Chronic Methamphetamine Causes Differential Expression of Immediate Early Genes in the Nucleus Accumbens and Midbrain of Rats

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Abstract

The present study investigated whether chronic methamphetamine (METH) would suppress METH-induced mRNA expression of immediate early genes (IEGs) in the rat brain. Rats were given METH or saline over two weeks. After an overnight withdrawal, saline- and METH-pretreated rats received an acute saline or METH challenge. The acute METH challenge increased expression of members of activator protein 1 (AP-1) and Nr4a IEG families in the nucleus accumbens (NAc) and midbrain of saline-pretreated rats. Chronic METH exposure attenuated the effects of acute METH challenge on AP-1 IEG expression in the NAc. However, chronic METH failed to attenuate acute METH-induced increases of Nr4a1 and Nr4a3 expression in the NAc. In contrast to observations in the NAc, chronic METH did not prevent acute METH-induced changes in IEG expression in the midbrain. These results suggest that these two brain regions that are implicated in neuroplastic effects of illicit substances might be differentially affected by psychostimulants.

Keywords
dopamine; c-fos; fosB; c-jun; Nurr77; midbrain; nucleus accumbens

1 Introduction

Methamphetamine (METH) abuse poses a major public health burden because of its medical, behavioral, and neuropsychiatric adverse effects [14]. Its acute administration is associated with euphoria, increased attention, and increased physical activity [17,33]. Chronic METH abuse is complicated by addiction, depression, and psychosis [8,29,32]. Moreover, chronic METH users show impairments in psychomotor, cognitive functions, and other neuropsychiatric side-effects [44,47] that are thought to be secondary to changes in brain structure and function [6,49].

METH induces its primary effects through the release of dopamine (DA) in reward-associated brain regions that include the nucleus accumbens (NAc), striatum, and midbrain [3,9,48]. DA release is followed by stimulation of DA receptors and increased expression of immediate early genes (IEGs) [4,5]. Similarly, acute cocaine [13,18] and amphetamine [13,25,39] produced increased IEG expression in the striatum and NAc. Interestingly, repeated injections of cocaine or amphetamine led to the blunting of acute effects of these drugs [18,25,34,39]. Acute injections of METH also cause increases in several members of activator protein 1 (AP-1) and nuclear receptor subfamily 4, group A (Nr4a) IEG families [2,5,22,26]. Similar to the observations with cocaine [34] and amphetamine [39], chronic METH was reported to suppress the acute effects of METH on IEG expression in the dorsal striatum [30].

The IEGs measured in these previous studies code for members of the family of AP-1 transcription factors that include fos and jun [18,25,34,39]. AP-1 transcription factors include c-Fos, FosB, Fra1, Fra2, c-Jun, JunB, and JunD [46]. They have been shown to respond rapidly to various exogenous stimuli [45,46] including administration of psychostimulants [4,21]. These transcription factors regulate transcription by forming Fos-Jun heterodimers or Jun-Jun homodimers that bind to specific DNA sequences [21,45,46]. Interestingly, chronic exposure to psychostimulants is associated with differential accumulation of AP-1 transcription factors [7,19] that have been implicated in the promotion and maintenance of addictive behaviors [36]. The effects of psychostimulants on the expression of the Nr4a IEG family [27] have been less well studied [30]. That family consists of Nr4a1 (Nur77), Nr4a2 (Nurr1), and Nr4a3 (Nor-1) [20,27]. Acute METH was shown to cause increases in their expression in the dorsal striatum whereas those stimulatory effects were blunted by chronic pre-exposure to METH [30].

The present study was conducted to test the regional generalizability of the chronic psychostimulant-induced mRNA blunting phenomena by investigating the effects of acute and chronic METH on the expression of AP-1 [46] and Nr4a [20] IEGs in the NAc and midbrain of rats. Herein, we report that acute METH administration increased the levels of several IEGs in both structures. Similar to the
observations in the striatum, there was a blunting of the acute METH-induced AP-1 IEG responses in the NAc of rats chronically exposed to METH. Surprisingly, only Nr4a3 (Nor-1) showed blunted responses to acute METH challenge after chronic METH exposure in the midbrain.

2 Materials and methods

2.1 Animals

Adult male Sprague-Dawley rats 330–370 g (Charles Rivers Laboratories, Raleigh, NC, USA) were used in the study. Animals were housed in an environmentally 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 NIDA-BRC Animal Care and Use Committee.

2.2 Drug treatment and tissue collection

Following habituation, rats were injected intraperitoneally with either (+)-METH-hydrochloride (NIDA, Baltimore, MD, USA) or an equivalent volume of 0.9% saline for a period of two weeks. METH dosing schedule is shown in Table 1 [30]. Following an overnight withdrawal (~18 h), METH and saline pretreated rats were further divided into two more groups and given a challenge injection. Rats received either METH (5 mg/kg) or equivalent volume of saline for the challenge injection. The various treatments correspond to four groups of rats: (1) saline-pretreated + saline-challenged rats (SS); (2) saline-pretreated + METH-challenged rats (SM); (3) METH-pretreated + saline-challenged rats (MS); (4) METH pretreated + METH-challenged rats (MM). All rats were euthanized by 2 h after the challenge injection. The brains were quickly removed and NAc and midbrain tissues encompassing the substantia nigra and ventral tegmental area were rapidly dissected on ice. Following dissection, tissues were flash frozen on dry ice, and stored at −80 °C until used in quantitative reverse transcriptase-PCR (qRT-PCR).

2.3 qRT-PCR

Total RNA was extracted from the rat NAc and the midbrain region encompassing ventral tegmental area and substantia nigra. Genes of interest were analyzed by qRT-PCR as described previously [30]. In brief, RNA obtained individually from 5–8 rats per group was reverse-transcribed with oligo dT primers and Advantage RT for PCR kit (Clontech, Palo Alto, CA, USA). PCR experiments were performed using BioRad SYBR Green master mix kit (BioRad, Hercules, CA, USA) according to the manufacturer’s protocol. Sequences for gene-specific primers corresponding to PCR targets were obtained using LightCycler Probe Design software (Roche, Indianapolis, IN, USA) and are shown in Table 2. The primers were synthesized and HPLC-purified at the Synthesis and Sequencing Facility of Johns Hopkins University (Baltimore, MD, USA) and were the same ones used previously [30]. PCR values were normalized using ornithine decarboxylase antizyme 1 (OAZ1) for the NAc and clathrin for the midbrain, then mRNA levels were quantified. The results are reported as fold-changes of each group in comparison to the SS group.

3 Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni’s Multiple

### Table 1: Schedule of chronic METH or saline injections.

<table>
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* denotes reference genes.
Figure 1: Effects of acute and chronic METH on the mRNA levels of fos family IEGs. Rats were treated with escalating non-toxic doses of METH or equal volume saline for two weeks prior to acute METH (5 mg/kg). The effects of METH for the NAc ((a), (c), (e), and (g)) and midbrain ((b), (d), (f), and (h)) are shown. The mRNA levels were measured as described in Section 2. Results are presented as means ± SEM of fold changes in comparison to the SS group (n = 5–8 rats per group). Statistical analyses were done by ANOVA followed by Bonferroni post-hoc test. Key to statistics: asterisk, comparison to SS group; number sign, comparison to SM group; dollar sign, comparison to MS group. Single sign, P < .05; double sign, P < .01; triple sign, P < .001.

Comparisons Test post-hoc (GraphPad Prism version 5.00 for MAC GraphPad Software, San Diego, CA, USA). Values are shown as means ± SEM. Criteria for significance were set at P < .05.

4 Results

Figure 1 shows the effects of METH on members of the fos family of IEGs in the NAc and the midbrain. Significant differences in c-fos mRNA levels between treatment groups were observed in the NAc [F(3, 27) = 82.71, P < .0001, Figure 1(a)]. Acute METH (SM) injections significantly increased c-fos mRNA levels (7.5-fold, P < .001) in the NAc compared to chronic saline injected rats (SS). The expression of c-fos was comparable between the MS and SS groups. Chronic METH exposure significantly blunted the acute METH-induced c-fos induction (P < .001) in comparison to the saline group. The c-fos induction was 7.5-fold versus 4-fold for SM and MM groups, respectively. In the midbrain, c-fos mRNA levels also differed significantly between treatment groups [F(3, 26) = 15.63, P = .0015, Figure 1(b)]. Acute METH caused 4.2-fold increases (SM group, P < .001) in c-fos mRNA levels compared to the chronic saline-treated group. There were no significant changes in midbrain c-fos mRNA levels in the MS group compared to the SS group. An acute METH challenge to METH-pretreated rats significantly increased c-fos mRNA levels (~ 4.3-fold, P < .001) compared to the SS and MS groups, with no differences between the SM and MM groups.

As illustrated in Figure 1(c), NAc fosB mRNA levels depended on the treatment group [F(3, 27) = 104.3, P < .0001]. The mRNA levels of fosB increased (4.2-fold, P < .001) in SM-treated rats compared to SS rats. The mRNA levels in MS-treated rats were not significantly different from the SS-treated rats. In contrast, the METH challenge significantly increased fosB mRNA levels in the MM compared to SS-treated rats (~2-fold, P < .001). The METH-induced increases in fosB mRNA levels in the MM group were attenuated by approximately 2-fold (P < .001) when compared to SM-treated rats. Significant variation in midbrain fosB mRNA levels were seen across treatment groups [F(3, 26) = 32.95, P < .0001, Figure 1(d)]. The mRNA levels of fosB increased significantly with acute METH treatment (2.7-fold, P < .001) compared to chronic saline treatment in the midbrain. The mRNA levels for the MS groups were comparable to SS-treated rats. In contrast, the METH challenge caused increases in fosB mRNA levels (P < .001) in METH-pretreated rats; these changes were similar to those observed in the SM group. Fra1 mRNA levels did not differ significantly between treatment groups in the NAc [F(3, 27) = 0.146, P = .93, Figure 1(e)] or the midbrain [F(3, 26) = 0.39, P = .94, Figure 1(f)].

The expression of Fra2 mRNA in the NAc is shown in Figure 1(g). There were significant changes in mRNA levels across treatment groups in the NAc [F(3, 28) = 104.3, P < .0001]. Acute METH challenge significantly increased (5.1-fold, P < .001) Fra2 mRNA levels. There were no significant changes in Fra2 mRNA levels in the MS group.
The mRNA levels of junB were significantly different between treatment groups in the NAc $[F(3,27) = 25.18$, $P < .001$, Figure 2(c)]. The SS and MS groups had similar levels of junB mRNA in the NAc. Acute METH caused significant increases ($2.5-$fold, $P < .001$) in junB mRNA levels in the NAc compared to rats in the SS group. An acute METH challenge also increased junB mRNA levels in the MM group ($1.9-$fold, $P < .001$) in comparison to the SS group, with these increases being blunted in comparison to the SM group. In the midbrain, junB mRNA levels varied according to treatment group $[F(3,26) = 11.61$, $P < .0001$, Figure 2(d)]. JunB mRNA levels increased ($1.7-$fold, $P < .001$) with acute METH injections compared to saline injections. MS rats had similar mRNA levels as the SS rats. The MM rats had mRNA levels that were also increased ($1.9-$fold, $P < .001$) compared to SS rats, with no differences between SM and MM groups.

Figure 3 illustrates the effects of METH injections on the mRNA levels of members of the Nr4a IEG family in the NAc and the midbrain. In the NAc, the treatment group had significant effects on the mRNA levels of Nr4a1 $[F(3,27) = 10.97$, $P = .005$, Figure 3(a)]. Acute METH significantly increased Nr4a1 mRNA levels ($2.1-$fold, $P < .01$) compared to SS-treated rats. Nr4a1 mRNA levels in the MS and SS rats were almost identical. Acute METH challenge also increased Nr4a1 mRNA levels in the NAc of the MM ($1.6-$fold, $P < .001$) compared to the SS group. The METH-induced changes in midbrain Nr4a1 mRNA levels also varied according to treatment group $[F(3,25) = 10.38$, $P < .0001$, Figure 3(b)]. MS-treated rats had similar levels of Nr4a mRNA as the saline-treated rats. Acute METH challenge increased midbrain Nr4a1 mRNA levels ($2.5-$fold, $P < .001$) compared to chronic saline injections. Acute METH challenge caused increases in midbrain Nr4a1 mRNA levels in the MM ($2-$fold, $P < .001$) compared to SS rats.

Figure 3(c) shows significant changes in mRNA levels of Nr4a2 in the NAc $[F(3,27) = 5.57$, $P = .004]$. The SM group had significant increases ($1.9-$fold, $P < .004$) in Nr4a2 mRNA levels compared to SS-treated rats. In contrast, chronic METH caused significant decreases ($50\%$, $P < .05$) in comparison to the SS-treated rats. However, the acute METH challenge after chronic exposure caused normalization of Nr4a2 expression in the MM group.

In the midbrain, we also observed significant changes in Nr4a2 mRNA levels according to treatment group $[F(3,27) = 5.90$, $P = .003$, Figure 3(d)]. An acute METH injection caused small increases ($1.4$ fold, $P < .05$) in Nr4a2 mRNA levels in comparison to SS rats.
Figure 3: Effects of acute and chronic METH on mRNA levels of Nr4A family IEGs. Rats were treated with escalating non-toxic doses of METH or equal volume saline for two weeks prior to acute METH (5 mg/kg). The effects of METH for the NAc ((a), (c), and (e)) and midbrain ((b), (d), and (f)) are shown. The mRNA levels were measured as described in Section 2. Results are presented as mean ± SEM of fold changes in comparison to the SS group (n = 5–8 rats per group). Statistical analyses were done by ANOVA followed by Bonferroni post-hoc test. Key to statistics: asterisk, comparison to SS group; number sign, comparison to SM group; dollar sign, comparison to MS group. Single sign, P < .05; double sign, P < .01; triple sign, P < .001.

MS group had small increases (~1.6-fold, P < .01) in comparison to the SS group. The acute METH challenge did not cause further increases in the midbrain of MM rats which showed similar changes to MS rats (Figure 3(d)).

As shown in Figure 3(e), treatment group significantly affected Nr4a3 mRNA levels [F(3, 27) = 43.19, P < .0001] in the NAc. Although mRNA levels in MS rats were comparable to saline rats, acute METH treatment (SM) caused substantial increases (4.2-fold, P < .001) in Nr4a3 mRNA levels compared to saline-treated rats. An acute METH challenge caused increases in Nr4a3 mRNA levels of the MM group (3.4-fold, P < .001) compared to the SS group. Figure 3(f) shows the significant [F(3, 25) = 29.87, P < .0001] changes in Nr4a3 mRNA levels in the midbrain. Acute METH injection increased Nr4a3 mRNA levels (2.9-fold, P < .001) in comparison to SS-treated rats (Figure 3(f)). Rats in the MS group had midbrain Nr4a3 mRNA levels that were similar to the SS group. The acute METH challenge also caused significant increases in the MM group (~2.2-fold, P < .001) in comparison to the SS group.

5 Discussion

Brain regions associated with the pathological features of addiction include midbrain nuclei and their dopaminergic projections to the NAc, striatum, and cortex [11]. Observations of psychostimulant-induced changes in IEG levels in some of these structures suggest that they participate in molecular events that lead to drug addiction [40,41]. In the current work, we have extended results of previous studies that have documented acute psychostimulant-induced increases in IEG mRNA levels in dopaminergic projection areas [18,30,34,39]. We found that, similar to observations reported in previous studies following repeated exposure to cocaine, amphetamine, or METH [18,30,34,39], prior chronic METH exposure attenuated acute METH-induced increases in the mRNA levels of c-fos, fosB, and c-jun in the NAc.

The fos and jun members of IEGs regulate gene transcription by forming heterodimers or homodimers that bind DNA at specific sites [23,46]. These IEGs are inducible by a variety of stimuli [16,23] that include stimulation of DA and glutamate receptors [2,31]. In the case of DA, DA D1 receptors appear to play a prominent role in inducing IEGs [35,43]. Subsequent to D1 receptor stimulation, there is activation of the cAMP/PKA pathway followed by CREB phosphorylation [37,38]. Phosphorylated CREB binds DNA consensus sequences to promote IEG transcriptional activation [21]. Related to this discussion, CREB is phosphorylated by acute METH-induced activation of the MAPK pathway [15] whereas chronic METH exposure decreased levels of CREB in the dorsal striatum [30]. It is therefore not farfetched to suggest that these decreases might play a role in suppressing acute METH-induced IEGs after chronic METH exposure. It is also to be noted that various combinations of fos and jun proteins have been shown to positively or negatively regulate their own transcription [16,21]. Interestingly, c-fos can also exert negative autoregulation because the c-fos protein can repress its own promoter [24,42].

An important novel finding of the current study is the demonstration that chronic METH pretreatment prior to the acute METH challenge failed to attenuate the acute METH-induced increases in mRNA levels of members of the Nr4a family of transcription factors [27] in the NAc.
These findings are in contrast to previous observations in the dorsal striatum [30]. Specifically, McCoy et al. [30] reported that chronic METH caused significant decreases in Nr4a1 levels, with acute METH challenge causing normalization of Nr4a1 levels. Since the repeated METH injections were identical to those used in the previous study [30], the differences in the two studies are probably due to differential molecular events that might regulate Nr4a responses in the NAc and dorsal striatum [10,12,28,50]. This idea is consistent with the fact that chronic D2 receptor antagonism with haloperidol was shown to increase Nr4a1 in the NAc core but not in the NAc shell, dorsal striatum or cortex [50]. Moreover, blocking of D2 receptors with the selective antagonist, ectoplopride, increased Nr4a1 and Nr4a3 but not Nr4a2 mRNA levels in the ventral tegmental area (VTA) [10]. When taken together, these reports suggest region-specific effects of stimulation of dopamine receptors and the signal cascades that regulate Nr4a family gene transcription and, possibly, their target genes.

Our observations of METH-induced changes in the midbrain are novel in that chronic METH failed to attenuate acute METH-induced increases in midbrain AP-1 IEG mRNA levels. Our results are also consistent, in part with, and extend those reported by others [1]. Specifically, these authors had reported that after 14 days of daily injections of METH (2 × 4 mg/kg) or saline and a further 21 days of withdrawal period, there was no blunting of the acute effects of METH on Nr4a2 expression [1].

In summary, the present work shows that an acute METH challenge increases mRNA levels for members of AP-1 and Nr4a families of IEGs in both the NAc and midbrain of rats. We found, in addition, that chronic METH differentially impacted the mRNA levels of members of AP-1 and Nr4a IEGs in the NAc and midbrain. Prior chronic METH exposure also differentially influenced IEG responses to an acute METH challenge. Therefore, the possibility exists that chronic METH-induced epigenetic changes in the NAc and midbrain might be responsible for these varied observations. Future studies will thus need to identify regional epigenetic changes that occur after chronic drug exposure.

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