Cocaine Regulates MEF2 to Control Synaptic and Behavioral Plasticity

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SUMMARY

Repeated exposure to cocaine causes sensitized behavioral responses and increased dendritic spines on medium spiny neurons of the nucleus accumbens (NAc). We find that cocaine regulates myocyte enhancer factor 2 (MEF2) transcription factors to control these two processes in vivo. Cocaine suppresses striatal MEF2 activity in part through a mechanism involving cAMP, the regulator of calmodulin signaling (RCS), and calcineurin. We show that reducing MEF2 activity in the NAc in vivo is required for the cocaine-induced increases in dendritic spine density. Surprisingly, we find that increasing MEF2 activity in the NAc, which blocks the cocaine-induced increase in dendritic spine density, enhances sensitized behavioral responses to cocaine. Together, our findings implicate MEF2 as a key regulator of structural synapse plasticity and sensitized responses to cocaine and suggest that reducing MEF2 activity (and increasing spine density) in NAc may be a compensatory mechanism to limit long-lasting maladaptive behavioral responses to cocaine.

INTRODUCTION

A major clinical challenge for effective treatment of drug addiction is its persistence even after long periods of drug abstinence. One of the longest-lasting neural correlates observed across several animal models of addiction is an increase in dendritic spine density on medium-sized spiny neurons (MSNs) in the nucleus accumbens (NAc) (Robinson and Kolb, 2004). Dendritic spines in the NAc are the primary sites of excitatory synapses from prefrontal cortex and other glutamatergic inputs. The necks of these dendritic spines receive dopaminergic inputs from the ventral tegmental area (Hyman et al., 2006). Therefore, altering the density of NAc MSN dendritic spines could have dramatic effects on the information processing from several upstream limbic structures and ultimately addiction-related behaviors. Although several groups have documented that repeated cocaine exposure increases NAc spine density (Lee et al., 2006; Li et al., 2003; Norrholm et al., 2003; Robinson et al., 2001; Robinson and Kolb, 1999a), the precise molecular mechanisms that control this process have remained elusive. Moreover, the cocaine-induced increase in NAc spine density has been hypothesized to contribute to the long-lasting behavioral sensitization that occurs after repeated cocaine exposure (Robinson and Kolb, 1999a), but direct evidence concerning the functional relationship between these two processes is lacking.

A recent study revealed that the cocaine-induced increase in NAc spine density is most stable in the D1 dopamine receptor-expressing neurons (Lee et al., 2006), suggesting that D1 receptor signaling plays a major role in long-lasting stabilization of altered spine density. Interestingly, the D1 receptor-expressing neurons correlated with the same population of NAc neurons that express high levels of the stable transcription factor, ΔFosB, which has been shown to play important roles in addiction-related behaviors (Colby et al., 2003; Hiroi et al., 1997; Kelz et al., 1999). A key ΔFosB gene target in the NAc is cyclin-dependent kinase 5 (Cdk5). Chronic cocaine increases the levels and activity of Cdk5 in the NAc (Bibb et al., 2001), and chemical inhibition of Cdk5 activity in this region blocks the cocaine-induced increase in dendritic spine density (Norrholm et al., 2003). These observations led to the hypothesis that ΔFosB accumulation up-regulates Cdk5, which presumably then phosphorylates key substrates to facilitate the increase in dendritic spine density.

One group of Cdk5 substrates in the brain is the myocyte enhancer factor 2 (MEF2) family of transcription factors (Gong et al., 2003). MEF2 proteins (MEF2A-D) are expressed in unique but overlapping patterns throughout the developing and adult brain (McKinsey et al., 2002; Shalizi and Bonni, 2005). They bind to DNA as hetero- and homodimers and recruit coactivators, such as p300, or corepressors, such as class II histone
MEF2 Transcription Factors in the NAc

RESULTS

MEF2 Transcription Factors in the NAc

To test the role of MEF2-dependent transcription in cocaine-induced NAc dendritic spine plasticity, we first analyzed the expression of MEF2 proteins in the adult striatum. We observed strong, nuclear MEF2A and MEF2D immunostaining throughout the striatum, including the NAc (Figures 1A and S1). MEF2A and MEF2D proteins appear to account for most of the MEF2 DNA-binding activity in the NAc, since NAc extracts incubated with either anti-MEF2A- or anti-MEF2D-specific antibodies (see Figures S2B and S2C) show completely “supershifted” consensus MEF2 response element (MRE)-binding activity in electrophoretic mobility shift assays (EMSA) (Figure 1B). This also suggests that MEF2A and MEF2D bind to DNA as heterodimers in the striatum. Moreover, expression of short-hairpin RNAs (shRNAs) against both MEF2A and MEF2D in cultured striatal neurons reduces the ability of K+ depolarization to induce endogenous MEF2-dependent transcription by ~90% (Figure 1C), further indicating that MEF2A and MEF2D account for most of the depolarization-dependent MEF2 activity in the NAc.

Chronic Cocaine Exposure Increases MEF2 Phosphorylation at Its Inhibitory Cdk5 Site

Since chronic cocaine administration increases Cdk5 activity in the NAc (Bibb et al., 2001) and Cdk5 phosphorylates MEF2 to suppress its activity in cultured cerebellar granule neurons (Gong et al., 2003), we hypothesized that MEF2 activity in the NAc might be attenuated by cocaine treatment. To test this idea, we injected adult rats daily for 7 days with saline (chronic saline) or cocaine (chronic cocaine), with acute cocaine-treated animals receiving 6 days of saline followed by a single cocaine injection on day 7. The animals were then analyzed 4, 24, or 48 hr after the last injection, and the striatum and cerebellum were isolated for western blot analysis with a phosphorylation site-specific antibody to P-Ser408/444 (MEF2A/2D) (Flavell et al., 2006), at 4 hr postinjection, we observed a robust increase in MEF2 P-Ser408/444 levels in striatal lysates of rats treated with chronic cocaine compared to those treated with saline or acute cocaine (Figure 2A). The chronic cocaine-induced hyperphosphorylation of MEF2 in the striatum persisted at 24 hr after chronic cocaine exposure (Figure 2B). Similarly, we observed a strong increase in P-MEF2 after a single acute injection of cocaine at 24 hr, suggesting that there is a delayed upregulation of P-MEF2 that is not yet observed at 4 hr after an acute injection. Moreover, these findings suggest that the increase of P-MEF2 at 4 hr after chronic cocaine represents a maintained level of MEF2 phosphorylation observed after the previous cocaine exposure. However, the inhibitory P-MEF2 levels returned to baseline conditions 48 hr after an acute or chronic course of cocaine (Figure 2B), suggesting that active, repeated drug exposure is needed to maintain suppression of MEF2 activity. In contrast to the striatum, neither acute nor chronic cocaine injections significantly altered MEF2 P-S408/444 levels in the cerebellum at any time point examined (Figure S4A and data not shown). Moreover, using an RNAi-based protein replacement assay (Flavell et al., 2006) in cultured striatal neurons, we found that MEF2 phosphorylation site mutants (i.e., S408A [MEF2A] or S444A [MEF2D]) were hyperactive compared to wild-type MEF2 (Figures 2C and S3A), suggesting that endogenous striatal MEF2 activity is normally regulated by phosphorylation at these Cdk5 sites. Taken together, these data suggest that cocaine-induced MEF2 phosphorylation at Ser408/444 in striatum, in vivo, suppresses its transcriptional activity.

MEF2 Regulates Dendritic Spine Density in the NAc

Since MEF2 activity regulates dendritic spine density and excitatory synapses in cultured hippocampal neurons (Flavell et al., 2006), we hypothesized that the cocaine-induced decrease in
MEF2 activity in the striatum may contribute to the increased density of dendritic spines seen after chronic cocaine exposure. To test this hypothesis, we generated RNAi-expressing adeno-associated viruses (AAVs) to reduce MEF2 levels in the NAc in vivo. As negative controls, we generated similar AAV-shRNA viruses that expressed MEF2 shRNA with point mutations that prevented their recognition of endogenous MEF2A/2D mRNAs. All AAV-shRNA constructs coexpressed EGFP or mCherry, which allowed us to clearly visualize dendritic spines after immunostaining. We coinjected MEF2A and MEF2D shRNA viruses into the NAc on one side of the brain and coinjected the mutant shRNA control viruses into the contralateral NAc. By 2–3 weeks postinjection, when transgene expression is maximal, we observed a strong, sustained reduction of MEF2A and MEF2D protein in the NAc (Figures 3A and S6A). In MEF2 shRNA- or mutant MEF2 shRNA-infected neurons, we observed no evidence of neuronal apoptosis (by nuclear or dendritic morphology or TUNEL staining, data not shown), suggesting that MEF2 activity is not required for NAc MSN survival in vivo.

Using an established cocaine-injection protocol to induce dendritic spines in the NAc (Norrholm et al., 2003), we injected mice once daily with cocaine or saline for 4 weeks before analyzing NAc MSN dendritic spine density. We analyzed dendritic spine density of GFP-positive MSNs using serial optical sections (z stacks) gathered by laser-scanning confocal microscopy, and spine density was manually determined by two independent investigators (blind to the conditions). For this study, detectable dendritic shaft protrusions were counted as dendritic spines; they were not analyzed for dendritic spine subtype or spine length/volume. We found that in both saline- and cocaine-injected mice, MEF2A/2D shRNA-expressing neurons had significantly higher NAc MSN dendritic spine density than the control neurons (Figures 3B and S6C). These results indicate that reducing MEF2 activity in NAc is sufficient to increase MSN dendritic spine density and reveal an important role for MEF2 in regulating basal dendritic spine density in the NAc in vivo.

Next we tested whether suppression of MEF2 activity by cocaine is required for the cocaine-induced increase in NAc MSN dendritic spine density. To this end, we produced AAVs that coexpress EGFP together with a constitutively active form of MEF2 (MEF2-VP16) or a DNA-binding-deficient negative control (AAV-MEF2ΔDBD-VP16). MEF2-VP16 is a fusion between the MEF2 DNA binding and dimerization domains and the basal transcription activation domain of the viral transcription factor VP16 (Black et al., 1996). In cultured striatal neurons, MEF2-VP16 increased MEF2-dependent transcription, whereas MEF2ΔDBD-VP16 or vector control had no effect (Figure S7). Similar to the
MEF2 RNAi experiments described above, we then injected either the AAV-MEF2-VP16 or the control AAV-MEF2ΔDBD-VP16 virus into the NAc on opposite sides of the brain. After a 3 week recovery and expression period, we injected the mice daily with either saline or cocaine for 4 weeks before analyzing NAc spine density of GFP-expressing neurons. Compared to the saline-injected mice, we found that cocaine significantly increased striatal P-MEF2 levels (mean ± SEM, ***p < 0.001, n = 4–5). P-MEF2 levels returns to control levels by 48 hr after the final dose (mean ± SEM, p > 0.05, n = 4–5).

Figure 2. Chronic Cocaine Upregulates Inhibitory MEF2 Phosphorylation at Ser408/444
(A) Western blots using a MEF2A/2D phospho-S408/444-specific antibody demonstrate that chronic cocaine administration significantly increases MEF2 P-S408/444 phosphorylation in striatum 4 hr after the last injection (mean ± SEM, *p < 0.05, n = 3).
(B) Quantification of MEF2 P-S408/444 western blotting at 24 hr after the last injection reveals that both acute and chronic cocaine significantly increase striatal P-MEF2 levels (mean ± SEM, **p < 0.01, n = 4–5). P-MEF2 levels returns to control levels by 48 hr after the final dose (mean ± SEM, p > 0.05, n = 4–5).
(C) RNAi-based protein replacement assays comparing wild-type and S444A MEF2D activity. Expression of MEF2D S444A results in significantly elevated basal and KCl-induced MEF2-dependent transcription in MEF2-luciferase assays (mean ± SEM, **p < 0.01 and ***p < 0.001, respectively, n = 3) (top). Similar effects are observed for the MEF2A phospho-mutant (S408A) (Figure S3 A). Anti-MEF2D western blots of HEK-293T total cell lysates of cultures transfected with equal amounts of expression plasmids (bottom).

MEF2 Activity in the NAc Modulates Behavioral Responses to Cocaine
Regulation of MEF2 activity has a potent effect on NAc dendritic spine density; therefore, we sought to test the functional relationship between MEF2-dependent NAc spine plasticity and sensitized behavioral responses to cocaine in vivo. We hypothesized that bilateral expression of MEF2-VP16, which blocked the cocaine-induced spine increase in the NAc, might reduce cocaine-induced behaviors. Contrary to this hypothesis, however, we found that NAc expression of constitutively active MEF2 enhanced locomotor responses to cocaine (Figures 4A–4C). Specifically, mice expressing MEF2-VP16 in the NAc responded normally to an initial injection of cocaine but demonstrated significantly higher locomotor responses to cocaine on day 2 compared to control mice (Figure 4A) and trended higher than the control mice for the duration of the injections. After 1 week of withdrawal, the MEF2-VP16-expressing mice remained significantly more sensitive to a cocaine challenge dose than the control mice (Figure 4B). Consistent with the sensitizing effect of MEF2-VP16 expression in the NAc on cocaine-induced locomotor behaviors, MEF2-VP16 expression also sensitizes mice to the rewarding effects of cocaine as measured by conditioned place

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preference. That is, mice overexpressing MEF2-VP16 in their NAc spend significantly more time in a cocaine-paired versus a saline-paired environment (Figure 4C). These findings suggest that enhanced MEF2 activity in the NAc, a condition that blocks cocaine-induced spine increases, promotes acquisition and maintenance of cocaine-induced locomotor sensitization as well as strengthens the rewarding effects of the drug.

As described earlier, cocaine induces the hyperphosphorylation of MEF2 in the NAc, which presumably suppresses its transcriptional activity. To explore the behavioral consequences of suppressing MEF2 activity in the NAc, we coinjected this region of adult mice bilaterally with AAV-MEF2A/2D shRNAs or mutant shRNA control viruses and tested their locomotor responses to repeated cocaine injections. Reducing MEF2 levels in the NAc, which was sufficient to increase dendritic spine density, both delayed the acquisition of cocaine-induced locomotor sensitization and attenuated sensitized behavioral responses to a cocaine challenge dose after 2 weeks of withdrawal (Figures 4D and 4E). These observations are in agreement with the opposite behavioral responses observed in mice with increased NAc MEF2 activity (Figures 4A and 4B).

In sum, these data reveal that MEF2 activity modulates the acquisition and maintenance of sensitized behavioral responses to cocaine. Unexpectedly, we find that enhanced MEF2 activity in the NAc, a condition that blocks the cocaine-induced dendritic spine density increase, actually enhances sensitized behavioral responses to cocaine. These findings suggest that the cocaine-induced increase in spine density is not required for
locomotor sensitization and reward learning, but may instead represent a compensatory process to limit maladaptive behavioral changes.

**Dopamine D1 Receptor Signaling Inhibits MEF2 Activity in Striatal Neurons**

Since cocaine exposure upregulates an inhibitory phosphorylation event on MEF2 in the striatum in vivo, we sought to explore molecular signaling events by which cocaine might regulate MEF2 activity in the striatum. To this end, we cultured dissociated striatal neurons and transfected them with a MEF2-luciferase reporter plasmid (MEF2-luc) in order to measure endogenous MEF2 activity. Since cocaine exposure has been associated with increased glutamatergic and dopaminergic neurotransmission in the striatum, we studied whether these stimuli regulate MEF2-dependent transcription. This activation of MEF2 required calcium influx through L-type voltage-sensitive calcium channels (LT-VSCCs) and calcineurin phosphatase activity (Figures S5A and S5B). While the stimulation of dopamine D1 receptors (10 μM SKF81297) alone had little effect on basal MEF2 activity, dopamine D1 receptor stimulation significantly attenuated the activation of MEF2 upon membrane depolarization (KCl) (Figure 5A). To test whether the suppression of MEF2 activity by dopamine D1 receptor signaling was due to elevation of cAMP, we treated the cultures with the adenylyl cyclase-activator, forskolin (10 μM), and observed a similar reduction of calcium-induced MEF2 activity (Figure 5B). This suggests that activated dopamine D1 receptors increase cAMP levels, which in turn antagonize calcium-dependent activation of MEF2 in striatal neurons.

As a point of comparison, we also analyzed endogenous CREB activity using a cAMP response element (CRE)-luciferase reporter plasmid. As expected, CREB activity is significantly lowered in response to cocaine challenge (Figure 5C).

**Figure 4. MEF2 Activity in the NAc Modulates Behavioral Responses to Cocaine**

(A) Viral-mediated expression of MEF2-VP16 in the NAc significantly increases sensitivity to repeated cocaine administration. Mice expressing MEF2-VP16 in the NAc have normal locomotor response to saline and the first cocaine injection (15 mg/kg) but are significantly more sensitive to the subsequent dose compared to the MEF2-VP16 DNA-binding mutant control (MEF2DBD-VP16) (mean ± SEM, *p < 0.05, n = 9–10, Student’s t test on day 2).

(B) Mice expressing MEF2-VP16 in their NAc remain significantly more sensitive to a challenge dose of cocaine (15 mg/kg) after 1 week of withdrawal (mean ± SEM, *p < 0.05, n = 9–10, Student’s t test).

(C) Mice expressing MEF2-VP16 in the NAc spend more time in a cocaine-paired (8 mg/kg) environment as measured by conditioned place preference (mean ± SEM, *p < 0.05, n = 13, Student’s t test).

(D) Viral-mediated knockdown of MEF2A/2D in the NAc significantly reduces sensitivity to repeated cocaine administration. Mice expressing shRNAs against MEF2A/2D in the NAc have normal locomotor responses to saline and the first cocaine injection (15 mg/kg) but are significantly more sensitive to the subsequent dose compared to mice expressing control shRNAs (mean ± SEM, *p < 0.05, n = 9–11, Student’s t test).

(E) Viral-mediated knockdown of MEF2A/2D in the NAc shows significantly less cocaine-induced locomotor activity in response to a challenge dose (15 mg/kg) given 2 weeks after the acquisition of cocaine sensitization (mean ± SEM, **p < 0.01, n = 9–11, Student’s t test). RNAi-mediated reduction of MEF2A/2D in the NAc has only a slight trend toward reducing locomotor responses to a challenge dose of cocaine (15 mg/kg) 1 week after acquisition of cocaine sensitization (mean ± SEM, p > 0.05, n = 9–11).
increased in our striatal cultures by either the dopamine D1 receptor agonist or upon membrane depolarization (Figure 5C). Unlike MEF2, however, combining membrane depolarization (KCl) with dopamine D1 receptor activation synergistically activated CREB-dependent transcription (Figure 5C). These findings suggest that calcium signaling and dopamine signaling cooperate to activate CREB activity in striatal neurons while combining to reduce activation of MEF2, which is consistent with recent findings in hippocampal neurons (Belfield et al., 2006). Interestingly, several studies suggest that cocaine-induced CREB activity serves to limit behavioral responses to cocaine (Carlezon et al., 1998; Dong et al., 2006). Therefore, dopamine signaling may differentially regulate CREB and MEF2 activities to limit sensitized behavioral responses to cocaine.

**Dopamine and Calcium Signaling Regulates MEF2 Activity through Calcineurin and RCS**

Since membrane depolarization stimulates MEF2 activity in striatal cultures through a calcineurin-dependent mechanism (Figure S5B), we speculated that dopamine signaling might regulate MEF2 activity by reducing calcineurin activity. To test this possibility, we transfected striatal neurons with constitutively active, Ca\(^{2+}\)/CaM-independent calcineurin (CaN\(\Delta\)CT) and measured the effect of cAMP signaling on calcium-dependent MEF2 activation. We observed that expression of CaN\(\Delta\)CT blocked the forskolin-dependent inhibition of MEF2 activity (Figure 5D), which indicates that cAMP signaling attenuates MEF2 in part by reducing Ca\(^{2+}\)/CaM-calcineurin activity.

Recently, the regulator of calmodulin signaling (RCS) was shown to negatively regulate calcineurin activity in striatal neurons (Rakhilin et al., 2004). Activation of the dopamine D1 receptor stimulates PKA-dependent phosphorylation of RCS at Ser55, which induces direct interaction of phospho-RCS with Ca\(^{2+}\)/CaM and competitive inhibition of calcineurin activity (Rakhilin et al., 2004). To test whether RCS regulates MEF2 activity, we expressed RCS in striatal neurons and found that, in the presence of forskolin, RCS inhibited calcium-stimulated MEF2 activity compared to the effect of forskolin alone (Figure 6A, right). Notably, RCS had no effect on calcium-inducible MEF2 activity in the absence of forskolin (Figure 6A, left), suggesting that RCS phosphorylation by PKA is necessary to inhibit MEF2 activity. Indeed, the cAMP-dependent inhibition of MEF2 activity by RCS required phosphorylation at Ser55 (PKA site) since a nonphosphorylatable RCS mutant (S55A) failed to suppress MEF2 activity in the presence of forskolin (Figure 6B). Importantly, the inhibition of MEF2 activity by cAMP/RCS is partial and might therefore indicate that P-RCS levels are limited and/or that additional cAMP-insensitive mechanisms regulate calcineurin/MEF2 activity.

To determine whether the cAMP/RCS signaling pathway might regulate MEF2 in the adult striatum in vivo, we generated phosphorylation-site-specific antibodies to P-Ser55 RCS (Figure S8A). We found that treatment of acute, adult striatal slices with either forskolin or SKF81297 increased levels of P-RCS (Figure S6C). Similarly, we analyzed striatal lysates from rats treated repeatedly with saline or cocaine for 1 week by western blotting. Similar to P-MEF2, chronic cocaine exposure increased P-RCS levels at 4 hr and 24 hr after the last injection (Figures 6D and S8B). Together, these findings suggest that cocaine regulates MEF2 activity in the striatum through activation of a dopamine- and cAMP-dependent signaling cascade that functions, at least in part, by RCS-dependent suppression of calcineurin activity.

**Figure 5. Dopamine D1 Receptor Signaling and cAMP Reduce Calcium-Dependent Activation of MEF2 in Cultured Striatal Neurons**

(A) Dopamine D1 receptor stimulation (SKF81297, 10 \(\mu\)M) significantly reduces calcium-dependent activation of MEF2-luciferase activity in cultured striatal neurons (\(p < 0.05; n = 15\), five independent experiments, Student’s t test).

(B) Forskolin (forsk) treatment (10 \(\mu\)M) of cultured striatal neurons significantly attenuates basal and KCl-induced MRE-luciferase activity. The inset shows the effect of forskolin on basal MRE-luciferase activity over a smaller scale (mean ± SEM, ***\(p < 0.001\); \(n = 21\), seven independent experiments, Student’s t test).

(C) Membrane depolarization (60 mM KCl) of cultured striatal neurons significantly increases CRE-luciferase activity (mean ± SEM, ***\(p < 0.001\); \(n = 21\), seven independent experiments).

(D) Constitutively-active calcineurin (CaN\(\Delta\)CT) blocks the inhibitory effect of forskolin on KCl-induced MEF2 activity (mean ± SEM, ***\(p < 0.001\) or N.S., \(p > 0.05\); \(n = 6\), two independent experiments, Student’s t test).
Genome-wide Analysis of MEF2 Promoter Binding Identifies Targets that Regulate Structural Plasticity

To identify MEF2 gene targets in vivo that ultimately mediate MEF2's effects on dendritic spine plasticity and behavioral responses to cocaine, we isolated NAc from chronic cocaine-treated mice and performed chromatin immunoprecipitation (ChIP) using a MEF2A antibody or IgG control. Immunoprecipitated DNA was then amplified and hybridized to genome-wide promoter arrays to identify potential MEF2 target genes. An example of the genome-wide MEF2-binding data is shown for chromosome 17 (Figure 7A). We found that, in cocaine-treated mice, MEF2 significantly binds to the promoters of 900 genes in the NAc at a significance level of p < 0.0001.

To explore the ascribed cellular processes that these MEF2-bound genes regulate, we performed Ingenuity pathway analysis on the significant MEF2-bound genes. We identified several highly enriched signaling pathways and cellular processes, including actin cytoskeleton signaling and cAMP signaling, which are known to have pronounced effects on dendritic structural plasticity and/or behavioral responses to cocaine (Figure S9A). For example, MEF2 is bound to the proximal promoters of the Wiskott-Aldrich syndrome proteins, N-WASP (Wasl), WAVE3 (Wasl3), and Profilin 1 (Pfn1) (Figures S9B and S9C), which have all been shown to regulate F-actin cytoskeletal remodeling in dendritic spines (Ackermann and Matus, 2003; Irie and Yamaguchi, 2002; Pilpel and Segal, 2005; Wegner et al., 2008).

Since chronic cocaine exposure appears to reduce MEF2 activity in the striatum, we compared MEF2-bound genes from our ChIP-chip arrays to a list of genes that are downregulated by chronic cocaine (24 hr after the last injection) as determined from gene expression microarrays performed with the NAc of cocaine-treated versus saline-treated mice (Renthal et al., 2007). We identified a subset of 82 MEF2-bound genes whose mRNA expression was downregulated >1.2-fold 24 hr after repeated cocaine administration (Figure 7B). From this group of cocaine-regulated and MEF2-bound genes, we focused our attention on one, the PI3-kinase gamma catalytic subunit gene, pik3cg.

PI3-kinase activity has been shown to be important for the expression of sensitized locomotor responses to repeated cocaine (Izzo et al., 2002), so we speculated that regulation of pik3cg could have an important impact on behavioral responses to cocaine. Using qRT-PCR, we confirmed that chronic cocaine...
exposure significantly downregulates Pik3cg mRNA in the NAc in an independent cohort of mice (Figure 7D). Using quantitative ChIP on NAc samples from chronic cocaine-treated mice, we found that MEF2 DNA binding to the pik3cg promoter was highly enriched at three near-consensus MEF2 response elements (MREs) (Figure 7C), suggesting that it could significantly regulate the expression of this gene. Consistent with this idea, PC12 cells transfected with a 4-hydroxytamoxifen (4OHT)-inducible MEF2-VP16-ERtm [Flavell et al., 2006] show significant increases in Pik3cg mRNA after 4OHT treatment (Figure 7E).

Since MEF2 regulates pik3cg transcription and chronic cocaine reduces both MEF2 activity and pik3cg levels, we speculated that repeated cocaine exposure might lower PI3-kinase activity in the NAc. Consistent with this idea, we found that chronic cocaine exposure significantly reduced the phosphorylation of Akt, a key PI3-kinase substrate, in the NAc (Figure 7E). Since PI3-kinase activity is important for facilitating expression of cocaine locomotor sensitization [Izzo et al., 2002], then the role of MEF2 in modulating the sensitized responses to cocaine might be due in part to regulation of this important signaling pathway. Interestingly, chronic morphine has been shown to reduce P-Akt levels in the ventral tegmental area [Russo et al., 2007], further supporting an important role for Akt signaling in drug addiction. Together, these data provide a genome-wide analysis of MEF2 target genes in brain and describe how an MEF2 target gene, pik3cg, might regulate important aspects of the complex biological events that underlie sensitized behavioral responses to drugs of abuse.

**DISCUSSION**

In this study, we find that repeated cocaine exposure regulates MEF2 transcription factors to control aspects of long-lasting synaptic and behavioral plasticity. Our findings suggest that chronic cocaine exposure reduces MEF2-dependent transcription to promote increased MSN dendritic spine density in the NAc. Surprisingly, this MEF2-controlled increase in dendritic spine density is associated with reduced behavioral sensitivity to cocaine, suggesting that the strong correlation between increases in NAc spine density and sensitized behavioral responses to cocaine may be functionally uncoupled processes. Our findings suggest that repeated cocaine exposure suppresses MEF2 activity in part by a cAMP/RCS-dependent...
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Figure 8. Model for How Cocaine Regulates MEF2 in the NAc to Alter Dendritic Spine Density and Behavioral Responses

that MEF2 potentially regulates NAc spine plasticity and, as a key target of Cdk5, may mediate some of its downstream effects on synapse and network plasticity. There are a number of interesting parallels between our MEF2 findings and reports in the literature regarding the function of Cdk5 activity in cocaine-induced spine plasticity and behavioral responses (Norrholm et al., 2003; Taylor et al., 2007). Specifically, chronic infusion of roscovitine into the NAc region, which we find increases MEF2 activity in striatal neurons (Figure S3B), blocks the chronic cocaine-induced increase in NAc MSN dendritic spine density (Norrholm et al., 2003). Similarly, we find that increasing MEF2 activity in the NAc (via MEF2-VP16 expression) also blocks the cocaine-induced increase in dendritic spine density (Figure 3D), suggesting that downstream inhibition of MEF2 by Cdk5 is a key component of roscovitine’s effect on spine plasticity in the NAc. Another interesting parallel between the Cdk5 and MEF2 studies is the recent findings that daily roscovitine injections into the NAc (Taylor et al., 2007) or conditional Cdk5 gene deletion in the NAc (Benavides et al., 2007) enhanced locomotor sensitization to repeated cocaine treatments. Consistent with these observations, we find that enhanced MEF2 activity in the NAc (via MEF2-VP16 expression) increased the locomotor responses to repeated cocaine injections (Figures 4A and 4B). Together, these studies provide evidence that experimental manipulations that block cocaine-induced increases in NAc dendritic spine density enhance sensitized behavioral responses to cocaine, suggesting that spine density increases are not required for locomotor sensitization and might instead be functioning to antagonize the process of sensitization. It is perhaps not surprising that spine increases are not required for behavioral sensitization, since chronic morphine exposure, which elicits long-lasting locomotor sensitization, actually decreases NAc dendritic spine density (Robinson and Kolb, 1999b). In our study, we cannot directly determine whether lower spine density actually causes increased cocaine sensitivity, per se, because the proximal manipulation in our study is MEF2—not the spines themselves. It is possible that MEF2 could function through independent mechanisms to control cocaine behavior and dendritic spine density. Nevertheless, one can conclude from this and previous reports that cocaine-induced increases in NAc dendritic spine density does not appear to be required for behavioral sensitization to cocaine. This functional relationship between cocaine-induced dendritic spines and behavioral responses suggests that the additional NAc dendritic spines may contribute to homeostatic adaptations that counteract the neural plasticity processes that cause sensitized behavioral responses.

reduction in calcineurin activity, which regulates MEF2 phosphorylation at the inhibitory Cdk5 sites, P-Ser408/444 (Figure 8). This inhibition of MEF2 through increased P-Ser408/444 levels is also likely influenced by the previously reported upregulation of Cdk5 levels and activity in the striatum after chronic cocaine exposure (Bibb et al., 2001). Finally, by combining in vivo MEF2 ChIP on genome-wide promoter arrays and gene expression microarrays, we identified a number of putative MEF2-target genes that likely contribute to aspects of cocaine-induced dendritic spine and behavioral plasticity in the NAc.

Cocaine administration increases the inhibitory Ser408/444 phosphorylation of MEF2A/2D in striatum with a complex time course. A single dose of cocaine is sufficient to induce MEF2 phosphorylation, but is only detected at 24 hr after the first injection. Repeated, daily cocaine injections appear to maintain the levels of MEF2 P-Ser408/444, as it is detected at 4 hr and 24 hr after the last cocaine injection (7 day injection regimen). However, in the absence of reinforcing cocaine injections, we observe that P-S408/444 levels return to baseline by 48 hr after the last injection in the 7 day injection regimen. This indicates that MEF2 inhibitory phosphorylation does not persist in the striatum for as long as the cocaine-induced spine changes that MEF2 activity controls. Moreover, it also suggests that suppression of MEF2 activity by cocaine likely regulates the initiation and maintenance of spine density changes observed during and shortly after chronic cocaine exposure, but may not play a significant role in the maintenance of those spine changes during extended withdrawal periods. In the future, it will be important to explore the regulation and potential role of MEF2 in the maintenance phase of spine plasticity during withdrawal.

Several recent reports have demonstrated an important role for Cdk5 activity in regulating chronic cocaine-induced spine and behavioral plasticity (Benavides et al., 2007; Bibb et al., 2001; Norrholm et al., 2003; Taylor et al., 2007). We show here...
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Neuron

How does regulation of MEF2-dependent transcription control NAc synapse density? In cultured hippocampal neurons, MEF2-dependent transcription is induced by glutamatergic synaptic activity to promote elimination of existing excitatory synapses (Flavell et al., 2006). As such, it is possible that suppression of MEF2 activity by cocaine in the NAc increases dendritic spine density by reducing the elimination rate of existing synapses rather than increasing the formation rate of new synapses. NAc neurons may therefore rely upon MEF2-dependent transcription rather than increasing the formation rate of new synapses. NAc neurons may therefore rely upon MEF2-dependent transcription to control homeostatic synaptic plasticity after repeated cocaine exposure (Turrigiano, 2007). Indeed, intrinsic neuronal excitability is reduced in the NAc of chronic cocaine-treated animals (Hu et al., 2004), and glutamatergic inputs to the NAc may be decreased after extended drug taking as a result of a decreased mPFC function (hypofrontality), which has been observed in both animal models of addiction and in human brain imaging studies of drug addicts (Jentsch and Taylor, 1999; Volkow et al., 2003). Therefore, it is interesting to speculate that increased NAc dendritic spine density may function homeostatically to compensate for reduced NAc excitability and ultimately limit an animal’s sensitivity to the cocaine.

Cocaine regulation of MEF2-dependent transcription ultimately mediates structural and behavioral changes in the NAc through the altered expression of downstream target genes. We utilized genome-wide ChIP-chip technology to identify nearly 900 gene promoters in cocaine-treated NAc tissue where MEF2A binding is enriched. Many of the target genes we identified cluster to cellular functions that regulate structural plasticity, such as F-actin remodeling. The MEF2 target genes that encode the proteins, N-WASP, WAVE3, and profilin 1, are all known to potently regulate cytoskeletal remodeling as well as dendritic spine density (Irie and Yamaguchi, 2002; Pilpel and Segal, 2005; Wegner et al., 2008). Importantly, dysregulation of actin polymerization with latrunculin A or a LIM-kinase peptide antagonist in the NAc promoted cocaine reinstatement behaviors in self-administering rats (Toda et al., 2006). Therefore, MEF2 gene targets that regulate actin remodeling may play important roles in sensitized cocaine responses, perhaps independent of the spine changes observed after chronic cocaine exposure.

Another functional group of MEF2-bound genes in the NAc clustered to the PI3-kinase/Akt signaling pathway (Figure S9A). Interestingly, a chemical inhibitor of PI3-kinase delivered intracerebroventricular (ICV) significantly blocked the expression of cocaine locomotor sensitization (Izzo et al., 2002), suggesting that MEF2-dependent regulation of this signaling pathway may play an important role in locomotor sensitization. Since cocaine suppresses MEF2 activity in the NAc, it was notable that a catalytic subunit of PI3-kinase was one of 82 MEF2 target genes downregulated by chronic cocaine exposure (Figures 7C and 7D). Pik3cg expression is also potently regulated by MEF2 activity in culture, suggesting that the cocaine-induced suppression of MEF2 activity directly contributes to the downregulation of this gene in the NAc. Consistent with a reduction in Pik3cg mRNA in the NAc after chronic cocaine exposure, we also observed a significant reduction in the phosphorylation state of Akt (Ser473), a key substrate of PI3-kinase activity, in the NAc after chronic cocaine. Moreover, RNAi-based reduction of MEF2 levels in the NAc, which would be expected to reduce Pik3cg expression and PI3-kinase activity, reduces behavioral sensitivity to cocaine, much like the infusion of a PI3-kinase inhibitor.

In this study, we found that cocaine administration regulates MEF2-dependent gene transcription in the NAc to control dendritic spine plasticity and behavioral responses to cocaine. We show here that chronic cocaine reduces MEF2 activity through a signaling mechanism involving Cdk5, calcineurin, and RCS. We find that reducing MEF2 activity in NAc in vivo is required for cocaine-induced increases in dendritic spine density. Our findings also suggest that behavioral sensitization to cocaine is functionally uncoupled from these cocaine-induced increases in dendritic spine density. Taken together, these observations implicate MEF2 transcription factors in the molecular mechanisms controlling cocaine-induced structural and behavioral plasticity and could ultimately lead to the development of improved treatments for drug addiction.

EXPERIMENTAL PROCEDURES

Plasmids

3X MRE-luciferase, pcDNA3-MEF2-VP16, pSuper-MEF2A(1234), pSuper-MEF2D(479), pcDNA3-MEFA (rat; wild-type and RNAi-resistant (RIR)), pcDNA3-MEF2D (rat; wild-type and RIR), pcDNA3-rMEF2A S408A (RIR), and pcDNA3-MEF2D S444A (RIR) were described previously (Flavell et al., 2006). To generate pcDNA3-Flag-RCS, we used PCR amplification from reverse transcriptase reactions of purified rat NAc mRNAs. The degenerate PCR primers incorporated unique BamHI (5') and NotI (3') sites, and the coding sequence for the N2 Flag epitope tag in-frame with the second amino acid of RCS. The PCR fragment was subcloned into pcDNA3, and the insert region was confirmed by sequencing.

Disassociated Striatal Cultures

Embryonic striatal neurons (E18/19) were cultured from Long Evans rats (Charles River Labs) as described previously (Ventimiglia and Lindsay, 1998), with modifications. Details can be found in the Supplemental Data.

Luciferase Assays in Primary Neurons

Disassociated striatal neurons were transfected at 8 days in culture using calcium phosphate as described previously (Flavell et al., 2006), then stimulated and harvested for dual luciferase activity (Promega). Details can be found in the Supplemental Data.

Animals

Adult male C57BL/6 mice (Jackson Laboratory) and adult male Sprague-Dawley rats (Harlen) were housed on a 12 hr light-dark cycle with access to food and water ad libitum. All procedures were in accordance with the Institutional Animal Care and Use (IACUC) guidelines.

Western Blots of In Vivo Samples and Immunohistochemistry

Details can be found in the Supplemental Data.

Generation of P-RCS Antibodies

Rabbits were injected with a synthesized P-RCS peptide encompassing RCS amino acids 50–60, where position S55 was phosphorylated (Covance). The injected peptide was conjugated to KLH via an N-terminal lysine residue.

Electrophoretic Mobility Shift Assay

Details can be found in the Supplemental Data.
Slice Pharmacology and Recombinant Adeno-Associated Viruses
Details can be found in the Supplemental Data.

Stereotactic Surgery
Details can be found in the Supplemental Data.

Dendritic Spine Analysis
Mice were unilaterally infused into their NAc with AAVs expressing MEF2A and MEF2D shRNAs or control shRNA viruses. For MEF2 overexpression studies, mice were unilaterally infused with AAVs expressing MEF2-VP16 or its respective control virus. Nineteen days post-op, mice were given daily cocaine (30 mg/kg) or saline 5 days/week for 4 weeks (20 total injections) and sacrificed 24 hr after the last dose. GFP-labeled neurons within the NAc core and shell regions were imaged at high resolution using a 100× oil-immersion lens on a Zeiss LSM 510 confocal microscope. PMT assignment, pinhole sizes, and contrast values were kept constant across different confocal sessions. Confocal stacks consisted of 31–178 sections at 0.23 μm in thickness imaged with a Z step of 0.1 μm. Images were taken at 1024 × 1024 pixel resolution to cover the entire Z dimension of the labeled neurons. Lengths of dendritic segments were measured using NIH ImageJ software. Spine densities were quantified by counting the number of spines along 30–100 μm segments of secondary dendrites (two to three dendrite segments/neuron). Spine densities were expressed as spines per 10 μm. Only spines appearing continuous with their parent dendrite shaft in maximum-intensity z projection were used for quantitative analysis. Mean spine densities were analyzed by pair-wise comparisons using the Student’s t test.

Cocaine-Induced Locomotor Sensitization
At the same time each day, mice were injected with saline or cocaine (IP) and were placed in standard plastic cages similar to their home cages for 2 hr. These cages were inside the Photobeam Activity System (San Diego Instruments, San Diego, CA), where five photobeams measured the mouse’s locomotor activity in 5 min bins. Mice received saline injections on days 1–3 to habituate them to the novel environment. The locomotor activity for their final saline day is displayed. On days 4–10, mice received cocaine injections (15 mg/kg). Challenge doses of cocaine (15 mg/kg) occurred on day 17 (1 week of withdrawal) and on day 24 (2 weeks of withdrawal; RNAi experiment only) of the experiment. Day 1 of the experiment was 21 days after viral delivery, a time point at which high levels of expression were verified. For each day, the sum of the first 30 min (MEF2-VP16) or 45 min (shRNA) of locomotor activity after injection is displayed.

Conditioned Place Preference
Eighteen to twenty-one days following stereotactic delivery of AAV-MEF2VP16 or its control virus into the NAc, mice were conditioned to cocaine in a standard three-chamber conditioned place preference box (gray side, middle, and stripped side). Using an unbiased 6 day paradigm, mice were pretrained on day 1 to balance pre-existing side bias. On days 2 and 4, mice received cocaine injection (8 mg/kg) and were confined to the appropriate chamber. On days 3 and 5, mice received a saline injection and were confined to the opposite chamber. On the final day, mice were placed again in the middle chamber with free access to all chambers and the time spent on each side was quantified. Data are expressed as time spent on the cocaine-paired side minus the time spent on the saline-paired side (CPP score).

RNA Isolation and Reverse Transcription PCR
Bilateral 14 gauge punches of rat nucleus accumbens were rapidly dissected and frozen at −80°C. Punches were thawed in TRIzol (Invitrogen), homogenized, and processed according to the manufacturer’s protocol. Total RNA was reverse-transcribed using SuperScript III (Invitrogen) and random hexamers.

ChIP-Chip Analysis
Details can be found in the Supplemental Data.
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