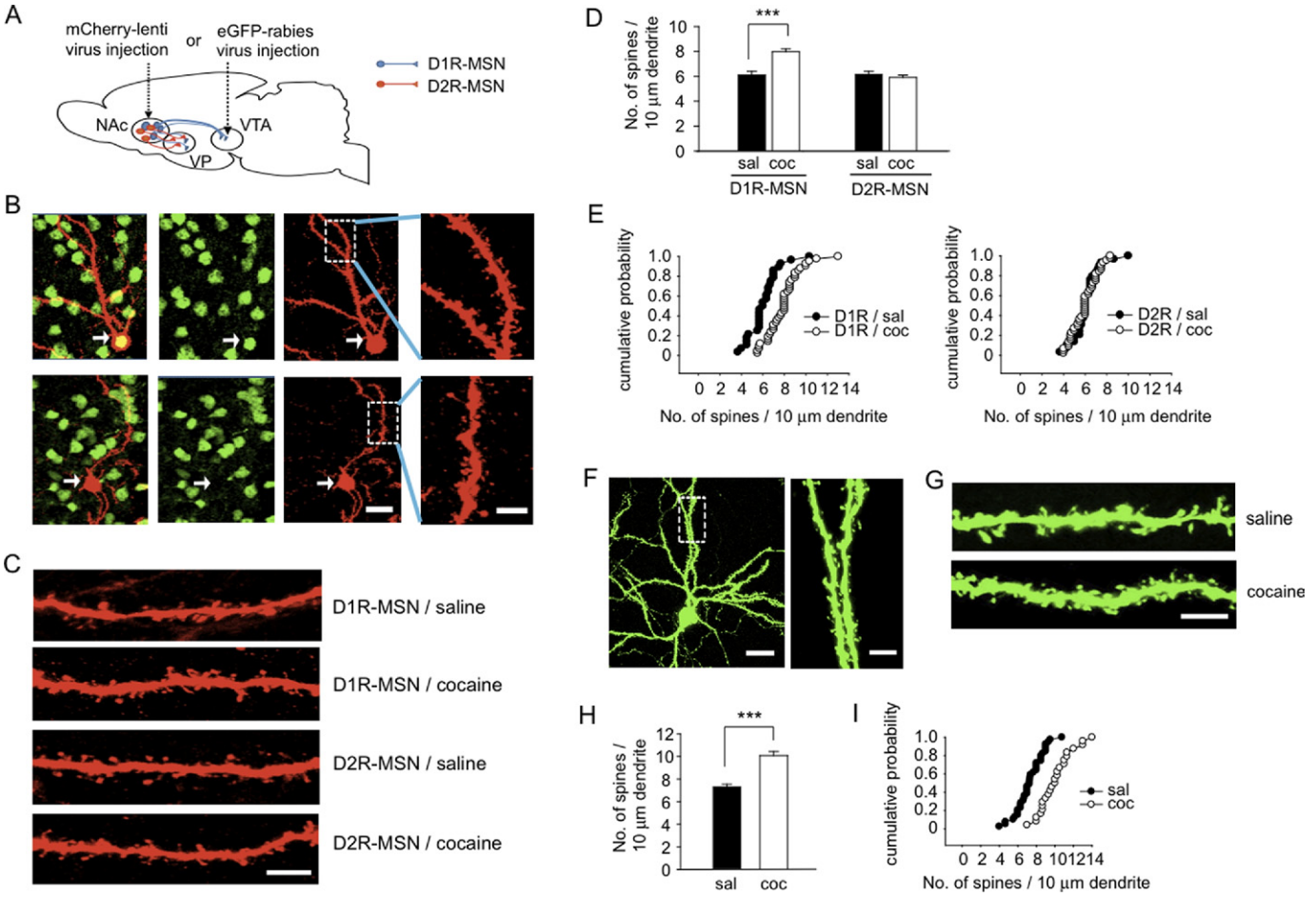


**Figure 5.** Spine density of D1R-MSNs but not of D2R-MSNs increases after repeated cocaine exposure. **(A)** Projection pathways of the two types of NAc MSNs and injection sites of either monomeric cherry red fluorescent variant (mCherry) lentivirus or enhanced green fluorescent protein (eGFP) rabies virus are schematically indicated. **(B)** Representative images of mCherry lentivirus-infected eGFP-positive (upper) and eGFP-negative (lower) MSNs. Arrows indicate the locations of virus-infected cells. The dendritic segments outlined with dotted lines are magnified to delineate spine morphology. Scale bars, 15 μm (third panel) and 5 μm (magnified panel). **(C)** Representative images of dendritic spines on the mCherry lentivirus-infected MSNs. Scale bar, 5 μm. **(D)** Bar plot for comparison of spine density between the saline and cocaine groups. D1R-saline, $n = 28$ cells, nine mice; D1R-cocaine, $n = 42$ cells, nine mice; D2R-saline, $n = 29$ cells, nine mice; D2R-cocaine, $n = 44$ cells, nine mice; $^{***}p < .001$ in D1R-MSNs. **(E)** Graphs plotting the cumulative probability of spine density of D1R-MSNs (left) and D2R-MSNs (right) are shown. **(F)** Representative images of the eGFP rabies virus-infected NAc MSNs. The dendritic segment outlined with a dotted line (left) is magnified to delineate spine morphology (right). Scale bars, 20 μm (left) and 5 μm (right). **(G)** Representative images of dendritic spines on the NAc MSNs infected with eGFP rabies virus through the ventral tegmental area. Scale bar, 5 μm. **(H)** Bar plot for comparison of spine densities between the saline and cocaine groups. Saline, $n = 39$ cells, five mice; cocaine, $n = 24$ cells, five mice; $^{***}p < .001$. **(I)** A graph for cumulative probability of spine density is shown. Other abbreviations as in Figure 1.

exposure in D1R-MSNs but not in D2R-MSNs, which do not have a D1R-mediated CDK5 activation cascade.

We found that AMPAR/NMDAR ratios were unaffected in either type of MSN 1 day after repeated cocaine treatment but increased in D1R-MSNs after 2 weeks of cocaine withdrawal. Unlike excitability or spine density, synaptic expression of AMPAR in the NAc MSNs remains unaffected but increases after long-term withdrawal (> 7 days) from cocaine (20–26). Therefore, the results of previous mixed and our cell type-specific assay suggest the following hypotheses: 1) AMPAR activity in the NAc glutamatergic synapses does not change at the time immediately after repeated cocaine administration, and 2) relapse and reinstatement to cocaine typically observed after long-term withdrawal from repeated cocaine treatment might be settled by the enhanced activity of AMPAR that gradually increases during the withdrawal period.

The increased frequencies of mEPSCs in D1R-MSNs but the corresponding decreases of mEPSC frequencies in D2R-MSNs might have physiological consequences involved in cocaine addiction. For instance, it was previously shown that that the inhibition of D2R activates glutamate receptor 1 phosphorylation (41), and the selective ablation of D2R-MSNs in the NAc increases amphetamine-conditioned place preference (42). Furthermore, we detected selective decreases in mIPSCs in D1R-MSNs after repeated cocaine treatment. Functionally, the reduction of mIPSCs in D1R-MSNs is likely to shift the ratio of excitatory and inhibitory drives to make excitatory effects dominant in D1R-MSNs. Hence, our findings, combined with previous reports (41,42), argue that the enhanced excitatory synaptic inputs to D1R-MSNs but decreased transmission to D2R-MSNs contribute to the neuroadaptations induced by psychostimulants.

The bidirectional modifications (i.e., reduced excitability vs. increased synaptic inputs) observed in D1R-MSNs might result from homeostatic regulation. Neurons adapt to chronic, experience-dependent stimuli by adjusting their synaptic efficacy or intrinsic plasticity within normal ranges (43,44). Activation and blockade of

NMDAR in the NAc MSNs lead to a homeostatic decrease and an increase in spike firing, respectively (16). It was also reported that reduction of excitability by overexpression of inwardly rectifying potassium channel in hippocampal neurons leads to a homeostatic increase in the frequency of mEPSCs, whereas the quantal amplitude is unchanged (45). Unlike Hebbian modification that occurs in an input-specific manner over a time scale of hours, homeostatic modification induces cell-wide changes over longer times. Both time (5 days) and direction of activity changes (i.e., reduced excitability and increased mEPSC frequency) arising in D1R-MSNs are matched to the general features of homeostatic adaptations observed in hippocampal neurons (45). Thus, the present and other previous findings support the notion that observed bidirectional changes in intrinsic properties and basal transmission in D1R-MSNs in response to chronic cocaine exposure are homeostatic adaptations for adjusting synaptic weights of the neural network in the reward-associated circuitry.

We found that, from intrinsic excitability to dendritic spine density, D1R-MSNs exhibit marked alterations in response to repeated cocaine administration, whereas D2R-MSNs remain relatively unaffected. Previous studies using D1R knockout mice have suggested a dominant role of D1R signaling in cocaine addiction (46,47). The phasic and tonic firing of VTA dopaminergic neurons are known to stimulate the low-affinity D1Rs and high-affinity D2Rs, respectively (48). A recent optogenetic study (49) demonstrated that the phasic but not the tonic activation of VTA dopaminergic neurons induces conditioned place preference behavior in freely behaving animals, suggesting the importance of D1R-MSNs in reward behavior. Moreover, a study using a reversible neurotransmission blocking technique (50) revealed that blockade of neurotransmission of D1R-MSNs significantly attenuated behavioral sensitization by repeated cocaine administration over 5 days, whereas blockade of D2R-MSNs did not. These studies highlight the predominant and unique roles of D1R-MSNs in cocaine addiction. Recent optogenetic studies segregated the opposed roles of "direct (D1R-MSN)" and "indirect (D2R-MSN)" pathways for motor (51) and cocaine-rewarding behaviors (52), and the bidirectional regulation of motor behavior was also confirmed by another genetic study (53) using cell type-specific deletion of DARPP-32 (dopamine- and cyclic-AMP-regulated phosphoprotein of molecular weight 32 kDa) in the striatum. Thus, a number of genetic studies suggest that D1R-MSNs take charge of excitatory drives, whereas D2R-MSNs exert basal inhibition in cocaine addiction, but the cell type-specific responses of NAc MSNs to repeated cocaine administration have not been empirically elucidated. In this study, we provide substantial experimental evidence for the first time that D1R- and D2R-MSNs respond differently to repeated cocaine administration, suggesting that these cell type-specific alterations are likely to be cellular mechanisms that contribute to drug addiction. Further studies of the distinct intracellular or synaptic mechanisms of both types of MSNs and their dynamics at various times during drug addiction are needed to understand the cell type-specific functions and physiological consequences of drug addiction.

*We thank Eric Nestler (Mount Sinai University, New York), Ming-Hu Han (Mount Sinai University, New York), Craig Bailey (Columbia University, New York), and Won-Kyung Ho (Seoul National University, Seoul, Korea) for critical comments and kind support throughout this study, and Ed Callaway (Salk Institute for Biological Studies, CA) for rabies virus. This work was supported by grants from the National Research Foundation of Korea (2010-0007293, 2010-0029402, & 2009K001247) and the Korea Healthcare Technology R&D Project (A080137) awarded to J-HK.*

*The authors report no biomedical financial interests or potential conflicts of interest.*

*Supplementary material cited in this article is available online.*

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