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Chronic methamphetamine exposure suppresses the striatal expression of members of multiple families of immediate early genes (IEGs) in the rat: normalization by an acute methamphetamine injection

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Abstract

Rationale—Repeated injections of cocaine cause blunted responses to acute cocaine challenge-induced increases in the expression of immediate early genes (IEGs).

Objectives—The aim of this study was to test if chronic methamphetamine (METH) exposure might cause similar blunting of acute METH-induced increases in IEG expression.

Results—Repeated saline or METH injections were given to rats over 14 days. After one day of withdrawal, they received a single injection of saline or METH (5 mg/kg). Acute injection of METH increased c-fos, fosB, fra2, junB, Egr1-3, Nr4a1 (Nur77), and Nr4a3 (Nor-1) mRNA levels in the striatum of saline-pretreated rats. Chronic METH treatment alone reduced the expression of AP1, Erg1-3, and Nr4a1 transcription factors below control levels. Acute METH challenge normalized these values in METH-pretreated rats. Unexpectedly, acute METH challenge to METH-pretreated animals caused further decreases in Nr4a2 (Nurr1) mRNA levels. In contrast, the METH challenge caused significant but blunted increases in Nr4a3 and Arc expression in METH-pretreated rats. There were also chronic METH-associated decreases in the expression of cAMP responsive element binding protein (CREB) which modulates IEG expression via activation of the cAMP/PKA/CREB signal transduction pathway. Chronic METH exposure also caused significant decreases in preprotachykinin, but not in prodynorphin, mRNA levels.

Conclusions—These results support the accumulated evidence that chronic administration of psychostimulants is associated with blunting of their acute stimulatory effects on IEG expression. The METH-induced renormalization of the expression of several IEGs in rats chronically exposed to METH hints to a potential molecular explanation for the recurrent self-administration of the drug by human addicts.

Keywords

Addiction; Arc; Preprotachykinin; Dynorphin; Molecular neuroadaptations

Introduction

Methamphetamine (METH) is an illicit psychostimulant that is widely abused throughout the world. The drug can cause persistent behavioral abnormalities that include the development of tolerance and dependence, paranoid states, and psychotic symptoms in human addicts (Comer et al., 2001; Suzuki et al., 2004). In animals, METH causes behavioral sensitization and is also self-administered (Akiyama et al., 1994; Krasnova et al., 2010; Mandym et al., 2008; Roth and Carroll, 2004). The acute behavioral effects of the drug are mediated mainly by the marked METH-induced increases in the amount of dopamine (DA) release in the brain (Bustamante et al., 2002; Comer et al., 2001; Kuczenski et al., 1995; Suzuki et al., 2004; Xi et al., 2009) and by the subsequent stimulation of DA receptors in various brain regions (Ujike et al., 1989). Activation of these receptors by direct or indirect agonists such as cocaine and amphetamine induces marked changes in the expression of several immediate early genes (IEGs) in the striatum (Cole et al., 1995; Gerfen et al., 1995; Graybiel et al., 1990; Young et al., 1991). These observations have prompted suggestions that the long-lasting enduring behavioral and cognitive effects of these two psychostimulants might depend on changes in IEG expression in various brain regions (Cadet, 2009; Cadet et al., 2009b; Harlan and Garcia, 1998; Persico et al., 1993). A similar argument could be made in the case of METH because acute injections of this agent also cause significant increases in the expression of several IEGs in the rat brain (Cadet et al., 2001; Hirata et al., 1998; Jayanthi et al., 2005; Jayanthi et al., 2009; Thomas et al., 2004; Umino et al., 1995; Wang and McGinty, 1995; Wang et al., 1995). These genes include *c-fos*, *Egr1*, and *Nur77*, among others. The majority of METH-induced changes in gene expression appear to occur via stimulation of post-synaptic DA D1 receptors (Beauvais et al., 2010; Cadet et al., 2010; Jayanthi et al., 2009; Wang and McGinty, 1995, 1996; Wang et al., 1995), with participation of glutamate NMDA and kainate receptors (Gross and Marshall, 2009; Wang and McGinty, 1996).

In contrast to acute transcriptional effects of psychostimulants, chronic administration of cocaine (Bhat et al., 1992; Hope et al., 1992; Unal et al., 2009; Zahm et al., 2010) and amphetamine (Jaber et al., 1995; Konradi et al., 1996; Persico et al., 1993; Renthal et al., 2008) has been shown to blunt the effects of a single dose of these drugs (summarized in Tables 1 and 2). For example, Hope et al. (1992) injected rats with cocaine (15 mg/kg) or saline twice daily for 14 days and then gave the rats an acute injection of the drug or saline after 18 hrs of withdrawal. The authors reported that acute cocaine caused significant increases in mRNA levels of *c-fos*, *fosB*, *c-jun*, *junB*, and *Egr1* in the nucleus accumbens whereas cocaine-pretreated cocaine-challenged rats showed no increases in IEG expression. Similarly, Moratalla and collaborators (1996) injected rats with saline or cocaine (25 mg/kg) twice daily for 4 or 7 days and then challenged them with an acute injection of cocaine (25 mg/kg) after 18 hrs of withdrawal. Acute cocaine caused increased expression of *c-Fos* and *JunB* proteins in the striatum. There were also significant time-dependent decreases in the responsiveness of *c-Fos* and *JunB* proteins to a cocaine challenge in animals pretreated with cocaine, with *c-Fos* protein levels showing no responses to the cocaine challenge after 7 days of repeated cocaine injections. Similar results have been reported following chronic treatment with amphetamine (summarized in Table 2). Specifically, Persico and others (1993) injected rats with either saline or amphetamine (7.5 mg/kg) twice daily for 14 days. The animals were then challenged with either amphetamine or saline. Acute injection of amphetamine caused substantial increases in the expression of *c-fos*, *fosB*, *c-jun*, *junB*, and *Egr1* mRNA levels in the striatum. Similar to cocaine, pretreatment with amphetamine caused significant inhibition of the acute effects of amphetamine. More recently, rats treated with saline or amphetamine (4 mg/kg) once per day for 6 days were also reported to show significant blunting of the effects of an acute amphetamine administration on *c-fos* mRNA levels in the striatum (Renthal et al., 2008).

Very few studies have assessed the effects of chronic exposure to METH on IEG expression. Namima and co-investigators (1999) reported that chronic treatment of mice with METH (2 mg/kg) given five times at intervals of 3 days attenuated the acute effects of the drug on c-Fos protein expression in the striatum. Hamamura and others (1999) also found that daily injections of METH (5 mg/kg) for 3 days also attenuated the acute effects of METH on c-fos mRNA levels in the cerebellum. Therefore, more experiments were necessary to identify the effects of chronic METH exposure on IEG expression in the striatum. This is important because these IEGs regulated the expression of downstream targets in the brain (Herdegen and Leah, 1998; O'Donovan et al., 1999). Therefore, we undertook the present experiments to investigate the effects of chronic METH administration on IEG expression in the striatum using a pattern of drug administration that does not cause any toxicity in the brain (Cadet et al., 2009a; Graham et al., 2008). We also tested the possibility that chronic METH injections might alter the acute stimulatory effects of the drug on IEG expression. We also tested potential functional significance of the effects of chronic drug exposure by measuring preprotachykinin (Tac1) and prodynorphin (Pdyn) mRNA levels in the striatum of METH-treated animals.

Materials and methods

Animals

Male Sprague-Dawley rats (Charles Rivers Laboratories, Raleigh, NC), weighing 330–370 g in the beginning of the experiment were used in the present study. Animals were housed in a humidity- and temperature-controlled room and were given free access to food and water. All animal procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care Committee.

Drug Treatment and Tissue Collection

Following habituation, rats were injected intraperitoneally with either ($\pm$) METH-hydrochloride (NIDA, Baltimore, MD) or an equivalent volume of 0.9% saline over a period of two weeks as described in Table 3. This dosing paradigm is similar with those used in our previous studies (Cadet et al., 2009a,b) and attempts to mimic the progressive increases in METH doses used by METH users (Cho and Melega, 2002). The saline- or METH-pretreated animals received a single injection of saline or METH (5 mg/kg) at 16–18 hrs after the last saline or METH pretreatment injection. This dose of METH does not cause any neurotoxic effects as much larger doses are required for pathological changes to appear in the rodent brain (Krasnova and Cadet, 2009). The delay of 16–18 hrs was chosen to allow for comparison with previous studies of the effects of cocaine (Hope et al., 1992; Moratalla et al., 1996). The four groups of animals were: saline/saline (SS), saline/METH (SM), METH/saline (MS), and METH/METH (MM). The animals were euthanized by decapitation 2 hrs later. Their brains were quickly removed, striatal tissues were dissected on ice, snap frozen on dry ice, and stored at -80°C until used in quantitative PCR experiments as described below.

RNA extraction and quantitative Real-time PCR

Total RNA was isolated using Qiagen RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA) and showed no degradation. The RNA extracted from the striatum was used to measure the expression of members of several classes of IEGs. Individual total RNA obtained from 6–8 rats per group was reverse-transcribed with oligo dT primers and RT for PCR kit (Clontech, Palo Alto, CA). PCR experiments were done using the Chroma4 RT-PCR Detection System (BioRad Hercules, CA USA) and iQ SYBR

Green Supermix (BioRad) according to the manufacturer's protocol. Sequences for gene-specific primers corresponding to PCR targets were obtained using LightCycler Probe Design software (Roche). The primers were synthesized and HPLC-purified at the Synthesis and Sequencing Facility of Johns Hopkins University (Baltimore, MD). The sequences for the IEG primers have been published previously (Beauvais et al., 2010; Jayanthi et al., 2005). The sequences for Tac1 were: Forward – 5' GGC ATG GTC AGA TCT CTC A 3', Reverse – 5' TGA ATA GAT AGT GCG TTA CAG G 3'. The sequences for Pdyn were: Forward – 5' CTT CAT CCT CCT CTG CTT A 3', Reverse – 5' CGG ACA CTG GAT GGA TT 3'. The sequences for NAB2 were: Forward – 5' ATC CCT GTT GAA GCT GA 3', Reverse – 5' TCA ACT CGT GCA AGC TAA 3'. The sequences for CREB were: Forward – 5' CCC GAT TTA CCA AAC TAG CAG TGG G 3', Reverse – 5' GAG GAC GCC ATA ACA ACT CCA GGG 3'. Quantitative PCR values were normalized using OAZ1 (ornithine decarboxylase antizyme 1) based on the paper by (de Jonge et al., 2007) who had reported that OAZ1 showed very stable expression in the mouse based on their analyses of 2,543 tissue samples hybridized to Affymetrix Mouse GeneChips after exposure to various experimental manipulations. The results are reported as relative changes calculated as the ratios of normalized gene expression data of each group compared to the SS group.

Statistical Analysis

Statistical analysis was performed using analysis of variance (ANOVA) followed by post-hoc analyses (StatView 4.02, SAS Institute, Cary, NC). Values are shown as means $\pm$ SEM. The null hypothesis was rejected at $p < 0.05$.

Results

Figure 1 shows the effects of METH on the mRNA levels for members of the fos family of transcription factors (Milde-Langosch, 2005). As expected, the acute injection of METH (5 mg/kg) to the saline-pretreated animals (SM) caused substantial increases (5.89-fold, $p < 0.0001$; Fig. 1a) in c-fos expression. Chronic administration of METH caused significant decreases in c-fos expression (MS) which reached 41% of the SS group ($p < 0.0254$). The acute METH challenge in the METH-pretreated (MM group) rats caused normalization of c-fos expression. The effects of METH on fosB expression are shown in figure 1b. The acute METH challenge caused significant increases (5.09-fold, $p < 0.0001$) in fosB mRNA levels in the saline-pretreated rats. Chronic METH caused 43% decreases ($p < 0.0096$) in fosB expression. METH-pretreated animals that received the acute METH challenge showed normalization in fosB expression. Interestingly, neither acute nor chronic METH treatment caused any significant changes in fra1 mRNA levels (Fig. 1c). There were, however, significant acute METH-induced increases (6.7-fold, $p < 0.0001$; Fig. 1d) in fra2 expression in saline-pretreated rats. Chronic METH administration caused significant reduction (–45%, $p < 0.0198$) in Fra2 mRNA levels which were also normalized by the acute METH challenge (MM group in Fig. 1d).

Figure 2 illustrates the effects of METH on the expression of the jun members of activator protein 1 (AP1) family of IEGs (Shaulian, 2010). The acute METH challenge did not cause any changes in c-jun mRNA levels in saline-pretreated rats (Fig. 2a). However, chronic METH injections caused marked reduction (–61%, $p < 0.0174$) in c-jun expression which was not impacted by the acute injection of METH, with c-jun mRNA levels being comparable in the MS and MM groups (Fig. 2a). The acute METH injection caused significant increases (2.35-fold, $p < 0.0291$; Fig. 2b) in junB expression in the saline-pretreated rats. Chronic METH caused decreases (–65%, $p < 0.0088$) in junB mRNA levels in comparison to the control group. Acute METH administration to the METH-pretreated rats caused almost complete normalization (up to 81% of control). Acute injection of METH did not cause any significant changes in junD mRNA levels (Fig. 2c). However, repeated

injections of METH caused substantial decreases (−64%, $p < 0.0032$) in comparison to the SS group. The acute METH challenge to the METH-treated rats caused small changes in this group which was up to 61% of the SS group).

The effects of METH on the early growth response (Egr) family of IEGs (O'Donovan et al., 1999) are shown in Figure 3. The METH injection caused significant increases (1.9-fold, $p < 0.0001$; Fig. 3a) in Egr1 expression in the striatum. In contrast, chronic METH caused significant decreases (−54%, $p < 0.0074$) in Egr1 in comparison to the SS group. Injection of METH to rats chronically pretreated with METH caused only small changes in Egr1 expression although the values in the MM group were no longer significantly different (67% of control, $p = 0.3245$) from the control group. The acute METH challenge also caused marked increases (5.2-fold, $p < 0.0001$) in Egr2 expression (Fig. 3b). Repeated injections of METH reduced Egr2 expression (−55%, $p < 0.0148$) in comparison to the control group. In contrast to the results for Egr1, the METH challenge caused complete normalization of the expression values in the MM group in comparison to the SS group. The METH challenge also caused increases (1.7-fold, $p < 0.0130$; Fig. 3c) in Egr3 expression in the striatum of animals pretreated with saline. Chronic METH caused significant decreases (−52%, $p < 0.0041$) in Egr3 mRNA levels in comparison to the SS group. The acute METH challenge had small effects on Egr3 mRNA levels in the MM group which increased to 65% of control. We also measured the effects of acute and chronic METH administration on the expression of NGFI-A-binding protein 2 (NAB2) because it is an Egr-inducible Egr co-repressor (Kumbrink et al., 2005; Svaren et al., 1996). Acute administration of METH caused no changes in NAB2 mRNA levels (Fig. 3d). However, chronic METH injections caused significant decreases (−63%, $p < 0.0008$; Fig. 3d) in NAB2 expression in comparison to the control group. Animals chronically treated with METH and challenged with METH also showed marked decreases (−67%, $p < 0.0055$) in NAB2 mRNA levels.

Figure 4 shows the effects of METH on the members of the nuclear receptor subfamily 4, group A (Nr4a) family of transcription factors (Hsu et al., 2004). Acute METH caused significant increases in Nr4a1 (2.0-fold, $p < 0.0036$; Fig. 4a) in comparison to the SS group. There were significant decreases (−70%, $p < 0.0102$) in Nr4a1 mRNA levels in the MS group. METH administration to the METH-pretreated animals caused normalization of Nr4a1 expression in the MM group. Figure 4b shows that the acute METH injection did not cause any significant changes in Nr4a2 expression in the saline-pretreated rats. Chronic METH did not significantly reduce Nr4a2 expression in the MS group. However, the acute METH challenge did cause significant reduction (−76%, $p < 0.0281$) of Nr4a2 expression in the METH-pretreated rats in comparison to the SS group. Acute METH administration caused significant increases (6.7-fold, $p < 0.0001$; Fig. 4c) in Nr4a3 mRNA levels. Chronic METH did not cause any significant changes in Nr4a3 expression whereas the acute METH challenge did cause significant but attenuated increases in Nr4a3 expression (3.6-fold, $p < 0.008$) in the MM group.

Figure 5 illustrates the effects of acute and chronic METH administration on cAMP responsive element binding protein (CREB) (Lonze and Ginty, 2002) and activity regulated cytoskeleton protein (Arc) (Bramham et al., 2008) mRNA levels. Because IEG expression is modulated by CREB via its binding to a cAMP response element (CRE), a *cis*-acting enhancer element on the promoter of several genes (Impey et al., 2004; Konradi et al., 1994; Lemberger et al., 2008), we tested the possibility that chronic METH treatment might affect CREB expression in a fashion similar to the effects of the drug on IEG expression. As shown in Figure 5a, the acute METH injection caused small decreases (−24%, $p < 0.0291$; Fig. 5a) in CREB mRNA levels. Repeated METH injections caused somewhat larger decreases (−43%, $p < 0.0268$; Fig. 5a) in CREB mRNA levels. Unexpectedly, the

chronically METH-treated rats that received the acute METH injections exhibited even greater decreases (-72% , $p < 0.0001$) in CREB mRNA levels.

We also quantified the expression of the effector IEG, Arc (Link et al., 1995; Lyford et al., 1995) because of its potential involvement in METH-induced synaptic plasticity. The acute METH challenge caused significant increases (4-fold, $p < 0.0001$; Fig. 5b) in Arc mRNA levels. Chronic METH administration alone did not cause any significant changes in Arc expression in the rat striatum but blunted the effects of the acute METH injection on striatal Arc expression (2-fold, $p < 0.0148$) in comparison to the observations in saline-pretreated animals (Fig. 5b).

We also measured the effects of acute and chronic METH administration on preprotachykinin (Tac1) (Almeida et al., 2004) and prodynorphin (Pdyn) (Schwarzer, 2009) mRNA levels because of their reported acute induction by METH (Bannon et al., 1987; Wang et al., 1995) and because of the presence of AP1/CRE binding sites on their promoters (Calin-Jageman et al., 2006; Messersmith et al., 1996). Figure 6a shows that the acute METH injection caused significant increases in Tac1 (~ 1.5 -fold, $p < 0.0267$). In contrast, chronic administration of METH caused significant decreases (-67% , $p < 0.0024$) in Tac1 expression. METH-pretreated animals challenged with the acute single METH injection showed further decreases (-82% , $p < 0.0006$) in Tac1 expression. In contrast to the effects on Tac1 expression, neither acute nor chronic administration of METH caused any changes in Pdyn mRNA levels (Fig. 6b).

Discussion

The main findings of this paper are that: (1) acute METH caused increases in several IEG mRNA levels; (2) chronic METH administration caused decreases in the baseline expression of several IEGs including members of the AP1, Egr, and Nr4a families of transcription factors; (3) chronic METH exposure blunts the acute stimulatory effects of METH on IEG gene expression; (4) repeated METH injections cause significant decreases in CREB mRNA levels which were potentiated by an acute METH injection; and (5) chronic METH exposure caused decreases in Tac1, but not in Pdyn, mRNA levels. Our paper extends previous data on the effects of chronic cocaine and amphetamine on IEG expression by reporting the effects of chronic METH exposure on several additional IEGs. The effects of acute METH injections have been well described and include induction of several IEGs in several brain regions (Cadet et al., 2001; Cadet et al., 2009a; Jayanthi et al., 2009; Thomas et al., 2004). Specifically, a single METH injection caused significant increases in c-fos mRNA in the rat striatum (Wang et al., 1995). Acute injection of METH also induces Nr4a1 which co-localizes with c-Fos immunoreactivity (Backman and Morales, 2002). Our present results are consistent with those previous observations. We found, in addition, that the acute METH injection caused increases in fra2 but not in fra1 mRNA levels. These observations are consistent with our previous report that repeated injections of METH (10 mg/kg, given 4 times at 2h intervals) also caused marked increases in fra2 but not fra1 transcripts (Beauvais et al., 2010). These findings are also consistent with those of a previous paper that demonstrated that binge administration of METH also caused increases in FRA2 protein in the mouse striatum (Pennypacker et al., 2000). Our previous study had suggested that the effects of METH on fra2 mRNA levels are mediated by stimulation of DA D1 receptors since pretreatment of the animals with the DA D1 antagonist blocked the increases in fra2 mRNA levels (Beauvais et al., 2010). Although the molecular mechanisms involved in the differential expression of fra1 and fra2 transcripts remain to be clarified, our present findings are consistent with the demonstration of differential expression of these genes in other models (Briese et al., 2005). Our results are also consistent with the fact that AP1

subunits play different regulatory roles in cellular proliferation, differentiation, and apoptosis (Ameyar et al., 2003; Hess et al., 2004; Shaulian and Karin, 2001).

The METH-induced increases in IEG expression are dependent on stimulation of dopamine (Beauvais et al., 2010; Cadet et al., 2010; Wang and McGinty, 1995) and glutamate (Gross and Marshall, 2009; Wang and McGinty, 1995) receptors. Injections of other indirect DA agonists, including cocaine and amphetamine, also cause increases in IEG expression in the striatum (Bertran-Gonzalez et al., 2008; Graybiel et al., 1990; Moratalla et al., 1992), with these increases being also mediated by stimulation of DA and glutamate receptors (Konradi et al., 1996; Torres and Rivier, 1993; Wang et al., 1994).

In contrast to their acute effects, chronic treatment with indirect DA agonists caused substantial decreases in the expression of several IEGs in DA-rich brain regions (Harlan and Garcia, 1998; Zahm et al., 2010) although little information had existed so far on the effects of chronic METH administration. For example, acute cocaine injections caused marked increases in c-fos, fosB, junB, and Egr1 mRNA levels in the nucleus accumbens but these acute cocaine-induced increases were no longer observed in animals pretreated with cocaine for several days (Hope et al., 1992). The basal levels for c-fos after chronic cocaine were reported to be comparable to those measured in the control animals although there was no mention of the baseline expression of other IEGs. Steiner and Gerfen (1993) had also reported that the effects of acute cocaine on c-fos mRNA in the striatum were inhibited after chronic administration of cocaine (15 mg/kg given twice daily). The authors also reported that the basal levels in rats chronically exposed to cocaine were comparable to those of control rats. Similarly, Couceyro and colleagues (1994) injected a single dose of cocaine (20 mg/kg) or cocaine for 12 days and found that striatal c-fos mRNA levels were induced after the single injection but unchanged after the chronic cocaine injections. Moratalla and others (1996) also reported blunting of acute cocaine-induced changes in c-Fos and c-Jun proteins in the striata of rats chronically exposed to cocaine. Similar to our present findings with chronic METH treatment, other investigators had reported that chronic cocaine administration can cause decreases in c-fos and Egr1 mRNA levels in comparison to baseline values (Bhat et al., 1992; Ennulat et al., 1994). The observed decreases in striatal IEG transcripts after cocaine are also consistent with reports that chronic amphetamine can also reduce the expression of c-fos, c-jun, junB, and Egr1 mRNA levels (Cole et al., 1995; Konradi et al., 1996). Thus, when taken together with our present observations that chronic METH can cause reduction in the basal levels of several IEGs while blunting their responses to an acute METH challenge, the accumulated evidence indicates that psychostimulant drugs can cause transcriptional adaptations in brain regions that receive extensive dopaminergic projections (Bjorklund and Dunnett, 2007). The manner by which these molecular adaptations occur probably involves important changes in the complex regulation of the expression of these IEGs. For example, the c-fos gene and other IEGs are inducible by an array of stimuli which include trophic factors, seizure activity, and DA agonists (Herdegen and Leah, 1998; Hughes et al., 1999; Treisman, 1995). Their acute induction appears to be mediated, in part, by activation of the adenylate cyclase/cAMP/PKA/CREB pathway (Changelian et al., 1989; Fisch et al., 1989; Gilman et al., 1986; Hamid et al., 2008; Konradi et al., 1994; Richards, 2001; Russell et al., 2003; Sassone-Corsi et al., 1988). Thus, the chronic drug-induced repression of IEG expression suggests possible alterations in common mechanisms that regulate basal IEG mRNA levels while also blunting their responsiveness to an acute METH challenge after chronic exposure to the drug. Because the cAMP/PKA/CREB-dependent pathway is involved in the acute effects of dopamine agonists such as amphetamine and cocaine (Konradi et al., 1994; Lemberger et al., 2008), we measured CREB mRNA levels and found that chronic METH also caused reduction of CREB expression which was further potentiated by the acute METH challenge (see Figure 5). The observations of chronic METH-induced decreases in CREB expression suggest that low

CREB levels might have played a role in the decreases in IEG expression found in the rats chronically exposed to the drug. To wit, CREB down-regulation might have led to the observed suppression of baseline IEG expression as well as the blunted acute METH-induced responses by decreasing the available pool of phosphorylated CREB that can bind the promoters of target genes such as the IEGs and Tac1. This idea is consistent with the report that cocaine did not induce IEGs in the striatum of mice lacking CREB and CREM (Lemberger et al., 2008). Our suggestion is also consistent with the demonstration that chronic administration of antidepressant drugs induces upregulation of CREB (Nibuya et al., 1996) which is associated with increased CRE-mediated gene expression in the brain (Thome et al., 2002) in contrast to the decreases in IEG expression observed in the present study. Nevertheless, because gene repression is known to be modulated by chromatin remodeling, changes in repressor proteins, and recruitment of co-repressors (Ballas and Mandel, 2005; Rojo, 2001; Wolffe et al., 2000), our present observations suggest that chronic METH administration might have also influenced these processes in the rat striatum. This suggestion is consistent with the recent report that chronic amphetamine-induced blunting of acute effects of the drug on c-fos expression is mediated by epigenetic desensitization due to accumulation of delta FosB (Renthal et al., 2008), a splice variant of FosB. Delta FosB can act as an activator or repressor of the expression of several genes implicated in drug addiction (Kelz et al., 1999; Nestler, 2008).

Long-term neuroadaptations after administration of psychostimulants are thought to play a role in the mediation of their addictive properties (Cadet, 2009; Dietz et al., 2009; White and Kalivas, 1998). Although the emphasis in these discussions has been mostly on the nucleus accumbens, it is clear that other brain regions, including the dorsal striatum, participate actively in the habitual manifestations of addictive disorders (Bardo, 1998; Everitt et al., 2008; Zahm et al., 2010). Therefore, our findings that chronic METH exposure can cause substantial decreases in striatal IEG expression and in their responsiveness to acute drug administration support the idea that addictive drugs can alter molecular dynamics via drug-induced transcriptional alterations in the brain (Cadet, 2009; Nestler, 2008). Indeed, this study suggests that METH-induced transcriptional alterations might involve multiple classes of transcription factors with potentially widespread consequences on striatal functions because these IEGs influence the expression of many overlapping and non-overlapping molecular pathways (Herdegen and Leah, 1998; Hughes et al., 1999; Impey et al., 2004). Interestingly, acute METH administration caused almost complete normalization of some IEG mRNA levels including c-fos, fosB, Fra2, and Egr2 in chronically METH-treated rats (MM group) that had exhibited low baseline expression when challenged with saline (compare MS to SS group). The normalization of IEG expression in these chronically treated animals by an acute METH injection is compatible with the observation that chronic METH administration is associated with corticostriatal depression that can be reversed by re-administration of the drug (Bamford et al., 2008).

The acute METH-induced stimulatory responses in Arc expression and the blunted responses to an acute METH injection after chronic METH pretreatment are also of interest. Since its initial discovery (Link et al., 1995; Lyford et al., 1995), Arc, an effector IEG, has been reported to play important roles in long-term potentiation, long-term depression, and memory formation (Bramham et al., 2008; Tzingounis and Nicoll, 2006). The acute METH-induced increases suggest that Arc might participate in acute plastic effects of the drug. However, the blunted METH-mediated responses observed after chronic METH exposure suggests the possibility that the chronic administration of the drug might have exerted negative influences on neuronal networks that regulate Arc-dependent synaptic processes. A similar argument could be made for the METH-induced structural changes in the brain (Jedynak et al., 2007). Nevertheless, our results suggest that chronic METH administration

results in alterations in transcriptional networks that might be at the core of the long-term pathological changes that occur in the addicted brain.

In order to test for potential consequences of the chronic METH-induced decreases in IEG expression, we also measured the expression of Tac1 and Pdyn because they had been reported to be induced acutely by METH (Adams et al., 2003; Bannon et al., 1987; Wang et al., 1995). Our present observations of METH-induced increases in Tac1 are consistent those of Bannon and colleagues (1987). However, we did not replicate the data on METH-induced Pdyn (Wang et al., 1995), possibly, because of the dose (5 mg/kg) used in our study since others have reported dose-dependent effects of METH with 15 mg/kg, but not 2 mg/kg, of the drug causing increases in Pdyn mRNA levels (Adams et al., 2003). The decreases in Tac1 mRNA expression observed after chronic METH are consistent with a previous report that toxic doses of METH can cause prolonged decreases in Tac1 (Johnson-Davis et al., 2002). However, since we did not use toxic doses in our study, the molecular processes that lead to the similar end-points in the two studies might actually be dichotomous. It is also of interest to note that the patterns of changes in Tac1 after chronic METH parallel those of CREB expression since both exhibited further decreases in expression when METH-pretreated rats were acutely challenged with an acute METH injection (compare Fig. 5a to 6a). These findings suggest an important role of CREB in regulating Tac1 expression. It was unexpected that chronic METH exposure could have resulted in such differential responses in Tac1 and Pdyn mRNA levels since both of their promoters contain AP1/CRE sites that bind members of the AP1 family of IEGs (Calin-Jageman et al., 2006; Cole et al., 1995; Messersmith et al., 1994; 1996; 1998). We had predicted that both Tac1 and Pdyn would show similar changes in expression after chronic METH exposure because they are localized in the same striatal neurons (Angulo et al., 1995) and because previous data suggested that they are both induced by METH (Bannon et al., 1987; Wang et al., 1995). Our present findings, although unexpected, thus, point to the possible existence of factors, other than AP1 transcription factors (Bronstein et al., 1996), which might play more essential roles in regulating Pdyn expression. Some of these factors might include differential chromatin modifications and/or accumulation of repressors and co-repressors (Ballas and Mandel, 2005; Rojo, 2001; Wolffe et al., 2000) on Tac1 and Pdyn promoters during chronic METH exposure.

In conclusion, an acute METH injection induces significant increases in IEG expression in the rat striatum. However, chronic exposure to the drug causes substantial decreases in the baseline expression of several IEGs including members of the AP1, Egr, and Nr4a families of transcription factors. Moreover, the acute METH-induced stimulatory effects on IEG expression were significantly blunted after chronic METH administration. Because these IEGs regulate the expression of many genes, it is possible that these chronic drug effects might trigger critical transcriptional responses that drive compensatory neuroadaptations. These molecular alterations might also serve to promote drug dependence and addiction that are characterized by periods of repeated and escalating use of drugs (Koob and Kreek, 2007). The repeated use of the drug, which might serve to recalibrate certain functions acutely, might also trigger a vicious cycle of further IEG suppression. Thus, further studies of the specific roles of individual genes will be necessary in order to better understand the varied molecular and cellular processes involved in METH-induced neuroadaptations. There is also a need to study the modifying effects of these IEGs on the regulation of target gene batteries that might work in concert to generate the phenotypic manifestations that constitute drug addiction. Molecular dissection of these pathways should allow for the development of therapies aimed at restoring the cognitive capacity of the addicted brain via normalization of the brain's transcriptional plasticity.

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Fig. 1. Effects of chronic METH treatment on the expression of fos members of AP1 family of IEGs

The rats were treated with saline or METH for two weeks. After a day of withdrawal, rats from both groups were given a single injection of either saline or METH (5 mg/kg). The animals were euthanized two hrs later. Total RNA was extracted from the striatum and used in quantitative PCR assays. The relative amounts of messenger RNA were normalized to OAZ1 (ornithine decarboxylase antizyme 1). Values from the four groups were compared by analysis of variance (ANOVA) followed by post-hoc analyses when ANOVA was significant. Key to statistics: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$, in comparison to the SS group; ### = $p < 0.001$, in comparison to the SM group.

Fig. 2. Effects of chronic METH treatment on the expression of jun members of AP1 family of IEGs

The rats were treated with saline or METH for two weeks as described in the text and table 1. Statistical analyses are described in figure 1 and in the text. Key to statistics: * = $p < 0.05$; ** = $p < 0.01$, in comparison to the SS group; # = $p < 0.05$; ## = $p < 0.01$; in comparison to the SM group.

Fig. 3. Effects of chronic METH treatment on the expression of Egr family members of IEGs
 The rats were treated with saline or METH for two weeks as described in the table. RNA extraction, PCR, and statistical analyses are documented in the text. Key to statistics: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$, in comparison to the SS group; ## = $p < 0.01$; ### = $p < 0.001$, in comparison to the SM group.

Fig. 4. Effects of chronic METH treatment on the expression of Nr4a family members of IEGs
The rats were treated with saline or METH for two weeks as described in the table. RNA extraction, PCR, and statistical analyses are documented in the text. Key to statistics: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$, in comparison to the SS group; # = $p < 0.05$; ## = $p < 0.01$; ### = $p < 0.001$, in comparison to the SM group; \$\$\$ = $p < 0.01$ in comparison to the MS group.

Fig. 5. Effects of chronic METH treatment on the expression of (a) Creb and (b) Arc mRNA levels

The rats were treated as described in the text. RNA, RT-PCR assays, and statistical analyses were described in the methods section and in figure 1. Key to statistics: * = $p < 0.05$; *** = $p < 0.001$, in comparison to the SS group; ### = $p < 0.001$, in comparison to the SM group; \$\$ = $p < 0.01$ in comparison to the MS group.

Fig. 6. Effects of chronic METH treatment on the expression of (a) Tac1 and (b) Pdyn mRNA levels

The rats were treated as described in the text. RNA, RT-PCR assays, and statistical analyses were described in the methods section and in figure 1. Key to statistics: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$, in comparison to the SS group; ### = $p < 0.001$, in comparison to the SM group.

Table 1

Summary of studies showing the acute and chronic effects of cocaine on IEG expressions.

Drug treatment regimen	Tissues	IEGs	Reference
Acute cocaine (25 mg/kg).	CPu, NAc	c-fos CPu mRNA c-Fos CPu and NAc protein	Graybiel et al., 1990
Chronic saline or cocaine (15 mg/kg, 2 times/day, for 5 days) followed by acute cocaine (15 mg/kg).	CPu, Ctx	Acute: Egr1 mRNA Chronic: blunted response	Bhat et al., 1992
Chronic saline or cocaine (15 mg/kg, 2 times/day, for 14 days) followed by acute cocaine (15 mg/kg).	NAc	Acute: c-fos, fosB, c-jun, junB, and Egr1 mRNAs Chronic: blunted responses	Hope et al., 1992
Chronic saline or cocaine (15 or 30 mg/kg, 2 times/day, for 4 days) followed by acute cocaine (30 mg/kg).	CPu	Acute: c-fos mRNA Chronic: blunted response	Steiner and Gerfen., 1993
Chronic saline or cocaine (6 mg/kg, 3 times/day, for 4 days) followed by acute cocaine (6 mg/kg).	CPu	Acute: c-fos and Egr1 mRNAs Chronic: blunted responses	Ennulat et al., 1994
Chronic saline or cocaine (25 mg/kg, 2 times/day, for 7 days) followed by acute cocaine (25 mg/kg).	CPu	Acute: c-Fos, Fra, and JunB proteins Chronic: c-Fos and JunB proteins	Moratalla et al., 1996
Cocaine self-administration (500µg/kg/nose poke, 2h/session/day) Acute, 1 session; Chronic, 6 sessions.	CPu	Acute: c-Fos protein Chronic: c-Fos protein	Zahm et al., 2010

Abbreviations: CPu, caudate-putamen; Ctx, cortex; NAc, nucleus accumbens

Table 2

Summary of studies showing the acute and chronic effects of AMPH on IEG expressions.

Drug treatment regimen	Tissues	IEGs	Reference
Acute AMPH (5 or 10 mg/kg).	CPu, NAc	c-Fos protein	Graybiel et al., 1990
Acute AMPH (0.5 - 15 mg/kg).	CPu, NAc	Egr1 mRNA	Moratalla et al., 1992
Chronic saline or AMPH (7.5 mg/kg, 2 times/day, for 14 days) followed by acute AMPH (7.5 mg/kg).	CPu, Ctx	Acute: c-fos, fosB, Fra1, c-jun, junB, and Egr1 mRNAs Chronic: blunted responses	Persico et al., 1993
Chronic saline or AMPH (5 mg/kg, 1 time/day, for 13 days) followed by acute AMPH (5 mg/kg).	CPu	Acute: c-Fos protein Chronic: blunted response	Jaber et al., 1995
Acute AMPH (5 mg/kg).	CPu, NAc	c-fos and Egr1 mRNAs	Wang et al., 1995
Acute AMPH (4 mg/kg).	CPu, NAc	Egr1 mRNA	Wang and McGinty, 1995
Chronic saline or AMPH (4 mg/kg, 1 time/day, for 11 days) followed by acute AMPH (4 mg/kg).	CPu	Acute: c-fos, c-jun, junB, and Egr1 mRNAs Chronic: blunted responses	Konradi et al., 1996
Acute AMPH (5 mg/kg).	CPu	JunB protein	Moratalla et al., 1996
Chronic saline or AMPH 4 mg/kg, 1 time/day, for 6 days) followed by acute AMPH (4 mg/kg).	CPu	Acute: c-fos mRNA Chronic: blunted response	Renthal et al., 2008

Abbreviations: AMPH, amphetamine; CPu, caudate-putamen; Ctx, cortex; NAc, nucleus accumbens

Table 3

Schedule of chronic METH injections and METH challenge

	Monday	Tuesday	Wednesday	Thursday	Friday
Week 1					
9:00					
10:00	0.5 mg/kg	1 mg/kg	1 mg/kg	1.5 mg/kg	
11:00					
12:00			1 mg/kg	1.5 mg/kg	
13:00					
14:00			1 mg/kg	1.5 mg/kg	
15:00					
16:00	0.5 mg/kg	1 mg/kg	1 mg/kg	1.5 mg/kg	
Week 2					
9:00					
10:00	1 mg/kg	1.5 mg/kg	2 mg/kg	2.5 mg/kg	
11:00					
12:00	1 mg/kg	1.5 mg/kg	2 mg/kg	2.5 mg/kg	
13:00					
14:00	1 mg/kg	1.5 mg/kg	2 mg/kg	2.5 mg/kg	
15:00					
16:00	1 mg/kg	1.5 mg/kg	2 mg/kg	2.5 mg/kg	
Week 3					
		Test Day			
9:00		5 mg/kg			
10:00	3 mg/kg				
11:00					
12:00	3 mg/kg				
13:00					
14:00	3 mg/kg				
15:00					
16:00	3 mg/kg				

Initially, the rats were divided into two groups, with one group receiving saline and the other group receiving chronic METH as described above. On the test day, the saline group received either saline (SS) or a single injection of 5 mg/kg METH (SM), the chronic METH group received either saline (MS) or a single injection of 5 mg/kg METH (MM). Animals were euthanized 2 hours after the acute injections.