Methylphenidate-induced dendritic spine formation and ΔFosB expression in nucleus accumbens

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Methylphenidate is the psychostimulant medication most commonly prescribed to treat attention deficit hyperactivity disorder (ADHD). Recent trends in the high usage of methylphenidate for both therapeutic and nontherapeutic purposes prompted us to investigate the long-term effects of exposure to the drug on neuronal adaptation. We compared the effects of chronic methylphenidate or cocaine (15 mg/kg, 14 days for both) exposure in mice on dendritic spine morphology and ΔFosB expression in medium-sized spiny neurons (MSN) from ventral and dorsal striatum. Chronic methylphenidate increased the density of dendritic spines in MSN-D1 (MSN-expressing dopamine D1 receptors) from the core and shell of nucleus accumbens (NAcc) as well as MSN-D2 (MSN-expressing dopamine D2 receptors) from the shell of NAcc. In contrast, cocaine increased the density of spines in both populations of MSN from all regions of striatum. In general, the effect of methylphenidate on the increase of shorter spines (class 2) was less than that of cocaine. Interestingly, the methylphenidate-induced increase in the density of relatively longer spines (class 3) in the shell of NAcc was bigger than that induced by cocaine. Furthermore, methylphenidate exposure increased expression of ΔFosB only in MSN-D1 from all areas of striatum, and surprisingly, the increase was greater than that induced by cocaine. Thus, our results show differential effects of methylphenidate and cocaine on neuronal adaptation in specific types of MSN in reward-related brain regions.

addiction | ADHD | cocaine | dopamine | striatum

Methylphenidate is the psychostimulant medication most commonly prescribed to treat attention deficit hyperactivity disorder (ADHD) (1, 2). Over the past 2 decades, the number of children, adolescents, and adults for whom methylphenidate has been prescribed has surged (2, 3). ADHD is associated with a dopamine imbalance, and methylphenidate likely helps ADHD patients by blocking dopamine reuptake and thereby increasing synaptic dopamine (4). Methylphenidate and cocaine have similar chemical structures and their pharmacological effects appear to be similar (5), prompting concern that methylphenidate may have addictive properties. Indeed, methylphenidate is widely abused for improving concentration and enhancing performance, or for recreational purposes (3, 6–10). Notably, a recent report has indicated that more than 7 million people in the US have abused ADHD stimulants, and as many as 750,000 teenagers and young adults may show signs of addiction (11). The increasing abuse of methylphenidate as well as the exposure of individuals through its therapeutic use prompted us to investigate possible long-term effects of methylphenidate on brain chemistry and neuronal structure.

Substantial evidence suggests that adaptive changes in dopaminergic function in the ventral tegmental area (VTA) and nucleus accumbens (NAcc) underlie psychostimulant-induced behaviors (12). In addition to dopamine, glutamate is required for the behavioral sensitization, drug seeking, and compulsive relapse in response to psychostimulants (13–16). Medium-sized spiny neurons (MSN) in ventral and dorsal striatum receive midbrain dopaminergic input, which serves to modulate excitatory glutamatergic input from prefrontal cortex. The initial site of interaction between dopamine and glutamate is within the dendritic spines of MSN, and notably chronic exposure to psychostimulants has been found to increase the number of dendritic branch points and spines of MSN in NAcc (17, 18).

GABAergic MSN, which represent 90–95% of all neurons in striatum, are comprised of 2 intermingled subpopulations. One subpopulation of MSN express high levels of dopamine D1 receptors (together with substance P and dynorphin) (MSN-D1), and the other MSN express high levels of dopamine D2 receptors (together with enkephalin) (MSN-D2) (19–22). Through the use of selective agonists and antagonists, both D1 and D2 receptors have been shown to be required for psychostimulant-dependent behavioral changes (23–28). Recent studies using bacterial artificial chromosome (BAC)-transgenic mice, where different proteins have been selectively expressed in either MSN-D1 or -D2, have shown distinct patterns of phosphorylation of signaling molecules (29, 30), gene expression (18, 30–32), and cocaine-induced increases of dendritic spine density (18) in the 2 subpopulations of MSN.

In this study, we compared dendritic spine morphology following chronic exposure to methylphenidate or cocaine in MSN-D1 and MSN-D2 from 3 different subregions of striatum: shell and core of NAcc, and dorsal striatum. In addition, we examined the effects of drug exposure on ΔFosB expression because previous studies have found that this transcription factor is involved in long-lasting regulation of gene expression, even after drug taking ceases (33–35). The results obtained indicate that methylphenidate, like cocaine, increases dendritic spine density and expression of ΔFosB in MSN, but that the precise pattern observed is distinct from that of cocaine.

Results

Heterogeneity in the Length of Dendritic Spines in MSN. Before analysis of the effects of drug exposure, we characterized in detail the morphology of dendritic spines from MSN of striatum, compared to spines of hippocampal pyramidal neurons (Fig. S1 *A* and *B*). The total density of dendritic protrusions of MSN from the shell of the NAcc (≈15.2/10 μm) was slightly less than that of pyramidal neurons from the CA1 region of hippocampus (≈19.6/10 μm) (Fig. S1*C*). Analysis of the relationship of width and length of individual spines indicated that the distribution of spine width was relatively comparable for MSN and pyramidal neurons, but that spines from MSN had a broader distribution in length and included a significant proportion of longer spines (Fig. S1 *D–G*). Thus in subsequent studies, we classified dendritic protrusions into 4 classes of spines based on their lengths (see *Methods*). The density of class 1 and 4 spines was found to be very low compared to class 2 and 3 (see examples in bar graphs in Figs. 1 and 2). The width distribution of class 2 and 3 spines were found to be indistinguishable indicating

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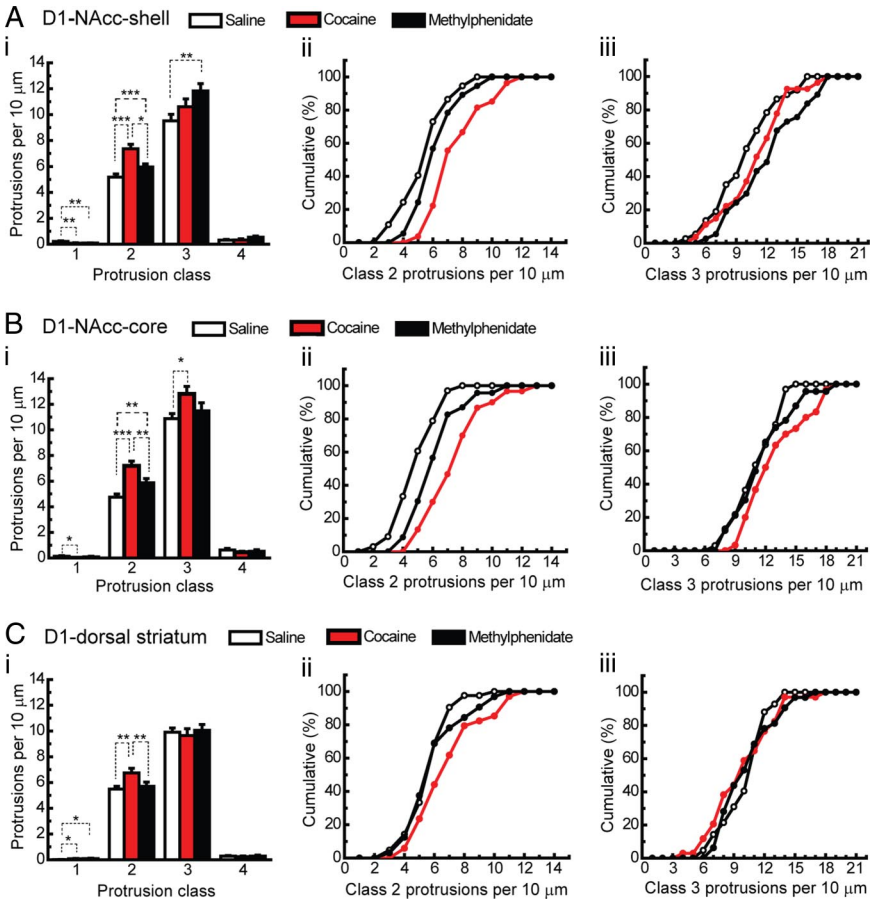

Fig. 1. Chronic methylphenidate- or cocaine-induced changes in spine density of MSN-D1 in NAcc and dorsal striatum. D1 dopamine receptor promoter driven (Drd1)-EGFP mice were injected daily with saline, cocaine (15 mg/kg) or methylphenidate (15 mg/kg) for 14 days. Two days after the last injection, mouse brains were processed for DiI labeling and immunohistochemistry. Dendritic protrusions of Drd1-EGFP positive MSN in shell (*A*) or core (*B*) regions of NAcc, or in dorsal striatum (*C*) were analyzed. (*i*) Density of 4 types of protrusions. Data are expressed as number of protrusions per 10-μm dendritic length (mean ± SEM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. One-way ANOVA with Newman-Keuls posttest. (*ii* and *iii*) Cumulative frequency plots showing the distribution of the density of class 2 (*ii*) and class 3 (*iii*) spines from individual dendrites that were analyzed in *i*. The total numbers of dendrites analyzed were 37 for saline-shell, 27 cocaine-shell, 40 methylphenidate-shell, 33 for saline-core, 30 cocaine-core, 23 methylphenidate-core, 42 for saline-dorsal striatum, 34 cocaine-dorsal striatum, and 32 methylphenidate-dorsal striatum.

that only spine length is a critical criterion to distinguish class 2 and 3.

Chronic Methylphenidate Increases the Density of Short (Class 2) and Long (Class 3) Spines in MSN-D1 in NAcc. Our previous studies using cocaine (30 mg/kg for 30 days) resulted in persistent spine formation in MSN-D1 but transient spine formation in MSN-D2 (18). In this study, we compared the effects of methylphenidate and cocaine on spine morphology in MSN-D1 and MSN-D2 using comparable doses of the 2 drugs. Chronic methylphenidate (15 mg/kg, 14 days) increased slightly the density of class 2 spines in MSN-D1 in the NAcc shell (115% of saline group; Fig. 1*Ai* and *ii*) as well as in the core (124% of saline group; Fig. 1*Bi* and *ii*). In general, the effect of methylphenidate on class 2 spines was less than that of cocaine (15 mg/kg, 14 days). Interestingly, the methylphenidate-induced increase in the density of relatively longer spines (class 3) in the NAcc shell (124% of saline group) was bigger than that obtained with cocaine (Fig. 1*Ai* and *iii*). However, methylphenidate had no effect on class 3 spines in NAcc core (Fig. 1*Bi* and *iii*). Chronic cocaine (123% of saline group) but not methylphenidate increased the density of spines significantly in dorsal striatum (Fig. 1*Ci* and *ii*). Notably, chronic cocaine (15 mg/kg for 14 days) increased the density of spines in MSN-D1 to a similar extent as chronic cocaine (30 mg/kg for 30 days) [125% of saline group in this study versus 128% in our previous study (18)]. There was no significant effect of either type of drug exposure on the width of spines in NAcc or dorsal striatum.

Chronic Methylphenidate Increases the Density of Short Spines (Class 2) in MSN-D2 in NAcc. Chronic methylphenidate or chronic cocaine exposure increased the density of class 2 spines in MSN-D2 in the NAcc shell (methylphenidate, 143% of saline group; cocaine, 158% of saline group; Fig. 2*Ai* and *ii*), but not in the core (Fig. 2*Bi* and *ii*). Chronic cocaine, but not methylphenidate, increased the density of class 2 spines significantly in MSN-D2 in dorsal striatum (120% of saline group; Fig. 2*Ci* and *ii*). In general the effect of methylphenidate on spines in MSN-D2 was less than that of cocaine. Chronic cocaine also increased the density of class 1 spines, but the presence of this class of spine is very low.

Chronic Methylphenidate Increases ΔFosB Expression in MSN-D1 from All Areas of Striatum. The transcription factor ΔFosB has been implicated in the addictive properties of psychostimulants (35). Previously we observed in response to chronic cocaine exposure that ΔFosB expression correlated with the formation and/or maintenance of dendritic spines of MSN-D1 and MSN-D2 in NAcc (18). We therefore compared chronic methylphenidate or cocaine exposure on ΔFosB expression. Surprisingly, given the generally larger

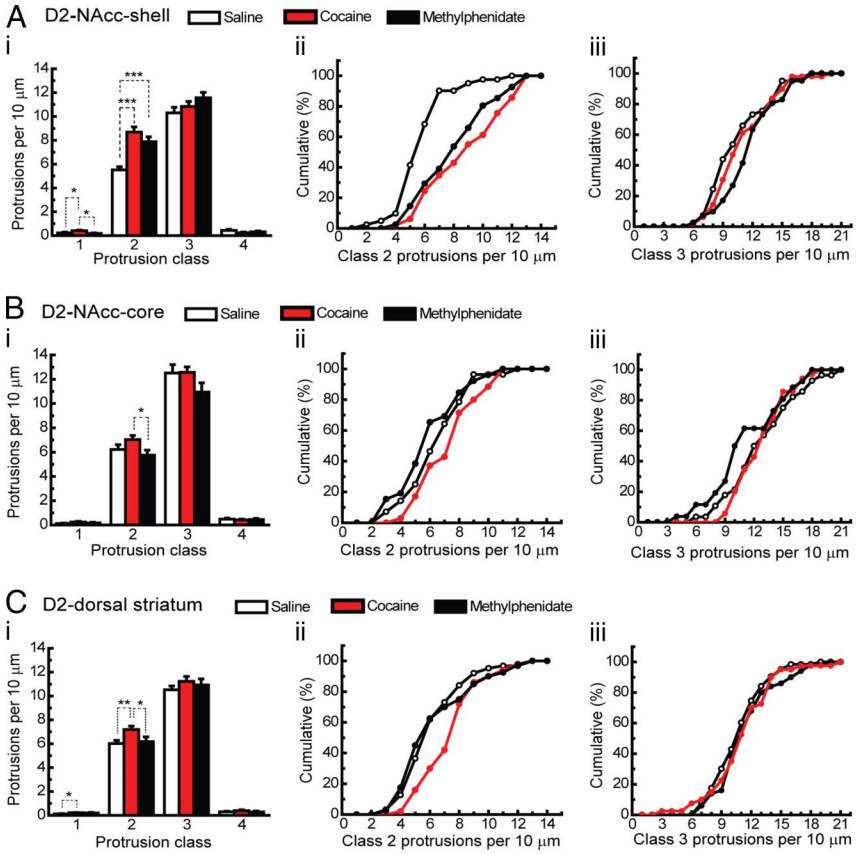

Fig. 2. Chronic methylphenidate or cocaine-induced changes in spine density of MSN-D2 in NAcc and dorsal striatum. D2 dopamine receptor promoter driven (Drd2)-EGFP mice were treated and mouse brains were processed as described in the legend of Fig. 1. Dendritic protrusions of Drd2-EGFP positive MSN in shell (*A*) or core (*B*) regions of NAcc, or in dorsal striatum (*C*) were analyzed. (*i*) Density of 4 types of protrusions. Data are expressed as number of protrusions per 10-μm dendritic length (mean $\pm$ SEM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. One-way ANOVA with Newman-Keuls posttest. (*ii* and *iii*) Cumulative frequency plots showing the distribution of the density of class 2 (*ii*) and class 3 (*iii*) spines from individual dendrites that were analyzed in *i*. The total numbers of dendrites analyzed were 41 for saline-shell, 50 cocaine-shell, 41 methylphenidate-shell, 28 for saline-core, 35 cocaine-core, 26 methylphenidate-core, 63 for saline-dorsal striatum, 50 cocaine-dorsal striatum, and 40 methylphenidate-dorsal striatum.

effect of cocaine on spine formation, chronic methylphenidate increased the number of ΔFosB-positive MSN-D1 to a greater extent than cocaine in the shell and core of NAcc as well as in dorsal striatum (Fig. 3). Chronic cocaine, but not methylphenidate, increased the number of ΔFosB-positive MSN-D2 significantly in the NAcc shell and core of NAcc (Fig. 4).

Discussion

Despite decades of clinical use of methylphenidate for ADHD, concerns have been raised that long-term treatment of children with this medication may result in subsequent drug abuse and addiction. However, meta analysis of available data suggests that treatment of ADHD with stimulant drugs may have a significant protective effect, reducing the risk for addictive substance use (36, 37). Studies with juvenile rats have also indicated that repeated exposure to methylphenidate does not necessarily lead to enhanced drug-seeking behavior in adulthood (38). However, the recent increase of methylphenidate use as a cognitive enhancer by the general public has again raised concerns because of its potential for abuse and addiction (3, 6–10). Thus, although oral administration of clinical doses of methylphenidate is not associated with euphoria or with abuse problems, nontherapeutic use of high doses or i.v. administration may lead to addiction (39, 40).

A major goal of the current study was to examine the effect of chronic exposure to methylphenidate on the structure of dendritic spines. Dendritic spines are highly heterogeneous in their density, length, and head width, and these variable structural properties are associated with synaptic activity and function (41). For instance, long-term potentiation increases the density of mature spines with bigger heads, whereas long-term depression results in spine retraction. In MSN of striatum, we found a relatively broad range in spine length. Notably, methylphenidate had a larger effect than cocaine on the formation of longer spines (class 3) in MSN-D1 from the NAcc shell, whereas cocaine had a larger effect on the formation of shorter spines (class 2) in MSN-D1 and -D2. The longer spines observed following methylphenidate exposure may be the thin or filopodia-like spines reported previously in NAcc core (42). Glutamatergic and dopaminergic synapses are found, respectively, on the head and neck of spines (43), and the variable morphologies of spines may influence the postsynaptic integration of glutamatergic and dopaminergic signaling. These morphological differences may also be associated with different roles for dopamine and glutamate in behavioral sensitization, drug seeking, and relapse (15, 16).

Persistent behavioral abnormalities associated with drug use have implicated long-term adaptive changes in gene expression as a cause for drug addiction. A number of studies have shown that methylphenidate regulates gene expression in corticostriatal circuits and that there were differences between its effects and that of cocaine or amphetamine (44). Among transcription factors implicated in the actions of addictive drugs, ΔFosB is probably the best characterized (35). Notably, the behavioral phenotype of mice that overexpress ΔFosB selectively in NAcc and dorsal striatum resembles that of mice following chronic drug exposure (35). ΔFosB overexpression was also found to enhance locomotor responses, sensitivity to its rewarding effects, and self-administration of cocaine.

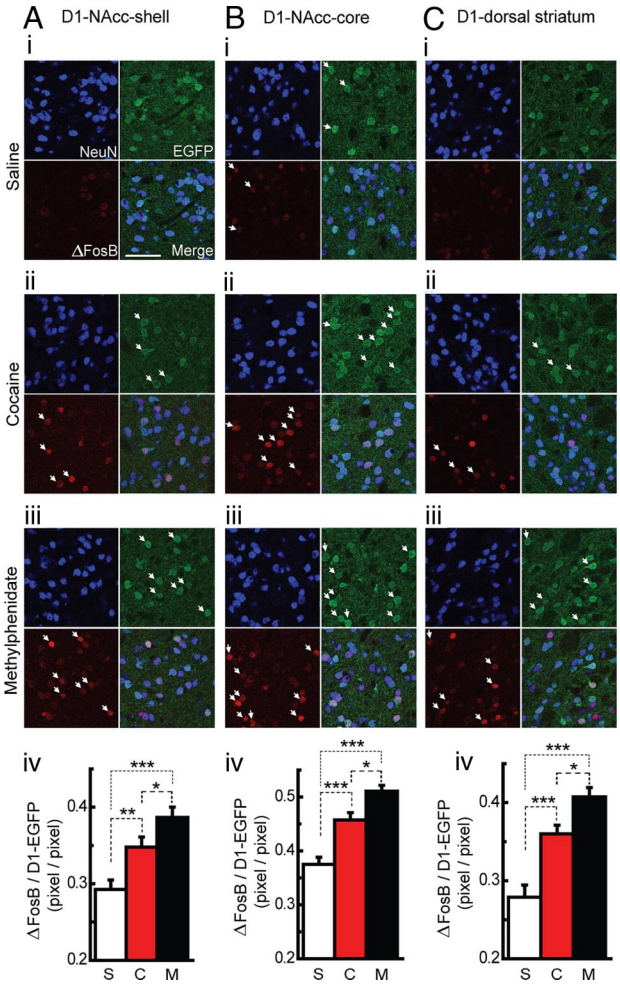

Fig. 3. Chronic methylphenidate- or cocaine-induced changes in ΔFosB expression in MSN-D1 from NAcc and dorsal striatum. Drd1-EGFP mice were treated with saline (*i*), cocaine (*ii*), or methylphenidate (*iii*) as described in the legend of Fig. 1. Two days after the last injection, the expression of EGFP (green, for identification of MSN-D1), and ΔFosB (red) were analyzed by immunohistochemistry in the shell (*A*) and core (*B*) regions of NAcc, as well as in dorsal striatum (*C*). The localization of NeuN (blue) was also analyzed to show the position of neuronal nuclei. NeuN, EGFP, and ΔFosB images were merged to examine their co-expression (Merge). Arrows indicate double-positive neurons for ΔFosB and EGFP. (Scale bar, 50 μm.) (*iv*) The fraction of ΔFosB-positive MSN-D1 was quantified as [the number of pixels containing both a red and a green signal divided by the number of pixels containing a green signal]. S, saline; C, cocaine; M, methylphenidate. Data are expressed as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; one-way ANOVA with Newman-Keuls posttest. A total of 80 images (227 × 227 μm)/group were used for quantification.

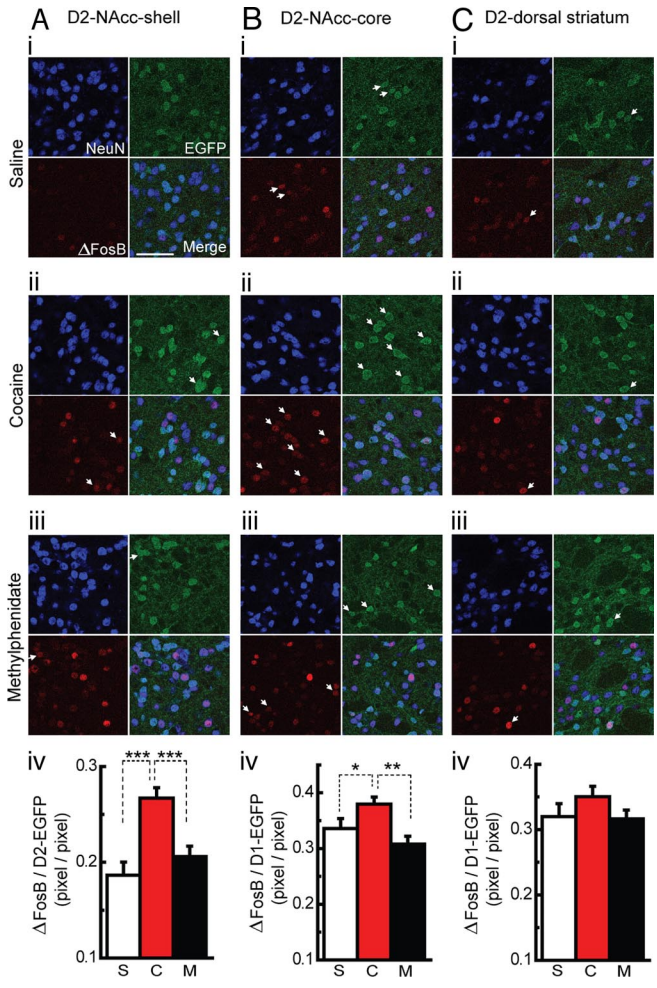

Fig. 4. Chronic methylphenidate- or cocaine-induced changes in ΔFosB expression in MSN-D2 from NAcc and dorsal striatum. Drd2-EGFP mice were treated with saline (*i*), cocaine (*ii*), or methylphenidate (*iii*) as described in the legend of Fig. 1. The localization of NeuN, EGFP and ΔFosB were analyzed in the shell (*A*) and core (*B*) regions of NAcc, as well as in dorsal striatum (*C*) as described in the legend of Fig. 3. Arrows indicate the double-positive neurons for ΔFosB and EGFP. (Scale bar, 50 μm.) (*iv*) Data are expressed as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; one-way ANOVA with Newman-Keuls posttest. A total of 80 images (227 × 227 μm)/group were used for quantification.

Our studies showed that chronic methylphenidate exposure increased the number of ΔFosB-positive MSN-D1 in NAcc shell and core and in dorsal striatum to a greater extent than chronic cocaine. ΔFosB acts mainly as a transcriptional activator, although it can also repress a small subset of genes (45), and this differential activity is regulated by the duration and level of its expression. Short-term expression and lower levels lead to more gene repression, whereas long-term expression and higher levels lead to more gene activation (35, 45). Several target genes for ΔFosB have been identified, among which cyclin-dependent kinase 5 (Cdk5), its cofactor p35, and NFκB, are known to be involved in dendritic spine formation (35, 46, 47). However our results indicate that the level of methylphenidate-induced expression of ΔFosB was not proportional to that of spine formation. For example, the effect of methylphenidate on class 2 spines in MSN-D1 was less than that of cocaine despite its stronger effect on the expression of ΔFosB. In addition, methylphenidate exposure increased the number of spines in MSN-D1 only in the NAcc, whereas it induced the expression of ΔFosB in MSN-D1 in all regions of striatum. Differences in the extent and duration of expression of ΔFosB induced by methylphenidate or cocaine in different regions of striatum, may differentially affect expression of Cdk5, p35, and/or NFκB, and therefore influence the exact pattern of spine morphogenesis.

Studies with animal models have demonstrated similar qualitative properties of methylphenidate and cocaine in terms of their effects on drug discrimination, self-administration, locomotor activity, and certain other behaviors (48). Methylphenidate was shown to have about twice the potency of cocaine in many of those studies. For instance, at the same dose (20 mg/kg, i.p. injection) and at the same degree of dopamine transporter occupancy, methylphenidate increased locomotor activity in mice to a greater extent than cocaine (48). In our current studies with the same doses of methylphenidate and cocaine, we observed differential effects of methylphenidate and cocaine on dendritic spine morphology, and on expression of ΔFosB in 2 types of MSN from 3 different regions

of striatum. The effect of methylphenidate was larger than that of cocaine on the formation of longer spines in MSN-D1 from the shell of NAcc, and on the expression of ΔFosB in MSN-D1 from all areas of striatum. In contrast, cocaine had a larger effect on the formation of shorter spines in MSN-D1 and -D2, and on the expression of ΔFosB in MSN-D2.

The IC_{50} value for binding of methylphenidate to the dopamine transporter is comparable to or slightly lower than that of cocaine (48, 49). Studies using positron emission tomography (PET) showed that the doses of methylphenidate and cocaine required for 50% occupancy of dopamine transporters in human and mice were very similar (40, 48). However, the half-life of methylphenidate (90 min) is much longer than for cocaine (10 min) in brain, and this influences the rate and duration of dopamine increase (40), and likely affects its reinforcing properties (50). The affinity for the norepinephrine transporter relative to the dopamine transporter is also approximately 3- to 4-fold higher for methylphenidate than for cocaine (48, 49). However, methylphenidate has very low affinity for the serotonin transporter compared to cocaine (49). Norepinephrine is critically involved in the effects of psychostimulants on locomotor sensitization, drug discrimination and reinstatement of drug seeking because these are decreased by noradrenergic antagonists or norepinephrine depletion (51). Serotonin is also known to be involved in the actions of cocaine on locomotor activity (52). Thus differences in the levels and kinetics of synaptic overflow of norepinephrine and (or) serotonin in different regions of NAcc and striatum together with differences in dopamine may explain the differential effects of methylphenidate and cocaine on the morphology of dendritic spines and the expression of ΔFosB.

The functional relationship between long-term structural modifications of MSN and any long-term behavioral effects of psychostimulants is an important issue. The presumption from initial observations has been that the increase in spine density observed following repeated exposure to psychostimulants might be directly linked to altered neuronal plasticity and that this in turn was involved in altered behavior (53). However, establishing a causal relationship between increased spine density and behavior has been limited by a lack of experimental methods to modulate dendritic spine number selectively. Notably, manipulation of the expression of the transcription factor MEF2 suggests that the psychostimulant-induced increase of spine density in NAcc is not required for locomotor sensitization (54). Instead it might be part of a negative feedback mechanism that antagonizes this type of behavioral sensitization. Other studies also question a direct relationship between altered spine density and locomotor sensitization. Chronic exposure to metamphetamine can increase spine density in some brain regions and decrease spine density in others (55, 56). Other studies have shown that inhibitors of the kinase, Cdk5, can prevent the effect of chronic cocaine on spine density (47), but a Cdk5 inhibitor or conditional Cdk5 knockout, leads to enhanced locomotor sensitization (57). Possibly, the increase of dendritic spines may be a homeostatic response to hypocorticostriatal glutamatergic input (54, 58). A variety of studies support altered prefrontal glutamate release into the NAcc as a critical mediator of drug seeking and the vulnerability to relapse (16). However, the precise role of glutamate during acute or chronic exposure to drugs, and in drug withdrawal and reinstatement remains to be established. Clearly more work is needed to identify the molecular processes involved in the increased spine density that accompanies chronic exposure to psychostimulants, and to understand the variable effects of cocaine, methylphenidate, and other classes of addictive drugs on synaptic structure and function.

Methods

Animals. The BAC transgenic mice carrying an enhanced green fluorescent protein (EGFP) transgene under the control of either the D1a or D2 dopamine receptor promoter (Drd1-EGFP mice or Drd2-EGFP mice) were used in this study (18, 59). The mice used in this study were 4–5 weeks old and were on a Swiss–Webster background. Mice were maintained in a 12:12-h light/dark cycle and housed in groups of 2–3 with food and water available ad libitum. For the analysis of dendritic spines, 12 mice/group were used and for the analysis of ΔFosB, 4 mice/group were used. All animal protocols were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Rockefeller University Institutional Animal Care and Use Committee.

Drug Treatment. Mice received one injection (i.p.) of 15 mg/kg methylphenidate or cocaine-HCl (or saline) each day for 14 consecutive days. Injections were carried out in the home cage. Two days after the last injection, mouse brains were processed for DiI labeling and/or immunohistochemistry.

Ballistic Labeling with the Fluorescent Dye, DiI. We followed the method described (18) with minor modifications. Mice were anesthetized with sodium pentobarbital and perfused transcardially with 5 mL PBS, followed by rapid perfusion with 40 mL of 4% paraformaldehyde in PBS (20 mL/min). Brains were quickly removed from the skull and postfixed in 4% paraformaldehyde for 15 min. Six serial sections [100-μm thickness each, from bregma 0.94 mm to 1.54 mm (64)] were used for spine analysis. Both hemispeheres of the brain were used. Brain slices (100 μm) were labeled by ballistic delivery of the fluorescent dye DiI (Molecular Probes) as described previously (60). The combined DiI labeling-immunohistochemistry method used a low concentration of detergent (0.01% Triton X-100 in PBS for 15 min), then incubation in 0.01% Triton X-100 and 10% normal goat serum in PBS for 30 min to minimize nonspecific labeling. Tissue sections were then incubated with 1% normal goat serum, 0.01% Triton X-100, and anti-GFP antibody (1:3,000; Abcam) for 2 h at room temperature, washed and incubated in a 1:1,000 dilution of FITC-conjugated secondary antibody (Molecular Probes). Sections were placed on microscope slides and coverslips were applied with mounting medium.

Immunohistochemistry. We followed the method described (18). Animals were anesthetized and perfused as described above. Brains were removed and post-fixed for 1 h in 4% paraformaldehyde at 4 °C. Brains were transferred to 30% sucrose in PBS solution for cryoprotection. Fifteen serial coronal sections [40-μm thickness each, from bregma 0.94 mm to 1.54 mm (64)] were cut on a freezing cryostat (Leica), then permeabilized in 0.3% Triton X-100 in PBS for 15 min and rinsed twice in PBS. Sections were preincubated in 2% normal goat serum in PBS for 30 min at 37 °C, exposed to primary antibodies (diluted in 1% normal goat serum in PBS) overnight at 4 °C, and then rinsed in PBS and incubated with secondary antibodies for 1 h at 37 °C. The following antibodies were used: rabbit anti-pan-FosB (SC-48, 1:500; Santa Cruz Biotechnology), mouse anti-NeuN (1:200; Chemicon), rabbit anti-GFP, FITC-conjugated anti-rabbit IgG, and rhodamine-conjugated anti-rabbit IgG (Molecular Probes). For triple labeling (ΔFosB, NeuN, and GFP), brain sections were first immunostained with anti-pan FosB antibody and anti-NeuN antibody, and then incubated with secondary antibodies (rhodamine-conjugated anti-rabbit IgG and cyan-conjuated anti-mouse IgG). Double-stained brain sections were further processed for GFP immunostaining using Zenon labeling technology (Zenon Alexa Fluor 488, Molecular Probes). The anti-pan-FosB antibody was raised to the N terminus of FosB and recognizes both ΔFosB and full-length FosB (61). ΔFosB, but not FosB or other Fos-related antigens, is known to be stably expressed following chronic cocaine treatment (35). We assume that the long-lasting increases in immunoreactivity represent stable expression of ΔFosB. However, the precise identity of the immunoreactive FosB signal observed in saline-treated mice is unknown.

Image Acquisition and Analysis. Fluorescent images were taken using a confocal microscope (Zeiss LSM 510) with an oil immersion lens (EC Plan-Neofluar 40×, 1.3 N.A., working distance of 0.2 mm) and a 7× digital zoom. DiI was excited using the helium/neon 543 nm laser line; EGFP using argon 488 nm; NeuN using helium/neon 633 nm. For dendritic spines, a stack of images was acquired in the z dimension with an optical slice thickness of 0.8 μm. We analyzed the EGFP and DiI images as described (18). EGFP expression in the Drd1 or Drd2-EGFP mice was used to stain neuronal cell bodies. Through careful comparison of the DiI stain and EGFP expression in the cell bodies of MSN, we identified both DiI- and EGFP-positive, or DiI-positive and EGFP-negative neurons. We analyzed dendritic morphology only in DiI- and EGFP-positive neurons, and all dendrites were densely spined, ranging from 9 protrusions/10 μm (minimum, found in saline group) to 28 protrusions/10 μm (maximum, found in cocaine group). Distal dendrites (2nd to 4th order dendrites) were examined. We collected 1–3 dendrites from the same neuron. The spine density of different dendrites from the same neuron was relatively constant. All measurements were made manually with Metamorph image analysis software (Universal Imaging Corporation).

All dendritic protrusions were included in the analysis. Protrusions from dendrites were classified into 4 types based on their length as described previously (62, 63). Class 1 protrusions, also called ''stubby protuberances'' were less than 0.5

μm in length, lacked a large spine head, and did not appear to have a neck; class 2, or ''shorter spines,'' were between 0.5 and 1.25 μm long; class 3, or ''longer spines,'' ranged between 1.25 and 3.0 μm; class 4, or ''filopodial extensions,'' were filamentous protrusions longer than 3.0 μm.

For ΔFosB images, the confocal images were processed as dual-layer images showing both ΔFosB expression (red fluorescence) and EGFP expression (green fluorescence). Both red and green layers were processed in MATLAB using a minimum threshold filter and then a median filter to remove noise. The parameters for the filters were adjusted automatically for each image, taking into account the distribution of pixel intensities for each image with the goal of maximizing the signal-to-noise ratio. The fractional colocalization for each image was computed as the ratio (colocalized pixels)/(green pixels), where (colocalized pixels) was computed as the number of pixels containing both a red and a green signal, and (green pixels) was computed as the number of pixels containing a green signal. Using sample images, we confirmed by manual counting that the values of the fractional colocalization were proportional to the percentage of ΔFosB-positive MSN.

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