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Cocaine Regulates MEF2 to Control Synaptic and Behavioral Plasticity

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1. Supplemental Methods

2. Supplemental Figures S1 – S9

Supplemental Methods:

Western Blots of *in vivo* samples: Sprague Dawley rats (male, 10 weeks old) were injected for 7 days with saline, acute cocaine (6 days saline followed by 1 dose of 20 mg/kg cocaine), or chronic cocaine (7 days of 20 mg/kg cocaine) and sacrificed 4, 24 or 48 hours after the last injection. Cocaine was purchased from Sigma. Animals were sacrificed by microwave irradiation aimed at the head (5kW, 1.5s, Murimachi Kikai Co. Ltd., Tokyo, Japan). Striatum was rapidly dissected and frozen on dry ice. Tissues were sonicated on ice in a SDS lysis buffer (1% (w/v) SDS, 300 mM sucrose, 1 mM EDTA) containing 1 mM activated sodium orthovanadate, 1 mM PMSF (Phenyl Methyl sulfonyl fluoride), 1 mM NaF (Sigma), 100 nM Okadaic acid (Calbiochem), 1 μM Cyclosporin A (Sigma), and 1X Complete Protease Inhibitor cocktail (Roche). Samples were then boiled for 5 minutes before centrifugation at 20,300 x g for 10 minutes at 4°C. Total protein concentration was determined by the DC assay kit (BioRad). For each sample, 30 μg of total protein were resolved on 10% SDS-PAGE and transferred to a nitrocellulose membrane and incubated overnight with either anti-MEF2A (C-21, Santa Cruz; 1:1000), anti-MEF2D (Transduction Labs; 1:2000), anti-P-Ser408/444 ((Flavell et al., 2006); 1:500), anti-β-tubulin (Sigma; 1:25,000), or anti-β-actin (Abcam; 1:2500) antibodies. Blots were developed with enhanced Chemiluminiscence western blotting detection system (ECL-plus, Amersham Pharmacia Biotech). Quantification of the autoradiographs was performed using optical densitometry and NIH ImageJ software.

Immunohistochemistry: Mice were terminally anesthetized with chloral hydrate and perfused transcardially with PBS followed by 4% (w/v) paraformaldehyde. Brains were post-fixed

overnight and then cryoprotected in 30% sucrose. Coronal sections (30 μm) were submerged in 100°C citric acid (pH 6.0) for heat-induced epitope retrieval and rinsed twice in PBS. The sections were blocked in 4% normal donkey serum for 1 hour at RT before overnight primary antibody incubation at 4°C. The signals were amplified by ABC-Elite kit (Vector Laboratories) following manufacturer's protocol. The labeling was revealed with Tyramide Amplification (TSA Perkin Elmer) followed by DAPI (VectaShield, Vector Labs) staining and dehydration. Biotinylated anti-Rabbit and anti-mouse secondary antibodies were obtained from Jackson Laboratories.

Dissociated Striatal cultures: Embryonic striatal neurons (E18/19) were cultured from Long Evans rats (Charles River Labs) as described previously (Lindsay, 1998) with modifications. The striatal tissues were digested with papain (10 unit/ml; Worthington) for 4 minutes (37°C) before dissociation with a plastic 5 ml pipet. For luciferase and immunocytochemistry experiments, the dissociated neurons were plated at 100,000/well (24 well plate; Corning) on PDL (Sigma)- and laminin (Invitrogen)-coated 12 mm glass coverslips (Bellco) in DMEM (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), penicillin (50 μg/ml)-streptomycin (50 units/ml; Sigma) and L-glutamine (4 mM, Sigma) and incubated at 37°C/5% CO_2 for 24 hours in a humidified incubator. Twenty-four hours after plating, the medium was changed to Neurobasal (Invitrogen), B27 supplement (2% (v/v); Invitrogen), penicillin-streptomycin (1X; Sigma) and L-glutamine (4 mM; Sigma). For western blotting and EMSA assays, "crude" striatal dissections were performed as described (Cowan et al., 2005), and plated at 8 x 10^6 cells per 10 cm plate (Corning).

Luciferase Assays in Primary Neurons: Dissociated striatal neurons were transfected using calcium phosphate as described previously (Flavell et al., 2006) at 8 days in culture. Approximately 42-48 hours after transfection, the cultures were stimulated in conditioned medium with isotonic depolarization solution (Flavell et al., 2006), indicated compounds or vehicle alone. All treatment conditions were administered simultaneously. Neurons were lysed 7-8 hours later and relative luciferase activity was determined using the dual luciferase assay (Promega). For the luciferase assays, CRE- or MRE-firefly luciferase activity was divided by TK-renilla luciferase activity, except for samples treated with SKF81297, where an independent effect on TK-renilla was observed. In these cases, the raw firefly luciferase data was averaged over 4-5 independent experiments to minimize effects of well-to-well transfection differences.

For RNAi-based protein replacement assays, pSuper vector or pSuper MEF2A(1234) and pSuper MEF2D(479) were co-transfected together with pcDNA3, pcDNA3-MEF2A (RiR) or pcDNA3-MEF2D (RiR) to simultaneously reduce endogenous MEF2 expression and replace with expression of wild type or mutant RNAi-resistant forms of MEF2A or MEF2D. For these experiments, 500 ng of each MEF2 shRNA plasmid was used together with 10-50 ng of pcDNA3-MEF2 (RiR). Initial titration experiments were performed to determine the optimal levels of plasmid that restore endogenous levels of MEF2 activity.

For experiments involving chemical inhibitors, the following reagents were diluted in conditioned medium at the indicated final concentrations: SKF81297 (10 μM; Tocris), forskolin (10 μM; Sigma), KN-62 (20 μM; Tocris), nimodipine (10 μM; Tocris), cyclosporin A (1 μM; calbiochem), roscovitine (10 μM; Tocris). For KCl depolarization, striatal neuron conditioned medium was supplemented with 0.5 volumes of isotonic depolarization medium (170 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.4) as previously described (Flavell et al., 2006).

Electrophoretic Mobility Shift Assay (EMSA): Tissue extracts from whole nucleus accumbens punches were prepared as described previously (Hope et al., 1992) with minor modifications. Specifically, NAc tissues were solubilized with lysis buffer (20 mM tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, 0.5% (v/v) NP-40, 1 μM cyclosporin A, and 1X Complete Protease Inhibitor cocktail (Roche)). 10-20 μg of NAc extract was incubated with ^{32}P-labeled MRE duplex oligos (McDermott et al., 1993) for 20 min at room temperature. Protein-DNA complexes were separated from unbound oligonucleotide on a 4% non-denaturing polyacrylamide gel containing 2.5% glycerol and 0.4X Tris-borate buffer (0.04M Tris borate, pH 7.5) and was visualized by phosphoimager (GE). Cold competitor MRE or mutant MRE was used at 50-fold excess. For antibody supershifts, 1 μl of specific antibodies to MEF2A (C-21, Santa Cruz), MEF2D (Signal Transduction labs), MEF2C or CREB (Signal Transduction Labs) were preincubated with cell lysates for 30 minutes at 37^{o}C prior to addition of labeled MRE DNA probes.

Recombinant Adeno-associated Viruses: For knockdown of endogenous MEF2A and MEF2D in the NAc, we subcloned specific MEF2A or MEF2D shRNA sequences (Flavell et al., 2006) into pAAV.shRNA (Hommel et al., 2003) under control of the U6 promoter. For MEF2A, pAAV.shRNA contained a CMV promoter driving expression of eGFP, and for MEF2D the CMV promoter drove expression of mCherry. To co-express MEF2-VP16 and EGFP *in vivo*, we modified the AAV shRNA vector (Hommel et al., 2003) such that the open reading frame of MEF2-VP16 (Black et al., 1996) was under control of the CMV promoter. The shRNA region was removed and replaced with an expression cassette with EGFP under control of the hEF2

promoter. Viruses were packaged as described (Hommel et al., 2003). Briefly, HEK-293 cells were cultured in either 10 x 15 cm plates or roller bottles and tranfected with pAAV.shRNA, pHelper and pAAV.Rc plasmids (Stratagene) with calcium phosphate. Viral purification was then performed as described previously (Hommel et al., 2003; Zolotukhin et al., 1999).

Slice Pharmacology: Male C57BL/6 mice (7–8 weeks old) were sacrificed by rapid decapitation, and the brains were rapidly removed and placed in ice-cold, oxygenated Krebs-HCO_3 2 buffer (124 mM NaCl, 4 mM KCl, 26 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 1.25 mM KH_2PO_4, 1.5 mM $MgSO_4$ and 10 mM D-glucose, pH 7.4). Coronal striatal slices (350 μm) were incubated for 1 hour at 30^0C under constant oxygenation with 95% O_2 / 5% CO_2 before addition of DMSO, SKF81297 (10 μM), or forskolin (50 μM). Slices were than frozen prior to boiling SDS (1% w/v) extraction (5 min.). Protein estimation was then performed using the BCA method (Thermo-Pierce). 60 μg of total cell lysate was then used to assess RCS phosphorylation by western blotting (15% SDS-PAGE).

ChIP-chip analysis: Mice were treated for 7 days with daily injections of cocaine (20 mg/kg) and sacrificed 24 hrs later. Chromatin from bilateral punches of NAc was immunoprecipitated with an antibody against MEF2A as described previously with minor modifications (Renthal et al., 2007). Chromatin was sonicated to an average of ~500 bp and immunoprepitated with antibody against MEF2A (Santa Cruz, sc-313) or an IgG control (Upstate/Millipore). Antibody-bound chromatin was precipitated using Protein A beads from Upstate (06-157), which were washed with low salt, high salt, and LiCl buffers to remove non-specific DNA binding. Eluted chromatin was reverse-crosslinked at 65^oC in the presence of proteinase K and EDTA. DNA was

purified by chloroform extraction/ethanol precipitation and the enrichment of specific promoters was either measured directly via real time PCR or amplified by ligation-mediated PCR for genome-wide analysis (Sikder et al., 2006). Amplified DNA was then labeled with Cy3 (input) or Cy5 (MEF2-enriched) and hybridized to Nimblegen (Madison, WI) MM8 mouse promoter arrays. Bilateral nucleus accumbens from eight mice were pooled for microarray analysis. All microarray data is deposited in the GEO database under accession GSM300354.

Stereotactic Surgery: Stereotactic surgery was performed on mice under general anesthesia with a ketamine/xylazine cocktail. Coordinates to target both the nucleus accumbens shell and core were, +1.6 mm A/P, +1.5 mm lateral, and -4.4 mm D/V from bregma (relative to dura) at a 10° angle. Virus was delivered bilaterally using Hamilton syringes at a rate of 0.1 μl/min for a total of 0.8 μl (0.4 ul AAV-MEF2A shRNA and 0.4 ul AAV-MEF2D shRNA were premixed). Viral placements were confirmed by staining for GFP, which was co-expressed in each virus.

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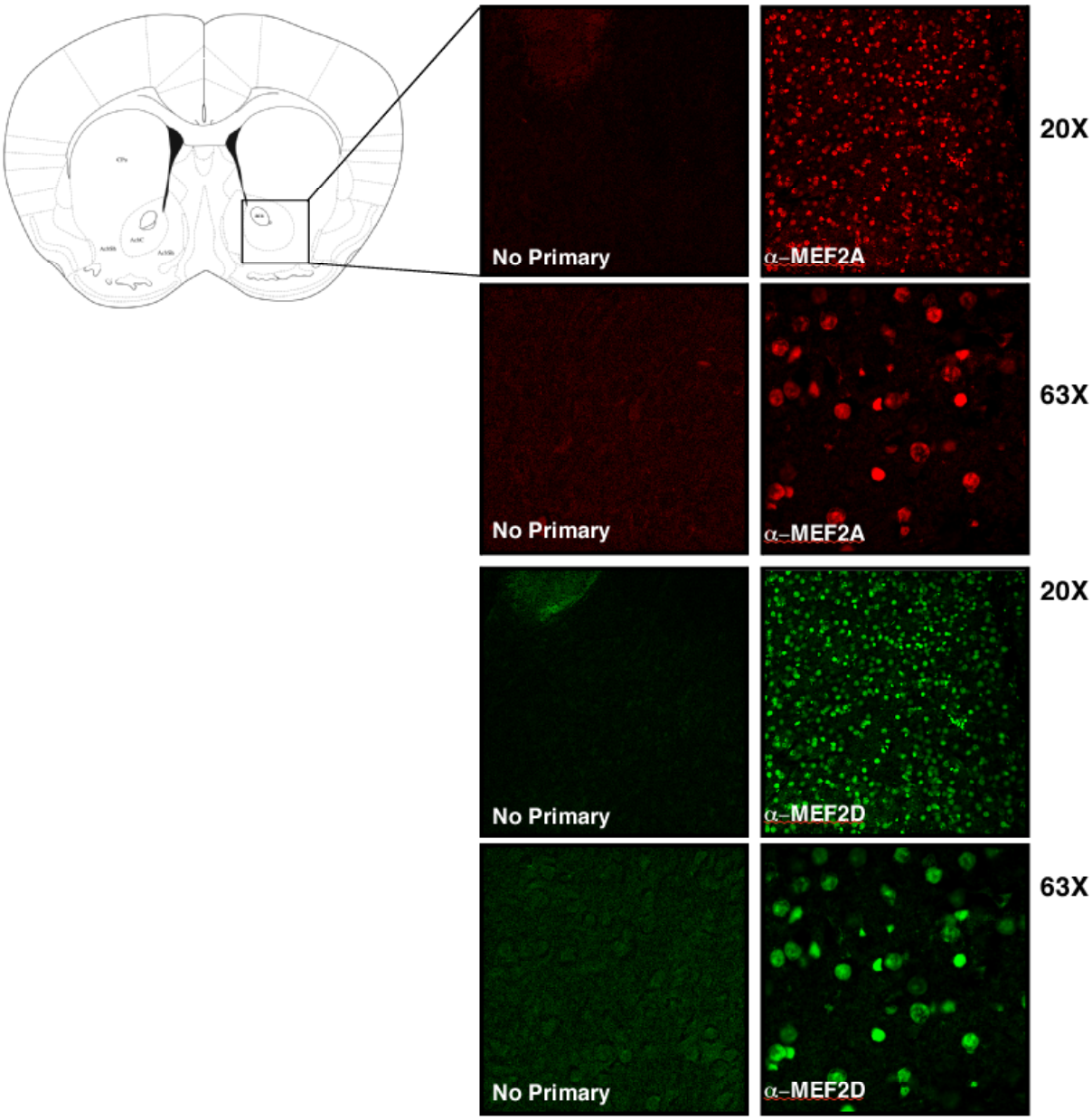
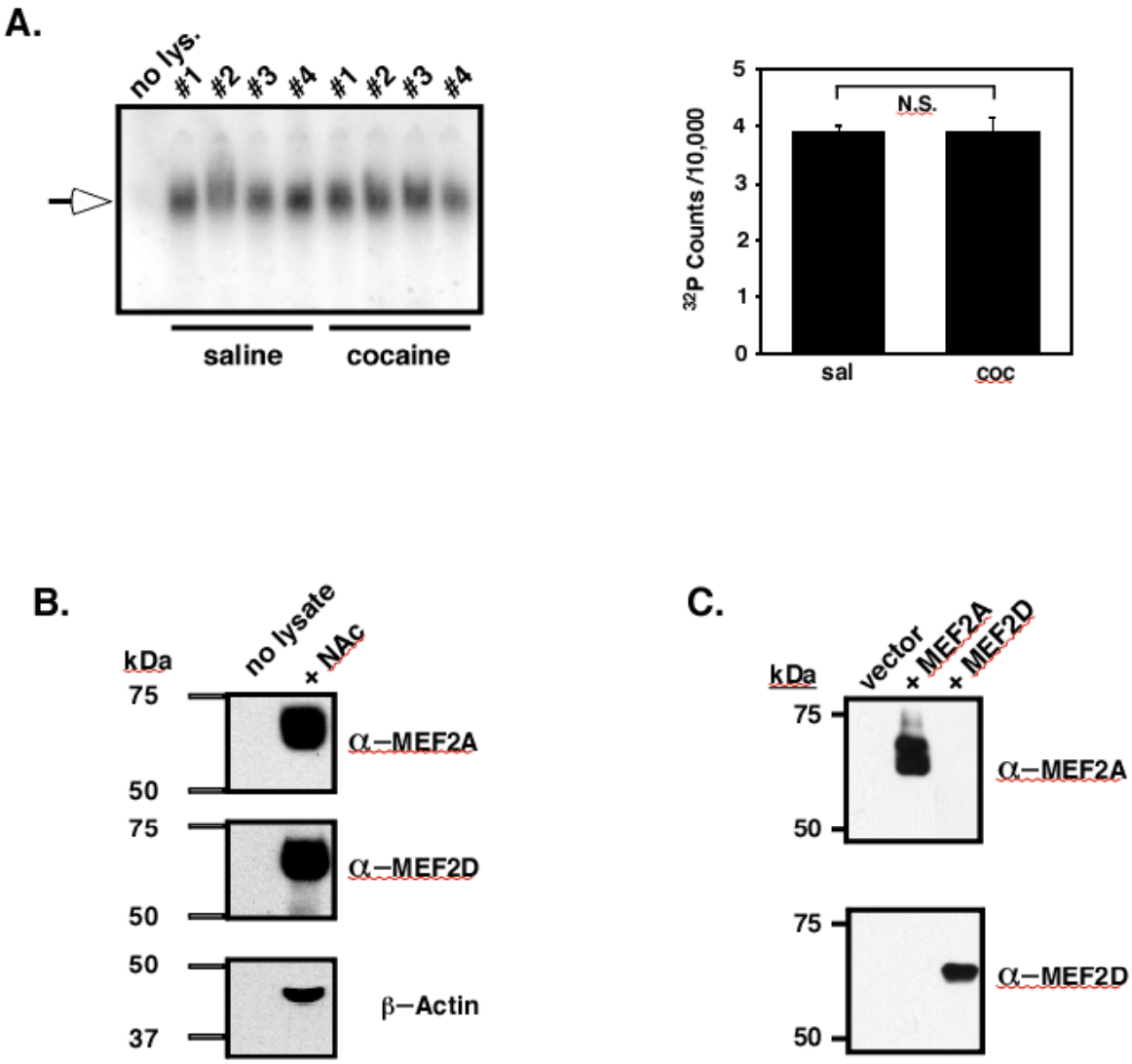
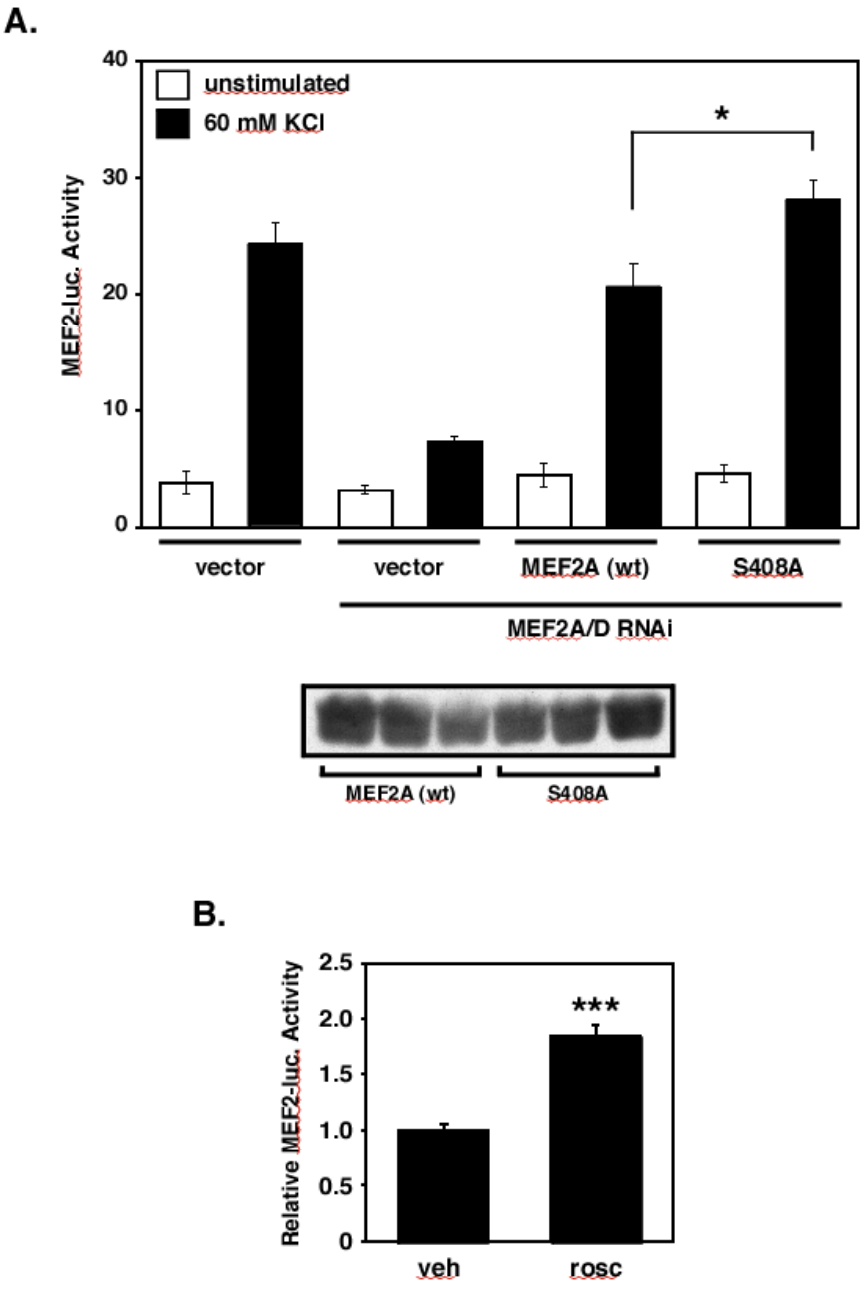
Supplemental Figures



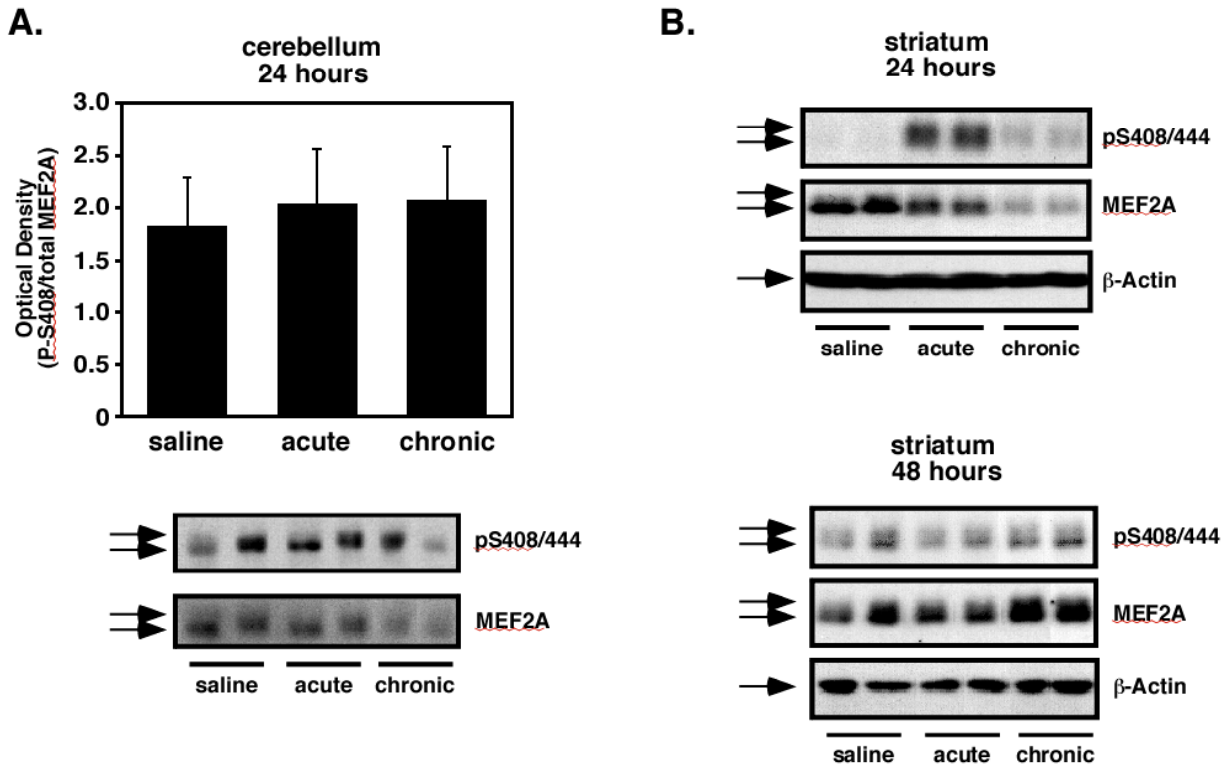
Supplemental Figure S1. MEF2 immunostaining in the NAc. Immunohistochemistry was performed with MEF2A or MEF2D antibodies, or no primary antibody as a control. MEF2A and MEF2D nuclear staining can be observed at 20X and 63X (right column), while only background fluorescence is observed with no antibody (left column).



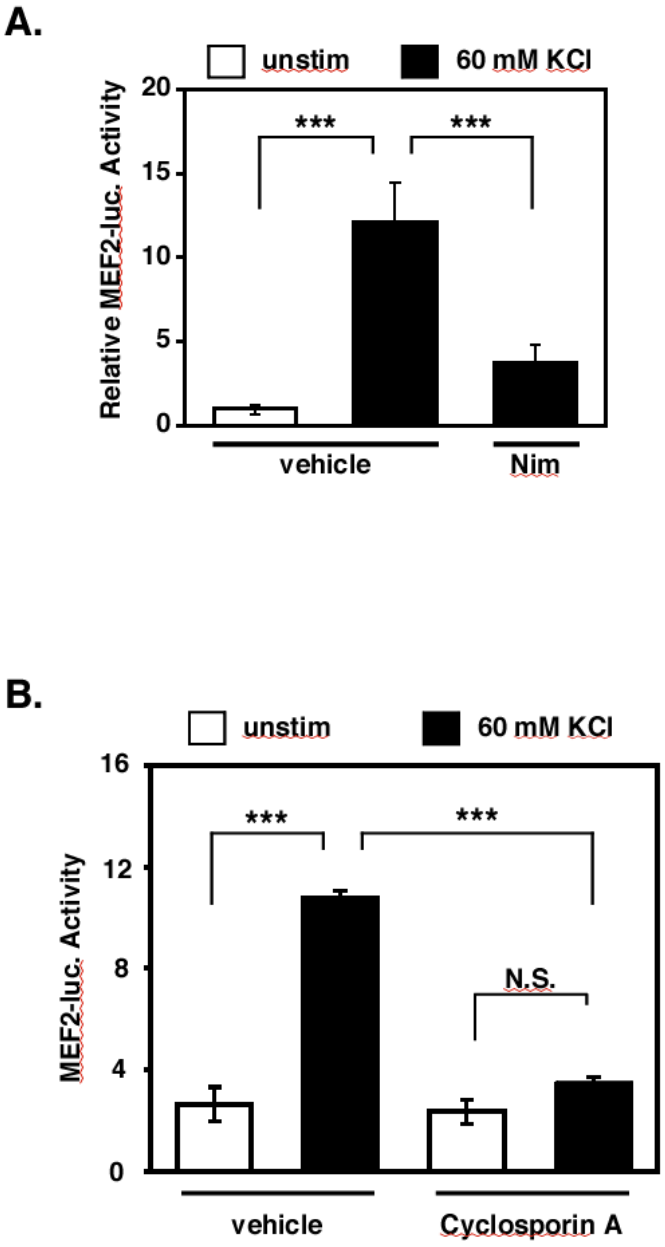
Supplemental Figure S2. Chronic cocaine does not alter MEF2 DNA binding activity in the NAc. (**A**) Chronic cocaine does not regulate DNA binding of MEF2. EMSA was performed on NAc lysates from mice injected daily for 7 days with cocaine or saline at 24 hours after the last injection. MEF2 binding to the radioactive probe (arrow) reveals no differences in DNA binding with replicate animals (4 per conditions) (left). Quantification of the amount of ^{32}P-labeled MRE shifted with NAc lysates from mice injected with saline or cocaine for 7 days (right, P>0.05, n=4). (**B**) NAc lysates used for EMSA were probed with antibodies against MEF2A, MEF2D, or β-actin, to verify protein integrity. No partial degradation products were observed for any of these proteins. (**C**) 293T cells were transfected with expression plasmids for MEF2A, MEF2D, or vector alone. Western blotting of these lysates demonstrates that the antibodies against MEF2A and MEF2D do not cross-react with the other family member.



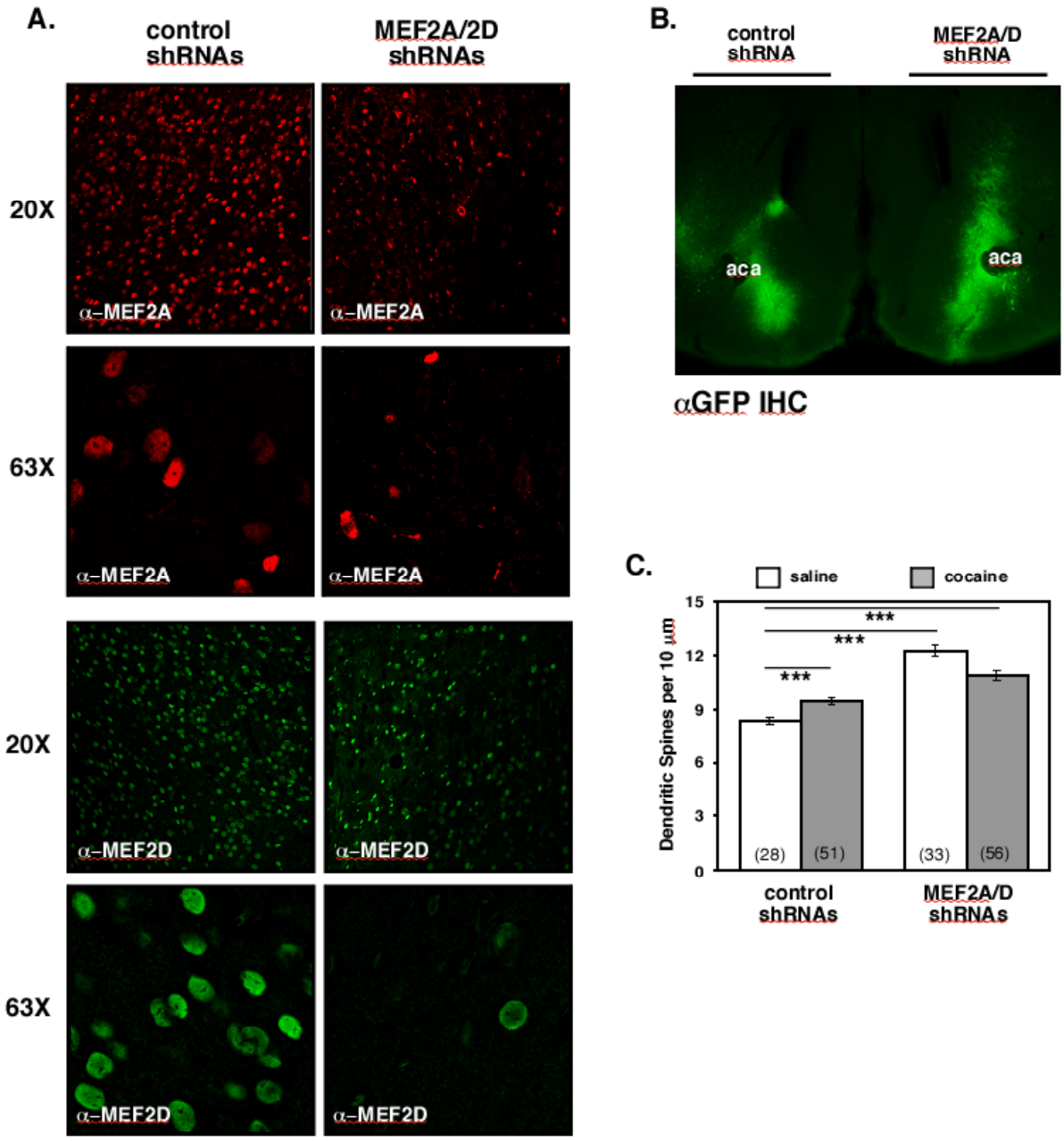
Supplemental Figure S3. (A) RNAi-based protein replacement assays reveal that phosphorylation site mutant of MEF2A (S408A) has elevated activity compared to wild-type MEF2A (representative experiment, $p<0.05$, n=3). Anti-MEF2A western blots of HEK-293T total cell lysates of cultures transfected with equal amounts of expression plasmid (bottom) demonstrate similar expression levels of the mutant and wild-type proteins. (B) The Cdk5 inhibitor roscovitine (10 μM) significantly increases basal MEF2-luciferase activity in cultured striatal neurons (***$p<0.001$, n=6, two independent experiments).



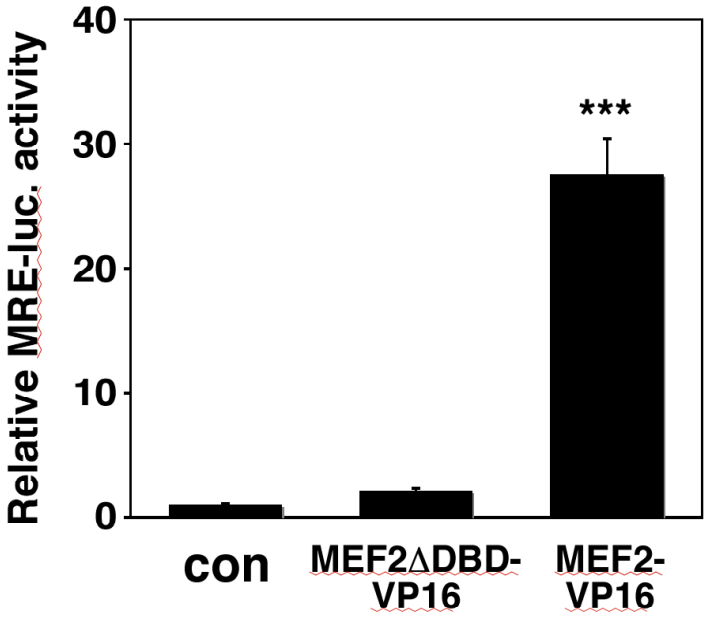
Supplemental Figure S4. **(A)** Effects of acute (1 day) and chronic (7 day) cocaine injections on cerebellar MEF2 phosphorylation at Ser408/444. Western blot using the MEF2A/2D phospho-408/444-specific antibody revealed similar levels of phosphorylation after saline or cocaine injections ($p>0.05$, $n=3$). (**B**) Representative western blots of striatal lysates from rats treated with saline, acute, or chronic cocaine and analyzed 24 hrs (top) or 48 hrs (bottom) later. Quantification of these blots is displayed in Fig. 2.



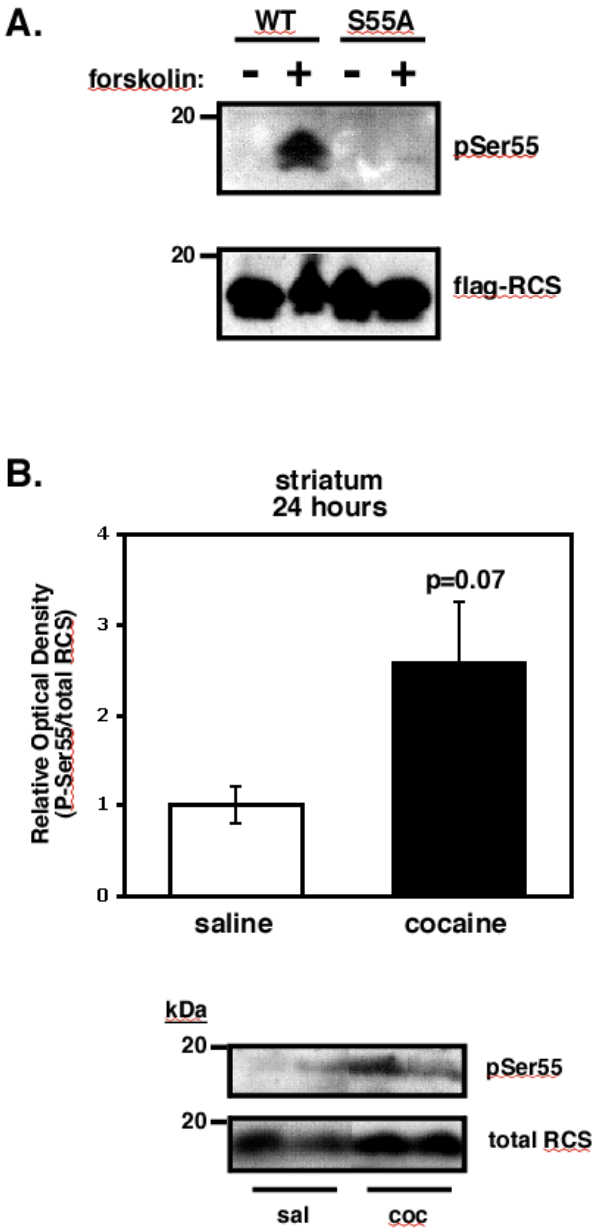
Supplemental Figure S5. (**A**) Nimodipine (10 μM), a specific L-type voltage-sensitive calcium channel antagonist, significantly blocks the KCl-induced activation of MEF2 in cultured striatal neurons (***p<0.001, n=3). The nimodipine was added simultaneously with the KCl simulation solution. (**B)** Cyclosporin A (CsA, 1 μM), a calcineurin inhibitor, significantly blocks the KCl-induced activation of MEF2 in cultured striatal neurons (***p<0.001, n=3). The CsA was added simultaneously with KCl stimulation solution (no pre-incubation).



Supplemental Figure S6. (**A**) Immunostaining of MEF2A (top) and MEF2D (bottom) in NAc infected with AAVs expressing control shRNAs (left) or MEF2A/D shRNAs (right). Confocal images taken at 20X (top) or 63X (bottom) magnification as indicated. AAV-infected regions show robust reduction of MEF2A (left, red) and MEF2D (right, green) nuclear protein levels in the NAc. (B) Representative anti-GFP immunostaining of NAc-containing coronal sections at 4 weeks after stereotactic AAV-MEF2A/2D shRNA injections. (**C**) Dendritic spine density quantification from AAV-shRNA-infected NAc neurons after 4 weeks of daily cocaine injections (once daily for 5 days/week at 20 mg/kg). MSNs expressing MEF2A/2D shRNAs had a significantly higher density of dendritic spines than control shRNA-infected neurons (***$p<0.001$, student's t-test). Data are from 4 mice injected with control and specific shRNA viruses on opposite sides of the brain. The number of secondary and tertiary dendritic segments analyzed is indicated in parentheses.

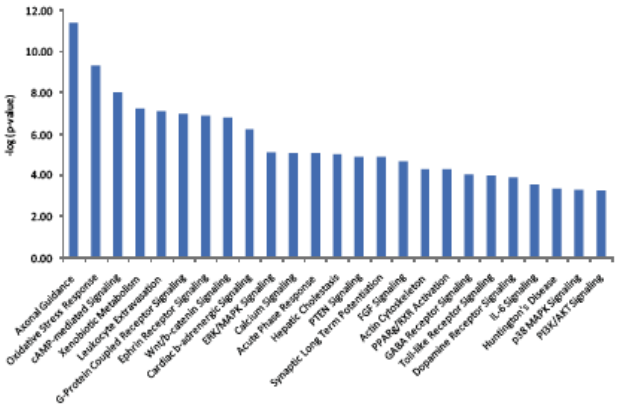


Supplemental Figure S7. MEF2-VP16 significantly increases MEF2 transcription in cultured striatal neurons (***$p<0.001$, n=6, two independent experiments). A DNA binding mutant of MEF2-VP16 (MEF2ΔDBD-VP16) showed no significant regulation of MEF2 activity in cultured striatal neurons.



Supplemental Figure S8. (A) Characterization of P-Ser55 RCS antibodies. HEK-293T cells were transfected with Flag-tagged wild-type or Ser55Ala RCS expression plasmids. Two days after transfection, the cells were treated with either DMSO or 10 μM forskolin for 30 minutes at 37°C. The total cell lysates were harvested with Sample buffer and separated by 13.5% SDS-PAGE, transferred to PVDF, and blotted using anti-P-Ser55 antisera (1:500 dilution in 3% BSA) or using anti-flag tag antibodies (1:2000 dilution). Specific signal was detected only in the lysates containing forskolin-treated, wild-type RCS, whereas the S55A phosphorylation site mutant RCS protein was not identified +/- forskolin treatment. (**B**) Repeated cocaine administration (7 daily IP injections of 20 mg/kg cocaine) increased RCS Ser55 phosphorylation in the rat striatum at 24 hours after the last injection (cocaine vs. saline, p=0.07, n=6, students t-test).

A.

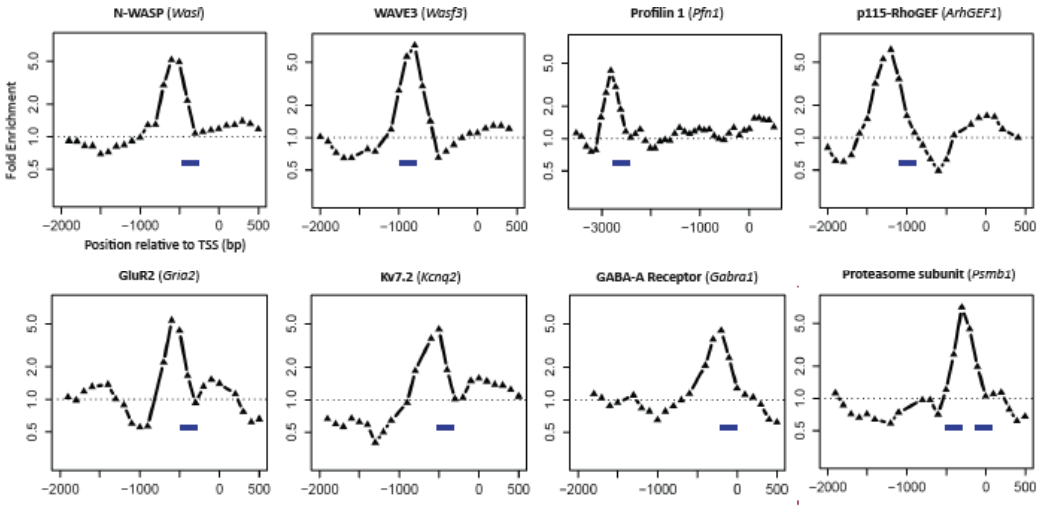


B.

MEF2 target genes implicated in dendritic spine plasticity and neuronal function

F-Actin Remodeling:	cAMP Signaling:	Excitability:	Ubiquitin Signaling:
N-WASP	Adcy7	GABAAR1	Ubiquitin D
WAVE 3	ADORA2A	GABAAR2	Usp18
WASP binding protein	PDE7A	GABABR1	Usp50
Profilin 1	ATF4	Kv1.1	Ube1l
p115-RhoGEF	GRK4	Kv4.2	Ube2m
PDZ-RhoGEF	Gαi	Kv4.3	Psmb1
RhoGAP 8	κOR	Kv7.2	
RasGRF2		GluR2	
RasGRP2		5-HT3A	
RasGRP3		N-AchR	

C.



Supplemental Figure S9. (**A**) Ingenuity pathway analysis of putative MEF2 target genes identifies several highly enriched pathways, which MEF2 may regulate. Notably, axonal guidance, cAMP-mediated signaling, actin cytoskeleton, and dopamine receptor signaling are among the most enriched pathways and are known to be involved in cocaine responses and dendritic and neuronal morphology. (**B**) Examples of putative MEF2 target genes involved in F-actin remodeling, cAMP signaling, neural excitability, and ubiquitin signaling that might influence cocaine-induced spine changes and sensitized behavioral responses to cocaine. (**C**) Representative genes where MEF2 is significantly bound in the NAc after chronic cocaine. The chromosomal location (x-axis) is displayed relative to the transcriptional start site (0). The blue bar underneath each plot represents a consensus or near-consensus MEF2 response element.