CREB Phosphorylation Regulates Striatal Transcriptional Responses in the Self-Administration Model of Methamphetamine Addiction in the Rat

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Abstract

Neuroplastic changes in dorsal striatum participate in the transition from casual to habitual drug use and might play a critical role in the development of methamphetamine (METH) addiction. We examined the influence of METH self-administration on gene and protein expression that may form substrates for METH-induced neuronal plasticity in the dorsal striatum. Male Sprague-Dawley rats self-administered METH (0.1 mg/kg/injection, i.v.) or received yoked saline infusions during eight 15-h sessions and were euthanized 2 h, 24 h, or 1 month after cessation of METH exposure. Changes in gene and protein expression were assessed using microarray analysis, RT-PCR and Western blots. Chromatin immunoprecipitation (ChIP) followed by PCR was used to examine epigenetic regulation of METH-induced transcription. METH self-administration caused increases in mRNA expression of the transcription factors, *c-fos* and *fosb*, the neurotrophic factor, *Bdnf*, and the synaptic protein, synaptophysin *(Syp)* in the dorsal striatum. METH also caused changes in ΔFosB, BDNF and TrkB protein levels, with increases after 2 and 24 h, but decreases after 1 month of drug abstinence. Importantly, ChIP-PCR showed that METH self-administration caused enrichment of phosphorylated CREB (pCREB), but not of histone H3 trimethylated at lysine 4 (H3K4me3), on promoters of *c-fos, fosb, Bdnf* and *Syp* at 2 h after cessation of drug intake. These findings show that METH-induced changes in gene expression are mediated, in part, by pCREB-dependent epigenetic phenomena. Thus, METH self-administration might trigger

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epigenetic changes that mediate alterations in expression of genes and proteins serving as substrates for addiction-related synaptic plasticity.

Keywords

methamphetamine; self-administration; dorsal striatum; ΔFosB; BDNF; pCREB

Introduction

Addiction to the illicit psychostimulant, methamphetamine (METH), is a serious public health problem worldwide because of severe medical and neuropsychiatric complications and high risks of relapse even after prolonged periods of abstinence (Callaghan et al., 2012; Sekine et al., 2008). These observations suggest that the transition from recreational drug use to addiction depends on long-lasting molecular adaptations in the brain secondary to repeated METH exposure. The adaptive changes may include alterations in gene and protein expression as well as structural modifications at dopaminergic and glutamatergic synapses (Robison and Nestler, 2011). The transition to addiction also appears to involve a shift of control over drug intake from ventral to the dorsal striatum when the drug user switches from casual to habitual and compulsive drug taking (Belin and Everitt, 2008; Everitt and Robbins, 2013; Wise, 2009).

Previous studies using chronic non-contingent amphetamine or METH administration have identified differential alterations in gene expression after repeated drug exposure in the rodent brain (Cadet et al., 2009; Konradi et al., 1996; Krasnova et al., 2011; McCoy et al., 2011; Renthal et al., 2008). They reported changes in the expression of immediate-early genes that include alterations in *c-fos* and *fosb* mRNA levels in the dorsal striatum after chronic amphetamine (Konradi et al., 1996; Renthal et al., 2008) or METH treatment (McCoy et al., 2011). Chronic METH exposure caused decreases in the expression of some immediate-early genes in animals euthanized 24 h after drug administration, with normalization of their expression after a single METH injection given to similarly treated rats (McCoy et al., 2011). However, the transcriptional alterations following METH self-administration have not been investigated. In the present study, we used METH self-administration model to decipher large-scale transcriptional changes in the dorsal striatum because of this region's role in habitual and compulsive aspects of drug addiction (Everitt and Robbins, 2013; Koob and Volkow, 2010). We also used chromatin immunoprecipitation (ChIP) followed by qPCR to identify potential epigenetic mechanisms underlying METH-induced changes in striatal gene expression. These experiments identified phosphorylated CREB (pCREB) as an important player in the regulation of changes in gene expression caused by METH self-administration.

Materials and Methods

Animals and METH Self-Administration

Male Sprague-Dawley rats (Charles River, Raleigh, NC), weighing 350–420 g, were housed individually under a reversed 12-h light/dark cycle with free access to food and water. Animal procedures were done according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of NIDA (ASP #09-BNRB-31). The extended-access METH self-administration procedure and apparatus have been described in details previously (Krasnova et al., 2010). In brief, rats underwent eight days of 15-h METH self-administration sessions with their yoked partners receiving saline injections when METH (0.1 mg/kg/infusion) was self-administered by a rat in an active chamber. Rats were euthanized at 2 h, 24 h and 1 month after the final self-

administration session and dorsal striata were isolated from the brains. Only animals with a total cumulative METH intake higher than 30 mg/kg over the eight daily sessions were used in further experiments. On the basis of these criteria, one or two rats from each group (<10% of animals) were excluded from the analyses.

RNA Isolation, Microarray Hybridization and Data Analysis

RNA extraction, RNA labeling and microarray hybridization were performed as previously described (Cadet et al., 2009). Microarray hybridization was done using RatRef-12 Expression BeadChips arrays (22 523 probes) (Illumina Inc.). A gene was identified as significantly changed if it showed increased or decreased expression according to an arbitrary cut-off at p 0.05, fold change 1.7. Functional annotation and classification analyses for significantly changed genes were performed using DAVID Annotation Tool (http://david.abcc.ncifcrf.gov) (Huang da et al., 2009) and were supported by literature searches. To focus on changes that could be related to gene function, we included only genes classified using DAVID Annotation Tool in further analyses and excluded all undefined (LOC and RGD) genes. Microarray results were extensively validated by qRT-PCR as described below.

Quantitative RT-PCR Analyses of Gene Expression

qRT-PCR experiments were performed as previously described (Krasnova et al., 2007; 2008). Unpooled total RNA (1μg) isolated from striatal samples (n=6 per group) was reverse-transcribed with oligo dT primers using Advantage RT-for-PCR kit (Clontech). qRT-PCR experiments were done using LightCycler technology and Light Cycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics). The primers were synthesized at the Synthesis and Sequencing Facility of Johns Hopkins University (Baltimore, MD). mRNA levels were normalized for each well to the clathrin mRNA levels. Purity of the qRT-PCR products was verified by melting curve analysis. The results are reported as fold changes calculated as the ratios of normalized gene expression data for each group in comparison to the control group.

Western Blot

Western blot experiments were conducted as described previously (Krasnova et al., 2011). Proteins were extracted from individual samples by sonication in lysis buffer A (10 mM HEPES, 10mM KCl, 1.5 mM $MgCl_2$, 1% Igepal CA) that contained protease and phosphatase inhibitors (Roche Diagnostics) and centrifugation at 14 000g for 5 min at 4°C. Supernatant was collected and used as cytosolic protein fraction. The remaining pellet was resuspended in lysis buffer B (20 mM HEPES, 25% glycerol, 840 mM NaCl, 1.5 mM $MgCl_2$, 0.4 mM EDTA) containing protease and phosphatase inhibitors and centrifuged at 15 000g for 15 min at 4°C. The supernatant was collected and used as nuclear protein fraction. 20–40 μg protein aliquots were separated by SDS-PAGE and transferred to PVDF membranes. Levels of D1 and D2 DA receptors, c-Fos, FosB, ΔFosB, BDNF and TrkB were measured in cytosolic protein fraction. Levels of CREB, pCREB and H3K4me3 were measured in nuclear protein fraction. After the transfer, membranes were blocked and incubated with antibodies against D1, D2 DA receptors (1:1000, EMD-Millipore), FosB (1:500, Cell Signaling), BDNF (1:500, Santa-Cruz Biotechnology, Inc.), TrkB (1:1000, EMD-Millipore), CREB, pCREB or H3K4me3 (1:1000, all from Cell Signaling). Immune complexes were detected with HRP-labeled second antibody and ECL+ chemiluminescence reagents (GE Healthcare). To confirm equal protein loading, blots were reprobed with anti-α-tubulin antibody. Optical densities were measured using Carestream Imaging System (Kodak). Each group contained samples from 5–9 animals that were run individually. The results are reported as % of control changes calculated as the ratios of protein expression for each METH group in comparison to control group.

Chromatin Immunoprecipitation (ChIP) Assay

Rats were subjected to METH self-administration as described above. Two hours after the cessation of METH self-administration animals were euthanized, dorsal striata were dissected and processed for ChIP as described by Tsankova et al. (2004). The tissue was minced and cross-linked in 1% paraformaldehyde/PBS for 15 min. Cross-linking was stopped by addition of glycine to a final concentration 0.125 M. Samples were washed in ice-cold PBS containing protease inhibitors (Roche Diagnostics), frozen on dry ice, and stored at −80°C. The fixed tissue was resuspended in SDS lysis buffer (EMD-Millipore) containing protease inhibitors and 1 mM PMSF and homogenized. Each sample was sonicated using the Bioruptor (Diagenode Inc.). The fragmentation of DNA was verified by gel analysis to confirm the sheared range of 300–600 bp. Dynabeads (Life Technologies) were incubated with 5 μg of antibodies against H3K4me3 (Cell Signaling) or pCREB (EMD-Millipore). 50 μg of chromatin lysate were diluted with ChIP dilution buffer (EMD-Millipore) to a volume of 1.5 ml. 100 μl of the preimmunoprecipitated lysate were saved as "input" for later normalization. The chromatin lysate was then immunoprecipitated with appropriate antibodies overnight at 4°C. The beads were washed with low salt, high salt, LiCl, and TE buffers (EMD-Millipore). DNA-protein complex was eluted from the beads with 500 μl NaHCO$_3$/SDS buffer. DNA and proteins were dissociated at 65°C for 4 h under high-salt conditions, followed by RNase A and proteinase K treatments. DNA was extracted with phenol/chloroform, precipitated with ethanol, and resuspended in 10 mM Tris pH 8.0.

qPCR was performed with primers specific for c-fos, fosb, c-jun, Nr4a1-3, Syp, Bdnf-pI, Bdnf-pIV, and *Bdnf-pVI* promoters (Table S1). qPCR for ChIP-derived DNA samples was done using a LightCycler thermal cycler (Roche Diagnostics), with SYBR Green monitoring. qPCR specificity was verified by melting curve analysis. All PCR reactions were performed in duplicate and included negative controls (no DNA). Threshold amplification cycle numbers (T_c) and LightCycler software were used to calculate IP DNA quantities as % of input controls.

HPLC Analysis

The levels of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT) and 5-hidroxyindoleacetic acid (5-HIAA) were analyzed in tissue extracts using HPLC with electrochemical detector (Krasnova et al., 2007). The data were expressed as ng/mg of tissue weight and reported as % of control concentrations.

Statistical Analysis

Results were expressed as means ± S.E.M.s and were considered significant at $p < 0.05$. Statistical analysis of the data was done using either ANOVA for repeated measures followed by Tukey's multiple comparison test, ANOVA followed by Fisher's protected least significant difference or Student's t-test (StatView 4.02). Correlations between microarray and qRT-PCR data were assessed using linear regression.

Results

METH Self-Administration and Intake

Previous studies on the actions of METH in animals have focused mainly on the effects of non-contingent experimenter-administered drug injections on monoaminergic systems in the brain (see Carvalho et al., 2012; Krasnova and Cadet, 2009; Marshall and O'Dell, 2012, for reviews). However, self-administration models provide a better tool for studying METH addiction, because they employ patterns of exposure more typical of human drug use that include voluntary drug taking behavior and escalation of METH intake during development of METH dependence (Cho and Melega, 2002). Thus, we used an extended-access model of

intravenous METH self-administration to study neuroplastic changes in the dorsal striatum. Rats self-administered METH during eight consecutive 15-h daily sessions as described previously (Krasnova et al., 2010). Control animals received yoked saline injections. Figure 1 shows a representative graph of METH intake for animals euthanized at 24 h after cessation of drug exposure. Rats self-administered an average 2.8 ± 1.0 mg/kg of METH during session 1. Access to METH self-administration caused an elevation of drug intake to 7.9 ± 0.8 mg/kg on session 2, which continued to increase and peaked at 14.2 ± 1.4 mg/kg on session 6 (Fig. 1). The escalation of METH intake was highly significant ($F_{(7, 80)} = 9.572$; $p < 0.001$). Total cumulative drug intake over eight sessions was 86.9 ± 8.6, 85.1 ± 8.7 and 88.5 ± 8.6 mg/kg for groups of rats euthanized at 2 h, 24 h and 1 month after cessation of METH exposure, respectively, and was not significantly different between the groups. To better mimic moderate-to-high drug use by METH addicted individuals, animals with total METH intake less than 30 mg/kg (one or two rats per group, <10% of animals) were excluded from further experiments.

METH-Induced Changes in Monoamine Levels and in the Expression of D1 and D2 DA Receptors

Using similar models, we (Krasnova et al., 2010) and others (McFadden et al., 2012a,b; Schwendt et al., 2009) have been able to replicate previous biochemical observations in human METH addicts (Volkow et al., 2001b; Wilson et al., 1996). These include depletion of DA, decreases in DA transporter and reductions in tyrosine hydroxylase expression in the striatum. In agreement with our previous findings (Krasnova et al., 2010), METH self-administration also caused significant depletion in DA levels in the dorsal striatum that persisted up to 1 month (−21%) after cessation of drug exposure ($F_{(3,32)} = 7.007$, $p = 0.0011$) (Fig. 2A). The concentrations of DOPAC were decreased at 2 (−27%) and 24 h (−23%) ($F_{(3,32)} = 3.503$, $p = 0.0369$), but returned to control levels at 1 month post-drug (Fig. 2B). The concentrations of 5-HT were reduced at 2 and 24 h (−19% and −29%, respectively) ($F_{(3,32)} = 4.128$, $p = 0.0139$), but normalized at 1 month withdrawal (Fig. S1). In addition, METH also caused a significant decrease in 5-HIAA levels up to 1 month post-drug (−22%) ($F_{(3,32)} = 7.978$, $p = 0.0004$) (Fig. S1).

Because METH addiction is associated with reductions in D2 DA receptors in the human caudate (Volkow et al., 2001a; Wang et al., 2011), we tested the possibility that similar changes might also occur in the dorsal striatum of rats exposed to METH self-administration. Fig. 2 shows the effects of METH self-administration and abstinence on striatal D1 and D2 DA receptor mRNA and protein levels. There were no significant changes in the expression of D1 or D2 DA receptor mRNAs (Fig. 2C, D). However, there were transient increases in D1 DA receptor protein levels (+47%) ($F_{(3,19)} = 3.390$, $p = 0.0443$) at 2 h that returned to control 24 h after drug cessation (Fig. 2E, F). Similar to observations in humans (Volkow et al., 2001a; Wang et al., 2011), D2 DA receptor protein levels were decreased (−24%) after 1 month of abstinence ($F_{(3,19)} = 3.401$, $p = 0.0389$) (Fig. 2E, G). Taken together, the behavioral and biochemical similarities between this rat model and the human addiction had provided us with the confidence necessary to pursue transcriptional and epigenetic studies in rats that self-administered METH.

Transcriptional Changes in the Dorsal Striatum Induced by METH Self-Administration and Short-Term Abstinence

In order to avoid *a priori* theoretical biases, we used a discovery platform, Illumina 22K Rat microarray, to measure transcriptional effects of METH self-administration in the dorsal striatum. We found that 543 and 266 transcripts were differentially affected (±1.7 fold, p < 0.05) in the striatum at 2 and 24 h after the last METH self-administration session,

respectively. Table 1 provides a partial list of these genes. Full lists of genes significantly changed at 2 and 24 h are shown in Tables S2 and S3.

2 h after cessation of METH self-administration, changes in gene expression were detected in several functional groups that included transcription factors, neurotrophic factors, neuropeptides, neurotransmitter and peptide receptors, transporters, and genes involved in neurotransmitter release (Table 1). Specifically, METH self-administration caused up-regulation of *c-fos, c-jun, Crem, fosb, Nptx1, Nptx2*, and *Nr4a1-3* (Table 1) that play roles in transcriptional regulation. The large number of receptors and transporters affected by METH self-administration suggests that adaptive neuroplastic responses might involve multiple striatal neurotransmitter systems. In addition, METH induced increases in the expression of several neuropeptides including cholecystokinin (*Cck*), neurotensin (*Nts*) and prodynorphin (*Pdyn*) that are modulators of striatal DA neurotransmission (Angulo and McEwen, 1994; Loonam et al., 2003; Muschamp and Carlezon, 2013). There was also up-regulation in the expression of genes coding for synaptic proteins, syntaxin 1A (*Stx1a*), synapsin II (*Syn2*), synaptophysin (*Syp*) and synaptojanin 2 (*Synj2*), which are involved in neurotransmitter release (Cesca et al., 2010; de Wit, 2010; Liu and Ju, 2001). Moreover, METH self-administration caused substantial increases in *Bdnf* mRNA levels in the striatum (Table 1). BDNF is a neurotrophic factor that participates in the regulation of synaptic plasticity and is affected by exposure to several drugs of abuse (Autry and Monteggia, 2012; Russo et al., 2009).

Validation of Microarray Data with qRT-PCR

We then used qRT-PCR to validate the expression of several genes identified in the microarray experiments as significantly changed by METH self-administration. We confirmed METH-induced changes in *c-jun, Crem, Nptx1, Nptx2, Nr4a1, Nr4a2*, and *Nr4a3* mRNA levels that were up-regulated 2 h but normalized after 24 h of drug abstinence (Fig. 3A). The expression of these genes is known to be altered by other illicit drugs, including cocaine and heroin (Graybiel et al., 1990; Horner and Keefe, 2006; McClung and Nestler, 2008).

The RT-PCR data also confirmed that METH self-administration caused increases in the expression of genes coding for several neuropeptides, including *Cck, Fst, Nmu, Nts, Pdyn* and *Pnoc* (Fig. 3B). Several of these neuropeptides interact with DA systems in the brain (Angulo and McEwen, 1994; Loonam et al., 2003) and participate in the regulation of reward mechanisms (Muschamp and Carlezon, 2013). In addition, we confirmed METH-induced increases in the expression of *Stx1a, Synj2* and *Syp* that label presynaptic terminals of functional synapses (Liu and Ju, 2001) (Fig. 3C). Finally, we verified that METH self-administration causes increases in the expression of *Bdnf, Vglut1*, and *Vglut2* (Fig. 4A). BDNF is known to play important roles in the regulation of synaptic plasticity in the adult brain (Autry and Monteggia, 2012; Russo et al., 2009) and in several neuropsychiatric disorders including affective disorders (Autry and Monteggia, 2012).

In order to quantify potential relationships between the microarray and qRT-PCR data (Fig. 4B), we performed a regression analysis and found a strong positive correlation between changes in gene expression obtained using both methods (Fig. 4C). This analysis validates the inclusion criteria for choosing genes with altered expression using the discovery array platform.

Regulation of cFos, FosB and ΔFosB by METH Self-Administration in the Dorsal Striatum

Because *c-fos* mRNA expression was up-regulated (+42%) at 2 h after cessation of METH self-administration ($F_{(3,19)} = 5.568$; $p = 0.014$) (Fig. 5A), and taking into account the

potential role of Fos transcription factors in the development of neuroadaptations that might underlie compulsive drug-taking (Nestler, 2012), we also measured the effects of METH self-administration and abstinence on FosB and ΔFosB mRNA and protein levels in the dorsal striatum. Similar to *c-fos*, METH caused increases (+45%) in *fosb* mRNA expression 2 h after cessation of drug exposure ($F_{(3,19)} = 3.69$, $p = 0.0315$), with normalization after 24 h (Fig. 5B). There were no changes in *Δfosb* mRNA levels (Fig. 5C). In contrast to increases in *c-fos* mRNA expression, METH self-administration did not affect cFos protein levels after 2 or 24 h of abstinence, but caused significant decreases (−42%) at 1 month withdrawal ($F_{(3,20)} = 3.91$, $p = 0.0238$) (Fig. 5D, E). METH did not change FosB protein levels up to 1 month after cessation of self-administration (Fig. 5D, E). Interestingly, ΔFosB protein levels were significantly increased 2 (+48%) and 24 h (+27%) after cessation of METH self-administration, but decreased expression (−67%) was detected after 1 month of abstinence ($F_{(3,20)} = 27.69$, $p < 0.0001$) (Fig. 5D, E).

Regulation of BDNF and TrkB Receptor by METH Self-Administration

To get a better picture of METH effects on *Bdnf* mRNA expression, we measured the time-course of METH-induced changes in the mRNAs for *Bdnf* and its receptor, *TrkB*. METH induced significant increases (+380%) in *Bdnf* exon I mRNA expression after 2 h, with normalization after 24 h and 1 month of abstinence ($F_{(3,19)} = 5.41$, $p = 0.0068$) (Fig. 6A). *Trkb* mRNA expression showed no significant changes at any time point (Fig. 6B). At 2 and 24 h after cessation of METH self-administration, BDNF protein levels were increased by 89% and 63%, respectively, compared to yoked saline controls (Fig. 6C, D). In contrast, after 1 month of abstinence, BDNF protein levels were significantly decreased (−59%) ($F_{(3,20)} = 29.71$, $p < 0.0001$) (Fig. 6C, D). We also measured the levels of TrkB protein after cessation of METH self-administration and found significant increases at 2 (+45%) and 24 h (+33%) (Fig. 6C, E). Similar to BDNF, TrkB protein levels were decreased (−33%) after 1 month of abstinence ($F_{(3,20)} = 22.13$, $p < 0.0001$) (Fig. 6C, E).

METH Self-Administration Caused Enrichment of pCREB, but not of H3K4me3, on *c-fos, fosb, Bdnf* and *Syp* Promoters

Gene expression is regulated, in part, by epigenetic modifications that include histone acetylation, methylation, and phosphorylation (Nelson and Monteggia, 2011; Wang et al., 2008). In order to investigate potential epigenetic mechanisms that might be involved in METH-induced changes in gene expression, we initially focused on the expression of histone 3 trimethylated at lysine 4 (H3K4me3) because this histone modification has been linked to activation of gene expression (Ng et al., 2009). We found that METH self-administration caused significant increases ($F_{(3,20)} = 3.565$, $p = 0.0363$) in H3K4me3 protein levels after 2 h (+60%) and 24 h (+51%), with normal levels measured after 1 month of abstinence (Fig. 7A, B). Next, we used ChIP-PCR to test if there might be increased enrichment of H3K4me3 on the promoters of some METH-upregulated genes of interest. These included *c-fos, fosb, c-jun, Nr4a1-3,* and *Syp,* which are involved in synaptic plasticity or regulated by synaptic activity (Fig. 7C). We also measured the binding of H3K4me3 on *Bdnf* promoters pI (*Bdnf-pI*), pIV (*Bdnf-pIV*), and pVI (*Bdnf-pVI*). There were no significant METH-induced changes in the enrichment of H3K4me3 on promoters of any of these genes (Fig. 7C).

In addition to histone modifications, inducible gene expression is regulated by a number of factors that include phosphorylation of CREB and subsequent activation of gene expression (Nestler, 2012; Robison and Nestler, 2011). Thus, we measured the expression of CREB and pCREB protein levels at various time-points following the cessation of METH self-administration. There were significant decreases in CREB protein levels (−40%) at 1 month ($F_{(3,20)} = 6.17$, p = 0.0038) (Fig. 7D, E), but increases in pCREB levels at 2 (+27%) and 24

h (+21%), followed by decreased levels (−19%) after 1 month of METH abstinence ($F_{(3,20)}$ = 15.232, $p < 0.0001$) (Fig. 7D, F). pCREB/CREB ratios were significantly increased at 2 and 24 h (+51% and +39%, respectively), but returned to basal levels at 1 month after cessation of drug exposure ($F_{(3,20)} = 4.074$, $p = 0.0226$) (Fig. 7G). To examine the role of CREB-regulated transcription in METH-induced gene expression, we used ChIP-PCR to measure the enrichment of pCREB on the promoters of the same genes listed above. These genes are known to contain CRE motifs (Fass et al., 2003; Tao et al., 1998). Four of these genes (*c-fos, fosb, Bdnf-pI, and Syp*) showed significantly increased enrichment of pCREB on their promoters 2 h after cessation of METH self-administration (Fig. 7H), results that are consistent with upregulation of their transcripts by METH.

Discussion

The transition from recreational drug use to addiction and recurrent relapse is hypothesized to involve a progressive shift from ventral to dorsal striatal control over drug intake as a result of long-term molecular and structural adaptations in mesostriatal dopaminergic pathways (Belin and Everitt, 2008; Everitt and Robbins, 2013; Volkow et al., 2011). These adaptive changes are secondary, in part, to drug-induced transcriptional and epigenetic alterations in the brain (Robison and Nestler, 2011). Indeed, we have found that METH self-administration was associated with transcriptional changes in genes that regulate transcription, synaptic transmission, and synaptic plasticity in the dorsal striatum. Specifically, there was increased ΔFosB protein expression 2 and 24 h after cessation of METH self-administration followed by a decrease after 1 month of abstinence. Our observations are consistent with recent findings of increased ΔFosB expression in the caudate-putamen of rats allowed to chronically self-administer METH (Cornish et al., 2012). A role for ΔFosB in METH self-administration is supported by a report that ΔFosB increases the motivation for cocaine self-administration (Colby et al., 2003) and by the finding that induction of ΔFosB is sufficient to increase locomotor and rewarding effects of cocaine (Kelz et al., 1999; Muschamp et al., 2012). The present data obtained at early stages of abstinence (↑ΔFosB) are consistent with the notion that the protein might regulate the transition from occasional drug use to increased drug-seeking and drug-taking behaviors and, possibly, the maintenance of addiction (Robison and Nestler, 2011). However, it is unlikely that ΔFosB is directly involved in relapse susceptibility because there were significant decreases in ΔFosB expression after 1 month of abstinence, a time at which animals might be more prone to reinstatement of drug-taking behavior (Shaham et al., 2003). Nevertheless, the protein could have had secondary effects by inducing the transcription of neurotransmitter receptors and other synaptic proteins, including ion channels and cytoskeletal proteins that might be involved in triggering relapse (McClung and Nestler, 2008; Renthal et al., 2008; Robison and Nestler, 2011). Indeed, given the consistency of the effects caused by various drugs on ΔFosB expression, it is very likely that this protein might be an important node that connects various networks involved in the early manifestations, maintenance of, and, perhaps, relapse to addiction.

In addition to changes in ΔFosB, we found significant increases in BDNF and TrkB 2 at 24 h after cessation of METH self-administration but decreases after 1 month of METH abstinence. It is of interest that METH increased TrkB protein, but not TrkB mRNA levels in the dorsal striatum. A similar dissociation between TrkB mRNA and protein expression has been previously reported in the rat nucleus accumbens after cocaine self-administration (Fumagalli et al., 2009; Graham et al., 2009). Together, these observations suggest that METH-induced increase in striatal TrkB protein levels is not due to up-regulated TrkB mRNA expression but is secondary to non-transcriptional mechanisms including increased translation and protein stability, and/or reduced protein degradation.

Although METH exposure increased both striatal BDNF mRNA and protein levels at 2 h, BDNF protein levels were increased at 24 h in the absence of changes in mRNA expression. Dissociations between the effects of psychostimulants on BDNF mRNA and protein levels have also been reported by other investigators (Graham et al., 2007; Fumagalli et al., 2007, 2013; Li et al., 2013). As with TrkB expression and at first glance, mechanisms other than increased mRNA levels appear to be responsible for METH-induced increased BDNF protein levels. These mechanisms might include increased stabilization of BDNF protein secondary to the early increases in BDNF mRNA and protein levels observed at 2 h after cessation of METH self-administration. Processing of pro-BDNF into mature BDNF has also been reported to increase in a Ca^{2+}-dependent manner via elevated secretion of tissue plasminogen activator (Gualandris et al., 1996). Another possible mechanism is METH-induced anterograde transport of BDNF protein from cortical neurons that project to the striatum and that also express high BDNF levels (Altar et al., 1997; Conner et al., 1997; Guillin et al., 2001; Lessmann et al., 2003). In support of this hypothesis, increases in BDNF protein levels have been shown in the prefrontal cortical target areas such as nucleus accumbens, basolateral amygdala and central nucleus of the amygdala after infusion of BDNF into rat dorsomedial prefrotal cortex (Berglind et al., 2007, McGinty et al., 2010). Finally, our observations of dissociations between the mRNA and protein levels for DA receptors, BDNF, and TrkB are consistent with the findings that correlations among individual mRNA and protein concentrations vary widely in the same organism as well as across species and are often surprisingly low (Anderson and Seilhamer, 1997; Unwin and Whetton, 2005; De Souse Abreu et al., 2009; Evguenieva-Hackenberg and Klug, 2011).

Our results showing increases in BDNF protein levels at 2 and 24 h after METH self-administration but decreases after 1 month of withdrawal are in agreement with clinical studies reporting elevated BDNF levels in the plasma of chronic METH users (Kim et al., 2005), with BDNF concentrations in METH addicts dropping with increased duration of abstinence (Hilburn et al., 2011). Our results are also consistent with the idea that BDNF signaling participates in the development of addiction (Russo et al., 2009). In this regard, BDNF can enhance the expression of a marker of functional synapses Syp (Liu and Ju, 2001); an effect that is blocked by BDNF and TrkB antibodies (Li and Keifer, 2008, 2012). Thus, our finding that METH also caused increase in *Syp* mRNA levels suggests that the drug might enhance the functionality of striatal synapses (Liu and Ju, 2001) and cause sustained changes in neurotransmission in METH-addicted rats. Increased BDNF expression is likely to enhance the activation of TrkB downstream pathways (Ji et al., 2010), possibly involving a cascade of events that include MAPK, phosphatidylinositol and/or PLC-γ pathways (Park and Poo, 2013). These signaling events might be responsible, in part, for METH-induced long-lasting morphological changes in the brain. This idea is consistent with observations of METH-induced dendritic spine formation on dorsal striatal neurons (Jedynak et al., 2007), an effect that is indeed dependent on BDNF-TrkB signaling (Rauskolb et al., 2010). Thus, it is possible that sustained BDNF accumulation is not necessary for its long-lasting impact on the density of dendritic spines, but only for the triggering of multiple cellular processes that facilitate persistent striatal reorganization of synaptic connectivity.

Note, however, that, after 1 month of abstinence, there were significant decreases in BDNF expression, suggesting the possibility that low BDNF levels might be conducive to relapse, as previously reported for ethanol drinking (Jeanblanc et al., 2009). These observations are in contrast to those reported for cocaine withdrawal, which is associated with progressive increases in BDNF levels in the nucleus accumbens up to 90 days of abstinence (Grimm et al., 2003). It is to be noted that Grimm et al. (2003) had measured BDNF in the nucleus accumbens and VTA, whereas we measured the expression in the dorsal striatum. It is also of interest to discuss the observations that there were non-significant decreases in *Ngf* and

Camkk2 mRNA expression at 2 h but increases in their mRNAs at 24 h. Together with BDNF, NGF belongs to secreted neurotrophins that regulate neuronal circuits and synaptic plasticity (Park and Poo, 2013). Therefore, increase in the expression of NGF could, in addition to BDNF, contribute to synaptic remodeling and neuroplastic changes caused by METH self-administration in the dorsal striatum. Similarly, CaMKK2 is highly expressed in the brain where it plays important role in activating intracellular responses to elevated Ca^{2+}. Up-regulated expression of CaMKK2 could contribute to increased phosphorylation of transcription factors, including CREB (Kokubo et al., 2009). Our observation of increased CREB phosphorylation in the present study suggests that CaMKK2 might also be involved in the regulation of gene expression observed after cessation of METH self-administration.

In eukaryotic cells, DNA exists as chromatin, whose basic unit is the nucleosome (Kornberg and Lorch, 1999). The nucleosome consists of 147 bp of DNA wrapped around a histone octomer that contains 2 of H2A, H2B, H3, and H4 histones. The histone tails protrude through that complex and form a scaffold for modifications such as acetylation, methylation, and phosphorylation that play very important roles in modulating gene expression (Nelson and Monteggia, 2011; Wang et al., 2008). As a first step towards testing the idea that METH-induced gene expression might be regulated by epigenetic modifications, we measured the levels of H3K4me3, a histone modification linked to activation of gene expression (Ng et al., 2009) and found METH-induced increases in H3K4me3 protein expression. Unexpectedly, none of the chosen genes of interest showed increased H3K4me3 enrichment on their promoters.

Gene expression is also dependent on combinatorial effects of transcription factors that bind on specific DNA sequences (Qiu and Ghosh, 2008). In the case of psychostimulants, they appear to exert their transcriptional and other long-term biochemical effects, in part, via stimulation of striatal D1 DA receptor subtypes, followed by increases in protein kinase A activity, phosphorylation of CREB, and consequent CREB-mediated transcription (Ares-Santos et al., 2012; Cadet et al., 2010; Granado et al., 2011; Muschamp and Carlezon, 2013). Specifically, it has been shown that D1 DA receptor antagonists (Beauvais et al., 2010; Jayanthi et al., 2009) or deletion of D1 DA receptors (Moratalla et al., 1996) can interfere with amphetamine- or METH-induced increases in c-Fos, FosB, and CREM gene and protein levels in the dorsal striatum, indicating that this receptor subtype might also play a key role in the control of gene and protein expression observed in the present study. We thus investigated the possibility that this cascade might also be involved in METH self-administration by testing whether pCREB might regulate the expression of some genes of interest and found, for the first time, that METH self-administration is accompanied by increased recruitment of pCREB on the promoters of *c-fos*, *fosB*,*Bdnf*, and *Syp*, all of which show METH-induced increases in mRNA expression in the dorsal striatum. Our findings, therefore, indicate that these genes are co-regulated at both epigenetic and transcriptional levels and that they might work in concert to maintain METH-addicted state.

In conclusion, our study shows that METH self-administration causes long-term changes of ΔFosB, BDNF and TrkB expression in the dorsal striatum after various lengths of abstinence. Increased ΔFosB, BDNF, and TrkB levels were prominent during early stages of abstinence from METH self-administration whereas decreased levels were observed 1 month after cessation of drug exposure. The latter time interval might be associated with increased chance of reinstatement of drug taking (Shaham et al., 2003). These observations suggest that, in the case of METH, animals might reinstate because of ΔFosB, BDNF, and TrkB deficits. Our demonstration of increased pCREB binding on *c-fos*, *fosb*, and *Bdnf* promoters suggests that CREB is indeed an important player in regulating METH-induced gene expression and synaptic plasticity. Future studies using epigenetic approaches such as ChIP followed by large-scale DNA sequencing (ChIP-Seq) should help to decipher the roles

of pCREB, H3K4me3 and other epigenetic modifications in the global effects of METH on gene transcription in the dorsal striatum.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

Rats underwent a 15-hour extended access to METH self-administration for 8 days

Changes in gene and protein expression were studied in the dorsal striatum

METH caused changes in ΔFosB, BDNF and TrkB expression up to 1 month of withdrawal

METH also produced increased pCREB binding on *c-fos*, *fosb*, and *Bdnf* promoters

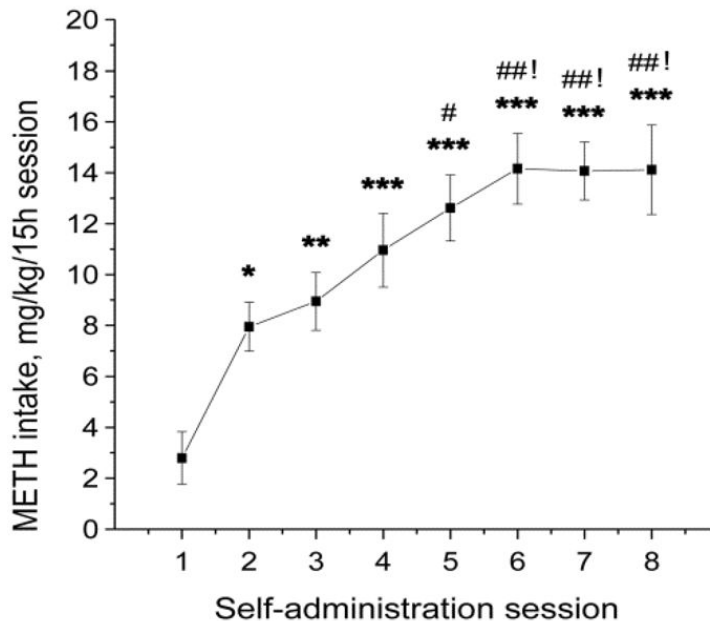METH self-administration-induced transcription is mediated, in part, by pCREB

Figure 1.
Rats escalated METH intake over the course of self-administration. Symbols show an average METH intake per 15-h session in the group of rats euthanized 24 h after cessation of self-administration. Rats increased METH intake on session 2 compared with session 1, and on session 5 compared with session 2. Animals further escalated their drug intake on session 6 in comparison to session 3 and stayed at this level until cessation of METH self-administration. METH intake by rats in the 24-h group reached 14.2 mg/kg/day. Data were analyzed by one-way ANOVA for repeated measures, followed by Tukey's multiple comparison test. N = 12. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with session 1; # $p < 0.05$, ## $p < 0.01$ compared with session2; ! $p < 0.05$ compared with session 3.
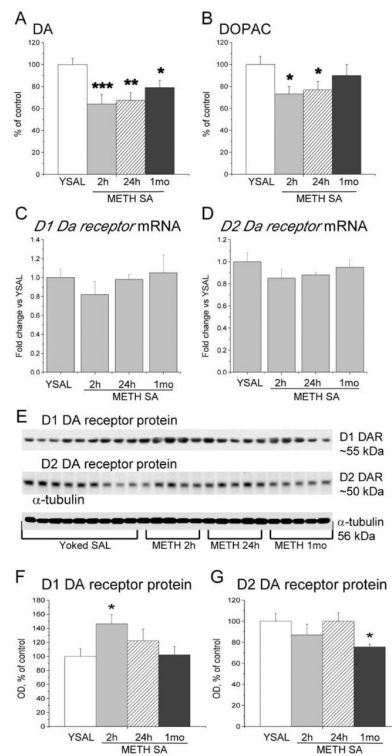
Figure 2.
Regulation of DA levels and D1 and D2 DA receptor expression in the dorsal striatum
following METH self-administration. Rats were allowed to self-administer METH for eight
15-h sessions and were euthanized at 2 h, 24 h or 1 month withdrawal. The dorsal striata
were dissected and processed for HPLC, qRT-PCR and Western blot as described in the
text. (A) METH caused persistent decreases in striatal DA levels up to 1 month post-drug;
(B) DOPAC concentrations were decreased at 2 and 24 h, but returned to control levels after
1 month of withdrawal. (C) D1 DA receptor and (D) D2 DA receptor mRNA levels are not
significantly changed at 2 h, 24 h, or 1 month after cessation of METH self-administration.
(E) Representative immunoblots of membrane D1 and D2 DA receptor protein levels. (F)
METH self-administration caused significant increases in D1 DA receptor protein levels.
(G) D2 DA receptor protein levels are decreased after 1 month. N = 5–9 per group. Data are
expressed as fold-changes (mRNA) or percent (protein, monoamine levels) of mean ± SEM
values of yoked saline group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significantly different
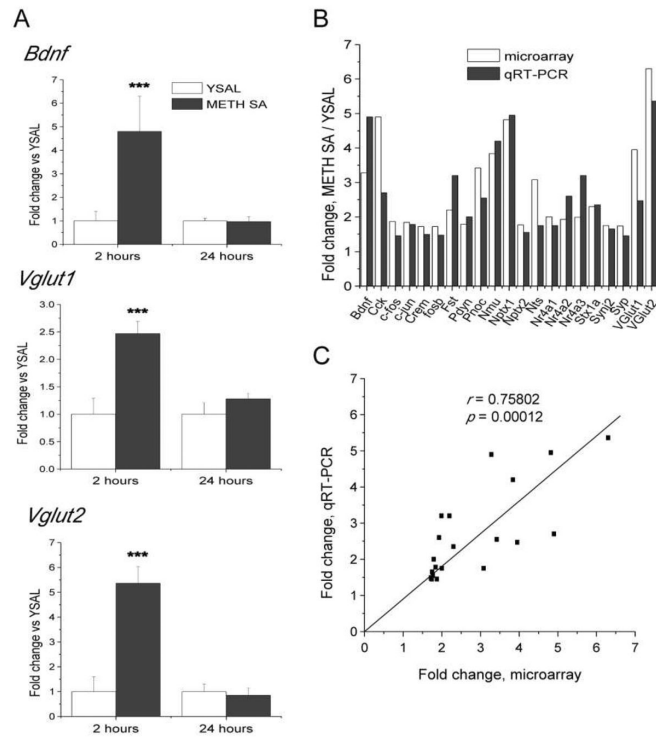from yoked saline.

Figure 3.
qRT-PCR validation of METH-induced changes in the expression of genes identified in microarray experiments. qRT-PCRs were performed with the same total RNA samples isolated from dorsal striata of rats that were used for microarray hybridization. Values represent means ± SEM in comparison to the respective yoked saline group. Consistent with microarray data, METH self-administration induced increases in the expression of (A) transcription factors, (B) neuropeptides, (C) genes involved in the synaptic functions at 2 h, which were normalized at 24 h after cessation of drug exposure. N = 6 per group. * $p < 0.05$, *** $p < 0.001$ in comparison to yoked saline (Student's t-test).
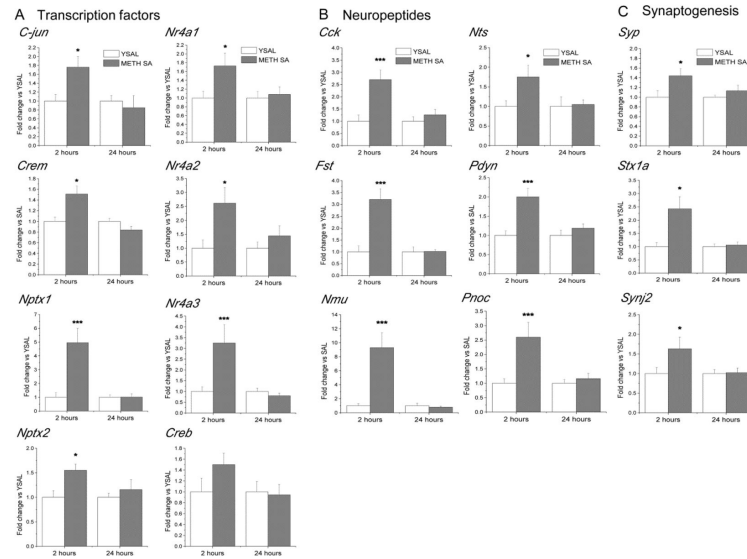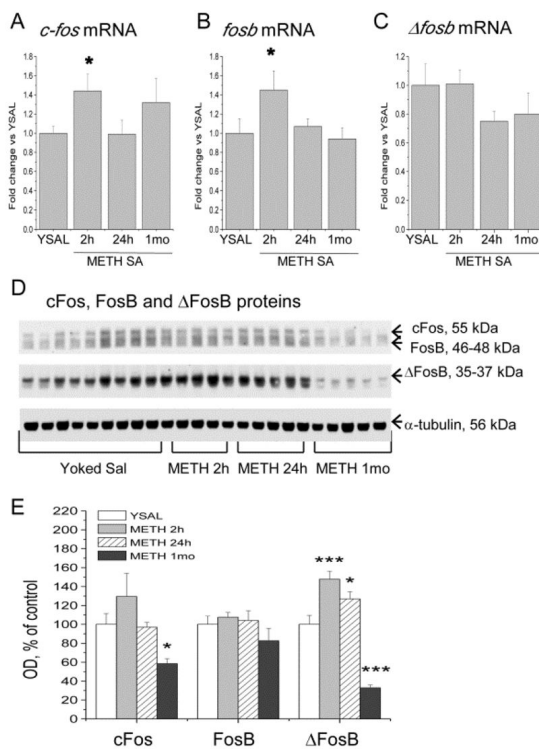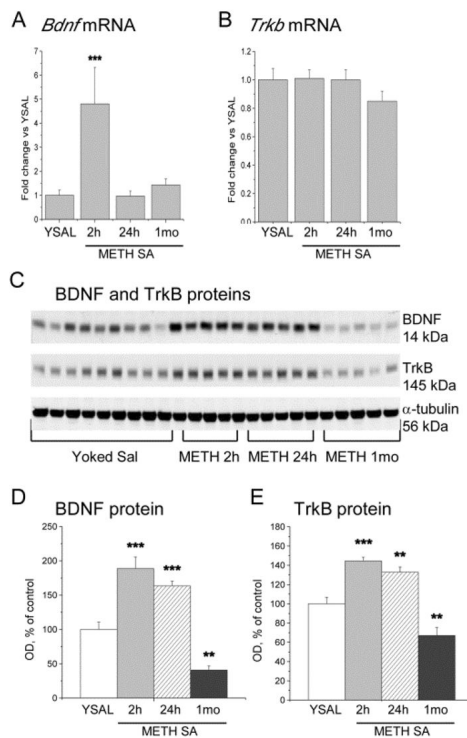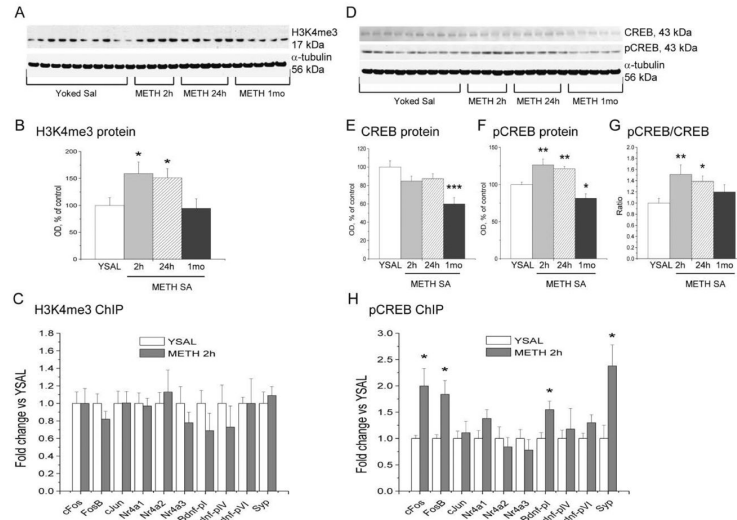
Figure 4.
Validation of METH-induced alterations in the expression of genes identified by microarray experiments using qRT-PCR. (A) METH self-administration caused increases in the expression of *Bdnf* and *Vgluts* in the dorsal striatum. (B) The increases in the expression of 21 genes obtained using microarray were validated with qRT-PCR. All fold-changes found with qRT-PCR were significantly different (p < 0.05). (C) METH-induced changes obtained using microarray analysis significantly correlated with qRT-PCR data. N = 6 per group. *** $p < 0.001$ in comparison to yoked saline (Student's t-test).

Figure 5.
Effects of METH self-administration and abstinence on *c-fos, fosb and Δfosb* expression in the dorsal striatum. METH induced increases in *c-fos* (A) and *fosb* (B) mRNA levels at 2 h, with normalization at 24 h and 1 month after cessation of self-administration. (C) METH self-administration did not cause significant changes in the expression of Δ*fosb* mRNA expression. (D) Analysis of c-Fos, FosB and ΔFosB protein levels in the dorsal striatum. Representative immunoblot showing the molecular weights of the proteins used to quantify levels of c-Fos (55 kDa), FosB (46–48 kDa) and ΔFosB (35–37 kDa). Signals were normalized to α-tubulin. (E) METH self-administration did not affect the expression of FosB, but caused decreases in the levels of c-Fos protein levels at 1 month of abstinence. METH induced increases in ΔFosB protein levels at 2 and 24 h followed by decreased expression at 1 month withdrawal. N = 5–9 per group. * $p < 0.05$, *** $p < 0.001$, significantly different from yoked saline.

Figure 6.
METH-induced changes in the expression of BDNF and its receptor, TrkB, in the dorsal striatum. (A) *Bdnf* mRNA expression (exon I) was increased 2 h after cessation of METH self-administration but returned to control level at 24 h and 1 month. (B) METH self-administration did not cause any changes in the expression of *Trkb* mRNA up to 1 month after cessation of METH self-administration. (C) Immunoblot analysis of BDNF and TrkB protein levels in the dorsal striatum. METH self-administration induced increases in (D) BDNF and (E) TrkB protein levels at 2 and 24 h but decreases at 1 month abstinence. Data are presented as means ± SEM. N = 5–9 per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significantly different from yoked saline control.

Figure 7.
Transcriptional responses in the dorsal striatum caused by METH self-administration are associated with increased enrichment of pCREB, but not H3K4me3, on promoters of genes related to synaptic plasticity. (A) Immunoblot analysis of H3K4me3 protein levels following cessation of METH self-administration. (B) METH caused increased H3K4me3 levels after 2 and 24 h abstinence from METH self-administration. (C) There were no changes in H3K4me3 enrichment on the promoters of METH-regulated genes. (D) Western blots of CREB and pCREB levels in METH self-administration and yoked saline rats. (E) Analysis of optical density showed decreases in CREB levels at 1 month abstinence. (F) METH induced increases in pCREB levels at 2 and 24 h, followed by decreases at 1 month after cessation of drug exposure. (G) pCREB/CREB ratios were increased at 2 and 24 h, but normalized at 1 month post-drug. (H) There was a significant increase in the levels of pCREB on promoters of *c-fos, fosb,Syp* and *BdnfpI*. Data presented as means ± SEM. N = 5–9 per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significantly different from yoked saline control.

Table 1

Partial list of genes affected by METH self-administration and withdrawal in the rat striatum.

Gene Name	Gene Symbol	Expression Ratio METH / Yoked SAL		p value (ANOVA)
		2h	24h	
Neurotrophic factors				
Heparin-binding EGF-like growth factor	Hbegf	4.44	3.45	0.0406; 0.0114
Brain derived neurotrophic factor	Bdnf	3.28	1.33	0.0379
Nerve growth factor, gamma	Ngfg	−2.31	2.22	0.0163
Synoptic proteins				
Syntaxin 1A	Stx1a	2.30	−1.02	0.0074
Synapsin II	Syn2	2.12	−1.11	0.0433
Synaptophysin	Syp	1.74	1.20	0.0044
Synaptojanin 2	Synj2	1.73	1.29	0.0482
Neuropeptides				
Cholecystokinin	Cck	4.93	1.68	0.0022
Neuromedin S	Nms	4.41	−1.01	0.0041
Neuromedin U	Nmu	3.84	1.35	0.0001
Cortistatin	Cort	3.53	1.19	0.0047
Prepronociceptin	Pnoc	3.42	1.23	0.0079
Neurotensin	Nts	3.08	1.05	0.0069
Follistatin	Fst	2.20	1.24	0.0001
Prodynorphin	Pdyn	1.79	1.26	0.0006
Neuropeptide Y	Npy	1.74	1.31	0.0112
Receptors				
Glial cell line derived neurotrophic factor receptor alpha 1	Gfra1	7.76	1.70	0.0048
Gastrin releasing peptide receptor	Grpr	7.08	−2.05	0.0057
Cholinergic receptor, nicotinic, alpha polypeptide 4	Chrna4	5.60	1.17	0.0484
Neuropeptide Y receptor Y1	Npy1r	2.35	−1.31	0.0440
Angiotensin receptor 1b	Agtr1b	2.10	−1.12	0.0048
Relaxin/insulin-like family peptide receptor 1	Rxfp1	1.88	1.27	0.0072
Cholinergic receptor, muscarinic 3, cardiac	Chrm3	1.82	1.29	0.0217
Fibroblast growth factor receptor-like 1	Fgfrl1	1.81	1.27	0.0104
Fibroblast growth factor receptor 4	Fgfr4	1.35	3.51	0.0132
Transporters				
Solute carrier family 8, member 1	Slc8a1	7.56	1.37	0.0012
Vesicular glutamate transporter 2	Vglut2	6.30	1.77	0.0001
Solute carrier family 37, member 1	Slc37a1	6.07	−1.05	0.0044
Solute carrier family 9, isoform 3 regulator 2	Slc9a3r2	5.60	−1.11	0.0035
Vesicular glutamate transporter 1	Vglut2	3.95	2.06	0.0138
Kinases/Phosphatases				
Ca2+/calmodulin-dependent kinase kinase 2, beta	Camkk2	−1.23	1.93	0.0342
Protein kinase C, delta	Prkcd	1.34	1.75	0.0388

Gene Name	Gene Symbol	Expression Ratio METH / Yoked SAL		*p* value (ANOVA)
		2h	24h	
Transcription factors				
Neuronal pentraxin 1	Nptx1	4.82	1.37	0.0027
Nuclear receptor subfamily 4, group A, member 1	Nr4a1/Nurr77	2.03	1.02	0.0015
Nuclear receptor subfamily 4, group A, member 3	Nr4a3	1.99	−1.01	0.0413
Nuclear receptor subfamily 4, group A, member 2	Nr4a2/Nurr1	1.92	1.51	0.0409
FBJ murine osteosarcoma viral oncogene homolog	c-fos	1.87	1.29	0.0424
Jun oncogene	c-jun	1.84	1.13	0.0155
Neuronal pentraxin 2	Nptx2	1.77	1.06	0.0213
cAMP responsive element modulator	Crem	1.72	1.02	0.0124
FBJ osteosarcoma oncogene B	fosb	1.72	1.27	0.0452