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## Differential effects of methamphetamine and SCH23390 on the expression of members of IEG families of transcription factors in the rat striatum

Genevieve Beauvais<sup>1,2</sup>, Subramaniam Jayanthi<sup>1</sup>, Michael T. McCoy<sup>1</sup>, Bruce Ladenheim<sup>1</sup>, and Jean Lud Cadet<sup>1,\*</sup>

<sup>1</sup>Molecular Neuropsychiatry Research Branch, NIH/NIDA Intramural Research Program, 251 Bayview Boulevard, Baltimore, MD 21224.

<sup>2</sup>University Paris Descartes, 4 Avenue de l'Observation, 75006 Paris France.

### Abstract

Methamphetamine (METH) is a psychostimulant that can cause long-lasting neurodegenerative effects in humans and animals. These toxic effects appear to occur, in part, via activation of dopamine (DA) D1 receptors. This paper assessed the possibility that the DA D1 receptor antagonist, SCH23390, might inhibit METH-induced changes in the expression of several members of immediate early genes (IEGs) which are known to control more delayed expression of other genes. We found that injections of METH (4×10 mg/kg, given at 2 hr intervals) caused significant increases in *c-fos* and *fra-2* expression which lasted from 30 min to 4 hr. Pre-treatment with SCH23390, given 30 min before each METH injection, completely blocked METH-induced expression of *c-fos*, but only partially inhibited *fra-2* mRNA expression. These results were confirmed by western blot analysis which showed METH-induced changes in c-Fos protein expression that were blocked by pretreatment with SCH23390. There were also delayed METH-induced DA D1 receptor-dependent effects on *fosB* mRNA expression. Even though *fra-1* expression was not affected by pretreatment with METH alone, the repeated injections of SCH23390 caused substantial decreases in *fra-1* mRNA expression in both the presence and absence of METH. The repeated injections of METH caused no changes in the mRNAs for *c-jun*, *junB* or *junD*. However, there were significant increases in the phosphorylation of c-Jun protein (ser63). Phosphorylation of c-Jun occurred in a delayed fashion (16 and 24 hours after the last METH injections) and was attenuated by SCH23390 pretreatment. Interestingly, SCH23390 given alone caused significant decreases in phospho-c-Jun at all time-points. The METH injections also caused delayed induction in the expression of members of the Egr family of transcription factors in a DA D1 receptor-dependent fashion. Repeated injections of SCH23390 caused substantial suppression of basal striatal *egr-1* and *egr-2* mRNA expression but not of that of *egr-3*. Both *crem* and *arc* mRNA levels were induced by METH in a SCH23390-sensitive fashion. Moreover, multiple injections of SCH23390 given alone caused marked inhibition of basal *arc* expression. These results show that multiple injections of METH can differentially affect the expression of several IEGs, some of which occurred in a DA D1 receptor dependent fashion. The SCH23390-mediated suppression of basal *fra-1*, *egr-1*, and *egr-2* mRNA levels suggests that their basal expression in the striatum might be dependent on tonic stimulation of the DA D1 receptor.

\*Corresponding Author: Jean Lud Cadet, M.D. Molecular Neuropsychiatry Research Branch DHHS/NIH/NIDA IRP 251 Bayview Boulevard Baltimore, MD 21224 (443) 740-2656 (Phone) (443) 740-2856 (Fax) jcadet@intra.nida.nih.gov.

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## Keywords

IEG; RT-PCR; SCH23390; signal transduction

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## Introduction

Methamphetamine (METH) is an addictive drug that causes long-term motor and cognitive abnormalities (Volkow et al., 2001; Gold et al., 2009). Imaging studies using positron emission tomography and post-mortem studies of the brains of METH-abusers have provided evidence for loss of striatal dopamine (DA) nerve terminals (Wilson et al., 1996; Volkow et al., 2001). Studies in rodents have shown that administration of toxic doses of METH, either as single or multiple injections, results in long-term changes in the brain (Krasnova and Cadet, 2009). These abnormalities include decreases in the levels of striatal DA and serotonin (5-HT) and of their metabolites as well as decreases in the activity of their biosynthetic enzymes (Kita et al., 2003). The use of toxic doses of METH can also cause neuronal apoptosis in the striata and cortices of rodents (Deng et al., 2002; Jayanthi et al., 2005; Thiriet et al., 2005; Cadet et al., 2005).

The pharmacological and neurotoxic effects of METH depend on the release of DA from striatal DA terminals and stimulation of DA receptors in the striatum which contains high densities of DA D1 and D2 receptors (Russell et al., 1992, O'Dell et al., 1991, 1993). The toxic effects of the drug depend, in part, on activation of D1 receptors because inhibition of DA D1 receptors protects against METH-induced toxicity in the striatum (Jayanthi et al., 2005; Xu et al., 2005). These protective effects of DA D1 antagonism might occur through inhibition of DA D1 receptor-mediated induction of genes that might be involved in pro-toxic cascades (Cadet et al., 2005; Jayanthi et al., 2005, 2009). To test the idea, we measured the effects of multiple METH injections on the expression of immediate early genes (IEGs), including several members of AP-1 and Egr families of transcription factors, in the absence and presence of the DA D1 receptor antagonist, SCH23390 (Iorio et al., 1983). We also measured the effects of SCH23390 on METH-induced changes in arc and crem mRNA levels because Arc is an effector gene involved in synaptic plasticity (Bramham et al., 2008) and because Crem participates in the regulation of neuroadaptive processes (Hughes and Dragunow, 1995). Our experiments show that multiple injections of METH doses which are known to cause long-term DA depletion and neuronal apoptosis (Deng et al., 1999; Ladenheim et al., 2000; Jayanthi et al., 2005) are associated with marked increases in the expression of several IEGs in a SCH23390-sensitive fashion.

## 2. Experimental procedures

### 2.1. Animals and Drug Treatment

Male Sprague-Dawley rats (Charles River Labs, Raleigh, NC, USA), weighing 250-300g were maintained in a room at temperature of 22 °C and had free access to food and water. They were divided into four groups of animals: [i) saline (C), ii) SCH23390 (0.5 mg/kg × 4 injections every 2 hr) (S), iii) METH (10 mg/kg × 4 injections every 2 hr) (M), and iv) METH plus SCH23390 (M + S)]. All injections were given intraperitoneally. The SCH23390 injections were given 30 min before each METH injection. For quantitative PCR, rats were euthanized at 30 min, 2, and 4 hr after drug injections. For Western blot analysis, animals were euthanized at 30 min, 2, 4, 16, and 24 hr after the last drug injection. All animal use procedures were according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care Committee.

## 2.2. Isolation of RNA

After animals were euthanized, striatal tissues were rapidly dissected, placed on dry ice and stored at  $-80^{\circ}\text{C}$  ( $n = 6$  per group). Total RNA for the 30 min, 2 hr and 4 hr samples was extracted with Qiagen RNeasy Midi kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Analysis of samples for quality and quantity was assessed using an Agilent 2100 Bioanalyzer 2 (Agilent, Palo Alto, CA, USA).

## 2.3. Reverse transcription and mRNA quantitation

The expression levels of several immediate genes were studied by real-time quantitative polymerase chain reaction (qPCR). Total RNA for each sample was reverse-transcribed using oligo (dT) into cDNA using Advantage RT for PCR kit (Clontech, Mountain View, CA, USA). For PCR amplification of cDNA, primer Sequences for rat were generated by the LightCycler probe design software v. 2.0 (Roche, Indianapolis, IN, USA) and purchased from Synthesis and Sequencing Facility of Johns Hopkins University (Baltimore, MD, USA). The primer list is shown in Table 1. PCR experiments were performed on Lightcycler 480 II (Roche, Indianapolis, IN, USA), using iQ SYBR Green Supermix (BioRad, Hercules, CA, USA). The relative amounts of messenger RNA were normalized to clathrin mRNA and then quantified.

## 2.4. Western Blot

Protein for striatal tissues were homogenized in a buffer containing 320 mM sucrose, 5 mM HEPES, 1  $\mu\text{g}/\text{ml}$  leupeptin, 1  $\mu\text{g}/\text{ml}$  aprotinin, and 1  $\mu\text{g}/\text{ml}$  pepstatin. Homogenates were centrifuged at 5000g for 5 min, and the supernatant fractions were subsequently centrifuged at 30,000g for 30 min. The resulting pellet was resuspended in the sample buffer (62.5 mM Tris-HCl, 10% glycerol, 2% SDS, 0.1% bromophenol blue, and 50 mM dithiothreitol). Protein concentration was quantified with the BCA protein assay kit (Thermo scientific, Rockford, IL, USA). The lysates were denatured in sample buffer at  $100^{\circ}\text{C}$ , and separated by SDS-PAGE. After the proteins were electrophoretically transferred on PVDF membranes, and membrane blocking, primary and secondary antibody incubations, and chemiluminescence reactions were carried out according to the protocol described by individual antibody suppliers. The membranes were incubated with c-Fos, c-Jun (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and phospho-c-Jun (Ser63) (New England Biolabs, Beverly, MA, USA) (1:1000) antibodies, at  $4^{\circ}\text{C}$  overnight. The blots were re-probed with  $\alpha$ -Tubulin antibody (1:4000; Sigma, 2 hr at room temperature). For quantification, the signal intensity was normalized over the signal intensity of  $\alpha$ -Tubulin. Signal intensity was measured densitometrically with LabWorks version 4.5 (BioImaging Systems analysis software, BioImaging System, UVP Inc., Upland, CA, USA).

## 2.5. Statistical Analysis

For analysis of the qPCR data, the values used consist of a ratio of the fluorescence values, normalized to the values of the endogenous gene clathrin. Values represent means  $\pm$  SE (6 animals/ group). The fold changes in gene expression were generated from normalized data from the various in comparison to the control group. Statistical analysis for the q-PCR and western blot data was carried by a one-way ANOVA followed by Fisher's protected least square difference (PLSD) test using StatView (SAS Institute, Cary, NC, USA). The null hypothesis was rejected at  $p < 0.05$ .

### 3. Results

#### 3.1 Multiple injections of METH caused differential changes in the expression of fos and jun families of IEGs

Fig. 1 shows the effects of METH and SCH23390 on members of the fos family of transcription factors. Repeated injections of SCH23390 alone caused no changes in c-fos expression (Fig. 1A). METH injections caused rapid and substantial increases in c-fos expression which were apparent at 30 min and lasted for the 4 hr duration of the study. Injections of saline before each of the four METH injections gave identical results to the injections of METH alone (data not shown). Injections of SCH23390 before each METH administration caused total inhibition of METH-induced c-fos expression (Fig. 1A). mRNA levels were measured according to a standard curve for each gene. We used 6 replicates for each reaction. These reactions yielded a standard curve with a slope of  $-3.3$  and the efficiency value was  $\approx 2$ . The amplification curve for a replicate of c-fos mRNA is shown in Fig. 1B. Similar curves were generated for each gene at all time points and are available on request. The protein level of c-Fos was also assessed and showed also increased at 30 min, 2 hr and 4 hr (Fig. 2). In contrast to the rapid METH-induced changes in c-fos expression, METH caused somewhat more delayed increases in fosB expression occurring at the 4 hr time point (Fig. 1C). Pretreatment of the animals with SCH23390 also blocked the METH effects of fosB expression (Fig. 1C). Unexpectedly, repeated injections of METH caused no changes in fra-1 expression in the rat striatum (Fig. 1D). In contrast, repeated injections of SCH23390 caused significant decreases in fra-1 expression at the 4 hr time point in both the absence and presence of METH, suggesting that the latter effects were due solely to DA D1 receptor antagonism. Fig. 1D shows that the METH-induced effects on fra-2 expression are somewhat similar to those observed for c-fos expression. Specifically, fra-2 expression was rapidly induced by METH, peaked at 2 hr then started to revert towards normal by the 4 hr time point (Fig. 1E). Interestingly, SCH23390 pretreatment blocked the effects of METH on fra-2 expression only at the 30-min time point, suggesting that METH might cause the more delayed increases via mechanisms other than stimulation of DA D1 receptors.

The repeated injections of METH caused no significant changes in the expression of c-jun (Fig. 3A), junB (Fig. 3B) and junD (Fig. 3C). Repeated injections of saline before each METH injection did not affect these results (not shown). Injections of SCH23390 alone caused no significant decreases in basal expression of these genes when compared to controls. As previously reported, c-Jun protein exerts its action via phosphorylation at serine-63 or -73 through the actions of c-Jun N-terminal kinases (JNKs) (Raivich, 2008), a process that seems to be involved in METH-induced toxicity since a single large toxic dose of METH can cause increased c-Jun phosphorylation at ser-63 (Jayanthi et al., 2002). Similarly, repeated injections of METH used in the present study also caused cJun phosphorylation at 16- and 24-hr after the last METH injection (Fig. 4). In contrast, SCH23390 caused significant decreases in the basal levels of phosphorylated c-Jun. These changes are not due to increased c-Jun protein expression since, similar to the PCR results, METH caused no changes in total c-Jun protein expression.

#### 3.2 Effects of toxic doses of METH on egr expression

The effects of METH on members of the Egr family of transcription factors are shown in Fig. 5. Repeated injections of SCH23390 alone caused significant suppression of the basal egr-1 expression (Fig. 5A). SCH23390 caused decreases in basal egr-1 expression at 60 min after the last injection of SCH23390 (30 min after the last METH injection) in both the absence and presence of METH. METH caused significant increases in egr-1 expression at the 4-hr time point. Injections of saline before each METH injection caused identical changes to those observed in the METH alone group (not shown). SCH23390 pretreatment blocked the METH-induced changes. Repeated injections of SCH23390 also suppressed basal egr-2 expression at

all the time points studied. METH caused time-dependent increases in *egr-2* expression which become significant at the 2-hr time point (Fig. 5B). Pretreatment with SCH23390 also blocked METH-mediated increases in *egr-2* expression. Injections of SCH23390 alone caused no significant changes in *egr-3* expression (Fig. 5C). METH caused time-dependent increases in *egr-3* expression. These increases were of greater magnitude than the changes observed in *egr-1* and *egr-2* expression (compare Fig. 5C to 5A and 5B). SCH23390 pretreatment also blocked the METH-induced increases in *egr-3* expression.

### 3.3 Effects of METH and SCH23390 on arc and crem expression in the striatum

Fig. 6 shows the effects of METH and SCH23390 on arc and crem mRNA levels. Administration of SCH23390 alone caused suppression of basal arc mRNA (Fig. 6A). METH injections caused small increases in arc expression at the 30-min and 2-hr time points, with reversal towards normal at the 4-hr time point. SCH23390 pretreatment blocked the METH-induced increases in arc expression (Fig. 6A). Interestingly, the levels of arc mRNA in the groups that got the combined SCH23390 and METH treatments were comparable to the levels observed in the SCH23390 alone groups (compare the S groups to the M+S groups in Fig. 6A).

Repeated injections of SCH23390 had no effects on basal crem expression in comparison to the control group (Fig. 6B). Repeated injections of METH caused increases in crem mRNA levels which were observable at the 30-min time-point and lasted for the duration of the study. Administration of SCH23390 prior to the injections of METH also blocked METH-mediated increases in crem expression (Fig. 6B).

## 4. Discussion

The main findings of this paper are that (i) multiple injections of METH caused substantial increases in the expression of multiple transcription factors in the rat striatum; (ii) the DA D1 receptor antagonist, SCH23390, blocked these METH-induced changes in a time-dependent fashion; and that (iii) repeated injections of SCH23390 can suppress the basal levels of *egr-1*, *egr-2* and arc mRNAs. Unexpectedly, the repeated injections of METH caused no changes in the levels of *c-jun*, *junB* or *junD* mRNAs. The observations of METH-induced increases in IEG mRNA levels and their dependency, for the most part, on stimulation on DA D1 receptors are consistent with the report that four injections of METH, given according to a schedule similar to the one used in the present study, can cause increases in DA efflux that stayed elevated for 16 hours after the last METH injection (O'Dell et al., 1991). The results of the present study are also consonant with and extend those of previous papers that have reported that single injections of METH (40 mg/kg) caused significant increases in *c-fos*, *fosB*, *fra-2*, *c-jun*, *junB* and *junD* in the mouse striatum (Cadet et al., 2001; Jayanthi et al., 2009). We also extended our previous results by showing that the effects of multiple METH injections occur mostly via stimulation of DA D1 receptor. These results are consistent with previous observations that acute administration of cocaine (Berretta et al., 1992) and amphetamine (Wang et al., 1995) can cause increases in a number of IEGs in a DA D1 receptor-dependent fashion.

The early induction of *c-fos* observed within 30 min of the last METH injection was not surprising, given the fact that *c-fos* is an IEG that is activated within minutes of neuronal stimulation (Hughes and Dragunow, 1995). This observation is consistent with those of other investigators who have reported that single injections of variable doses of METH can cause rapid increases in the expression of *c-fos* in the rodent brain (Wang et al., 1995; Thiriet et al., 2001). Because METH toxicity is exacerbated in *c-fos* knock-out mice (Deng et al., 1999), we had proposed previously that METH-induced increases in *c-fos* expression might constitute an attempt for the organism to protect striatal neurons against METH-induced neuronal apoptosis. The observation that the repeated injections of the DA D1 receptor antagonist, SCH23390, can block METH-induced increases in *c-fos* is consistent with observations of other authors who

have reported that cocaine and METH-induced c-fos protein expression occurs via DA D1 receptor-mediated mechanisms (Young et al., 1991, Yoshida et al., 1995). Our observations of METH-induced increases in fosB and fra-2 expression are also consistent with previous demonstrations that cocaine can also affect the expression of other fos-related proteins (Nye et al., 1995) and that METH can cause increases in fosB expression in mice (Cadet et al., 2002). Similar to c-fos, the METH-induced increases in fosB might be involved in a neuroprotective loop since METH toxicity is exacerbated in fosB knockout mice (Kuroda et al., 2009).

Our observations of METH-induced increases in fra-2 expression are consistent with the previous demonstration that administration of METH doses, similar to those used in our study, caused substantial increases in Fra-2 protein in the mouse striatum at 3 days post-drug (Pennypacker et al., 2000). The timing in protein expression reported in the Pennypacker paper was, however, more delayed than the changes in mRNA levels as might be expected. The current study extends those results further by showing that the early but not the delayed effects of METH on fra-2 mRNA are dependent on stimulation of striatal DA D1 receptors. The lack of inhibiting effects of SCH23390 on METH-induced changes on fra-2 at the 2-hr and 4-hr time points suggests that METH might induce fra-2 via other mechanisms which involve oxidative stress (Kranova and Cadet, 2009). When taken together, these results suggest the possibility that several members of the fos family of transcription factors might work in tandem to protect the brain against METH toxicity.

Fos proteins are known partners of Jun members of the AP-1 family of transcription factors (Raivich, 2008). These transcription factors participate in the regulation of neurotransmission via their influence on genes that code for various neurotransmitters (Hughes and Dragunow., 1995). Although our PCR results did not show any effects of METH on the expression of c-jun, junB and junD, we found that repeated injections of METH did cause c-Jun phosphorylation at ser-63 which is an important activating mechanism for that protein (Derijard et al., 1994; Raivich, 2008). We identified significant increases in phospho-c-Jun (Ser63) at the 16- and 24-hr time points, which were dependent on DA D1 receptor stimulation since pretreatment with SCH23390 blocked these changes. The manner by which stimulation of DA D1 receptors can cause phosphorylation of c-Jun might occur via its effects on the cAMP/PKA cascade which is known to interact with the SAPK pathway to activate JNK (Disa et al., 2000; Zhen et al., 1998).

Egr family transcription factors are expressed in several brain regions including the striatum and can be induced in responses to various stimuli to promote memory formation, tolerance to the effects of drugs, and to promote synaptic plasticity (Alberini, 2009). As shown above, SCH23390 treatment alone caused significant decreases in basal egr-1 and egr-2 expression, findings that are consistent with those of Maillieux et al (1992) who had used in situ hybridization technique and reported that SCH23390 can cause significant decreases in basal egr-1 expression in the rat striatum. These observations, taken together, indicate that basal levels of egr-1 and egr-2 mRNA are dependent on baseline stimulation of DA D1 receptors. Our observations that repeated injections of METH caused significant time-dependent increases in egr-1, egr-2 and egr-3 expression are compatible with those of earlier studies that had reported METH-induced egr-1 expression after single injections of METH (Thiriet et al., 2001, Wang et al., 1995). Jayanthi et al (2005) had previously reported that a single large dose of METH can also cause changes in the expression of these IEGs and that they were involved in the activation of the FasL/Fas death pathway in the rat striatum. Our present observations that multiple injections of METH can also result in egr expression are consistent with these results and support the idea that these transcription factors might indeed play a role in mediating METH-induced degeneration of intrinsic striatal cells (Jayanthi et al., 2005). Nevertheless, because these IEGs are known to influence processes that lead to synaptic plasticity and

memory formation (Bozon et al., 2002; Ko et al., 2005, Li et al., 2007), it is possible that these transcription factors might also be involved in METH-induced neuroadaptive changes in the brain. This discussion is also consistent with the fact that Egr-1 and Egr-3, which are induced by METH, can regulate the expression of Arc (Li et al., 2005), an METH-inducible effector gene that is involved in long-term potentiation (LTP) and neuronal plasticity (Bramham et al., 2008). It is thus not farfetched to suggest that the METH-induced increases in striatal arc mRNA expression might play a role in the development of structural plasticity observed after chronic exposure to psychostimulant (Robinson and Kolb., 2004).

The regulation of the expression of several transcription factors that are influenced by direct and indirect DA agonists is mediated, in part, by phosphorylation of the cAMP Response Element Binding (CREB) protein (Hyman et al., 1995, Hughes and Dragunow., 1995) although there are many IEGs whose expression is independent of stimulation of DA D1 receptors (Jayanthi et al., 2009). CREB function is regulated, in part, by ICER, which is an inducible product of the CREM gene (Borlikova and Endo., 2009, Mioduszevska et al., 2003). ICER is a powerful repressor which is important for the transient nature of cAMP- induced gene expression (Mioduszevska et al., 2003). Thus, the observed METH-induced changes in CREM expression might serve to maintain cellular homeostasis by suppressing CREB-mediated gene expression.

In summary, the present data show that administration of METH caused differential changes in the expression of several IEGs, for the most part, in a DA D1 receptor-dependent fashion. The time course of these changes and their dependency, for the most part, on stimulation of DA D1 receptors, are consistent with the fact that repeated injections of METH can cause prolonged elevation (up to 16 hr after the last METH injection) of the levels of DA in the extracellular space (O'Dell et al., 1991). In any case, the observations that SCH23390 can block the METH-induced increases in the expression of the majority of the mRNAs measured in the present study provide a partial explanation for the protective effects that pretreatment with the DA antagonist, SCH23390, affords against METH-induced cell death in the striatum (Xu et al., 2005). Finally, these observations further implicate DA D1 receptor-mediated mechanisms as important targets for therapeutic interventions against METH addiction and toxicity.

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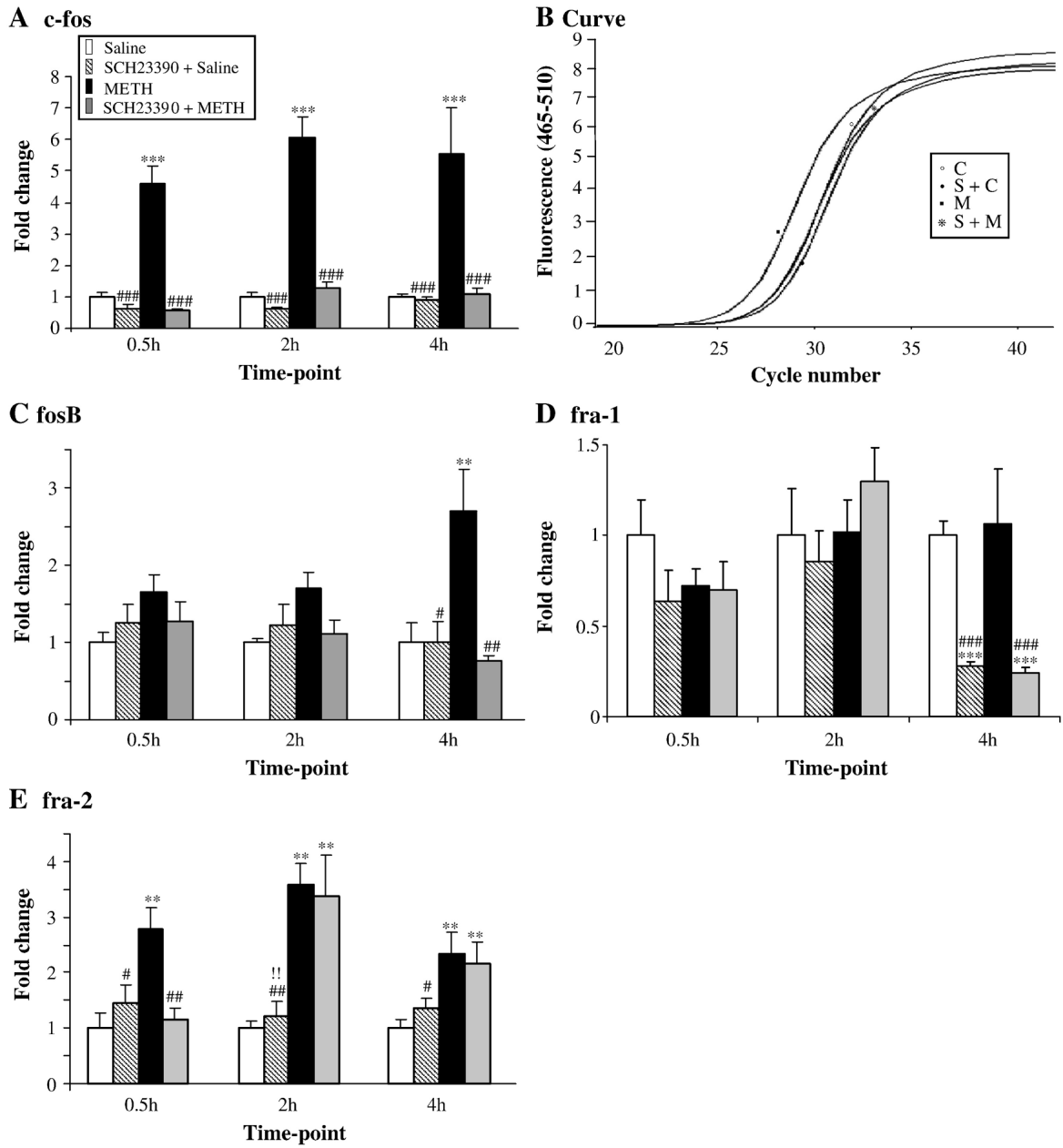
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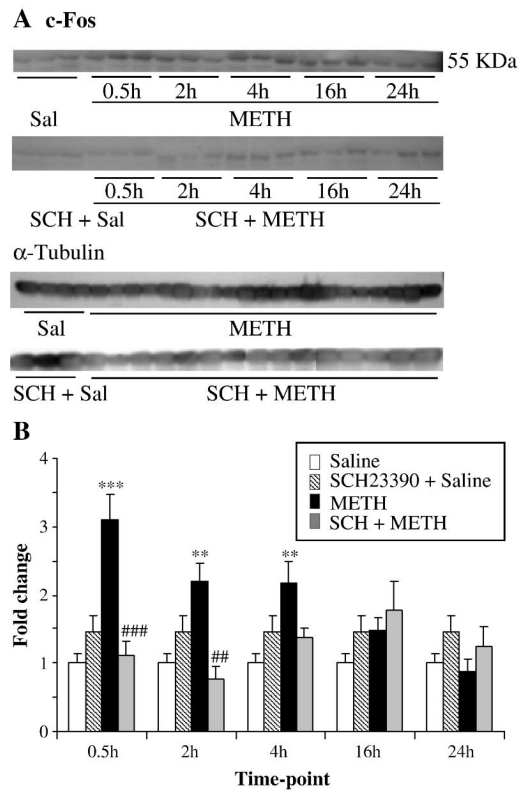


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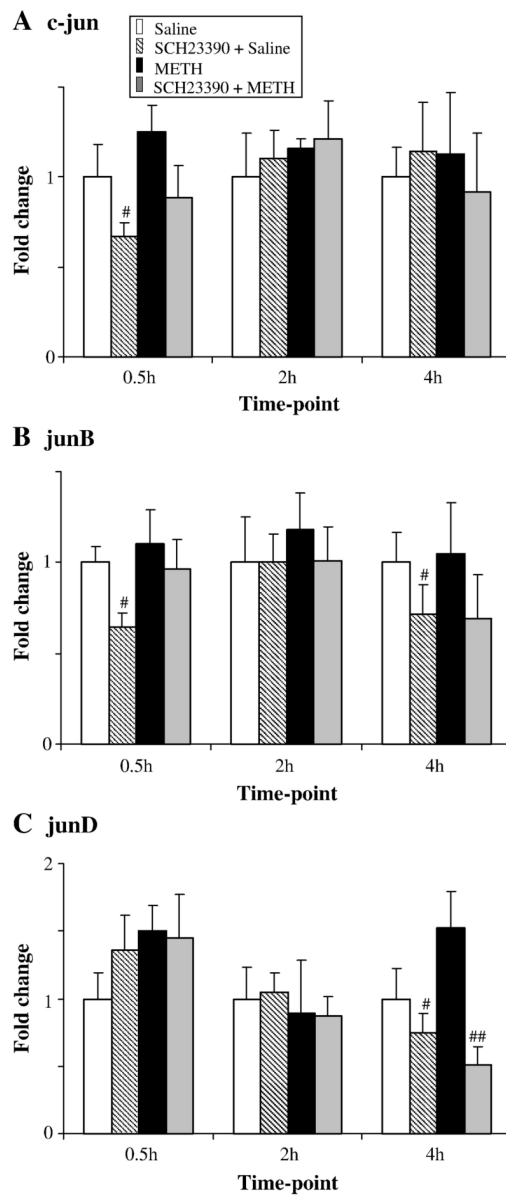


**Fig. 1. Effects of METH and SCH23390 on the expression of the fos family genes**  
 METH administration caused induction of (A) c-fos, (C) fosB and (E) fra-2, but did not influence fra-1 expression (D). Amplification curve for c-fos gene expression for the 30 min time-point is shown in fig. 1B and was reproducible. The levels of mRNA were normalized to clathrin mRNA levels. Data were obtained from RNA isolated from six animals per group and quantitation determined individually. Statistical significance was determined by ANOVA followed by protected least-squares difference (PLSD). Key to statistics: \*, \*\*, \*\*\* =  $p < 0.05$ , 0.01, 0.001, respectively in comparison to the control (C) group. #, ##, ### =  $p < 0.05$ , 0.01, 0.001, respectively in comparison to the METH (M) group.

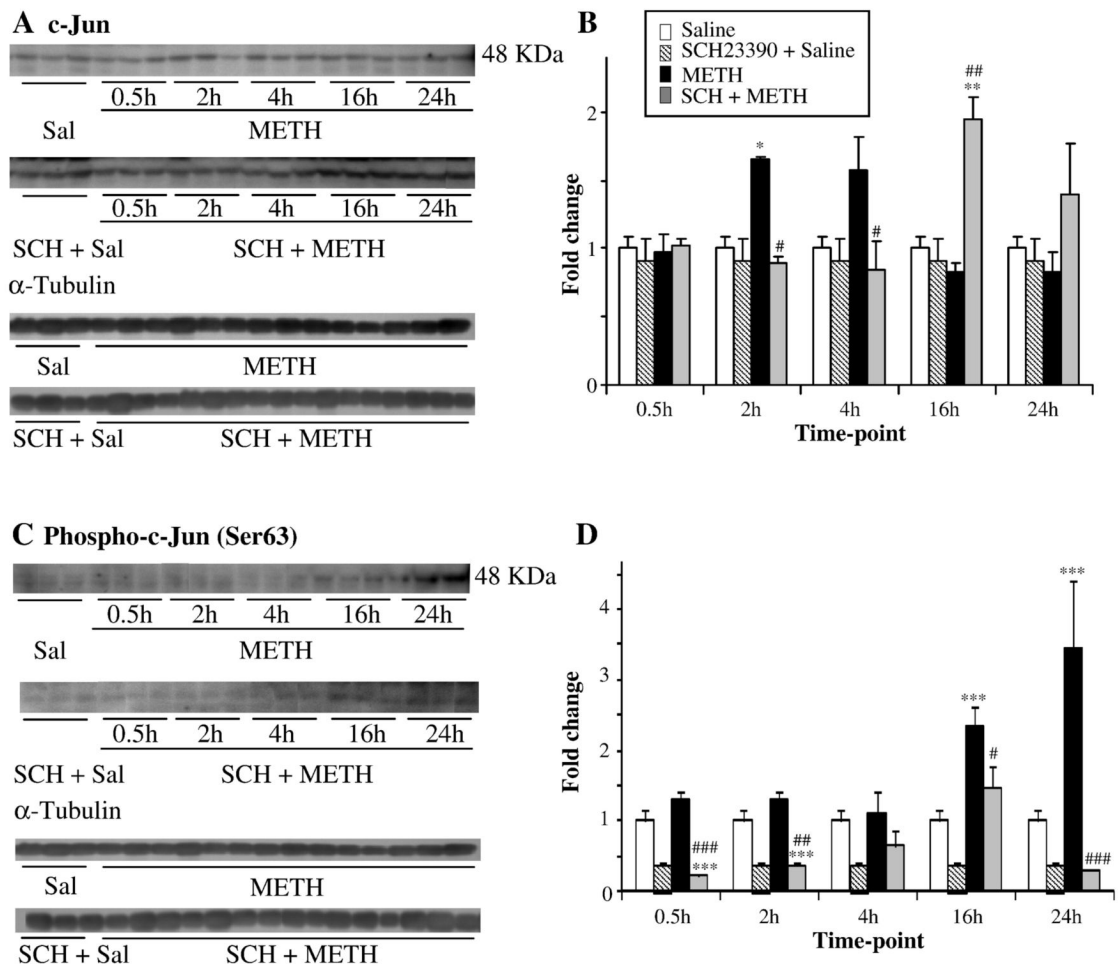


**Fig. 2. Effects of METH and SCH23390 on c-Fos protein level**

(A) Representative photomicrograph of results showing the effects of METH and SCH23390 pre-treatment on striatal c-Fos protein levels at different time-points. (B) The quantitative data of the western blots represent means  $\pm$  SEM ( $n = 3$ ). The experiments were repeated three times for each protein. For quantification, the signal intensity was normalized over the signal intensity of  $\alpha$ -tubulin. (B) METH caused significant DA D1 receptor-dependent increases in c-Fos protein. Statistics are as described in Fig. 1. Key to statistics: \*, \*\*, \*\*\* =  $p < 0.05, 0.01, 0.001$ , respectively in comparison to the control (C) group. #, ##, ### =  $p < 0.05, 0.01, 0.001$ , respectively in comparison to the METH (M) group.

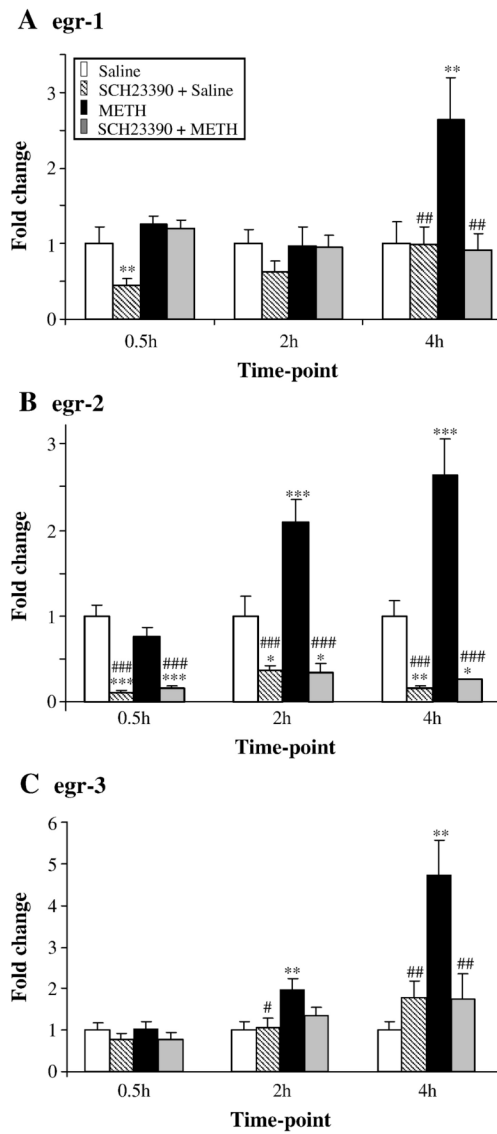


**Fig. 3. Effects of METH and SCH23390 on the levels of jun transcripts**  
 The expression of (A) c-jun, (B) junB and (C) junD mRNA expression was not affected by METH treatment although SCH23390 pretreatment caused significant decreases in junD at 4 hr post drug treatment (C). Normalization, quantification, and statistics are as described in Fig. 1. Key to statistics: #, ## =  $p < 0.05$ ,  $0.01$ , respectively in comparison to the METH (M) group.



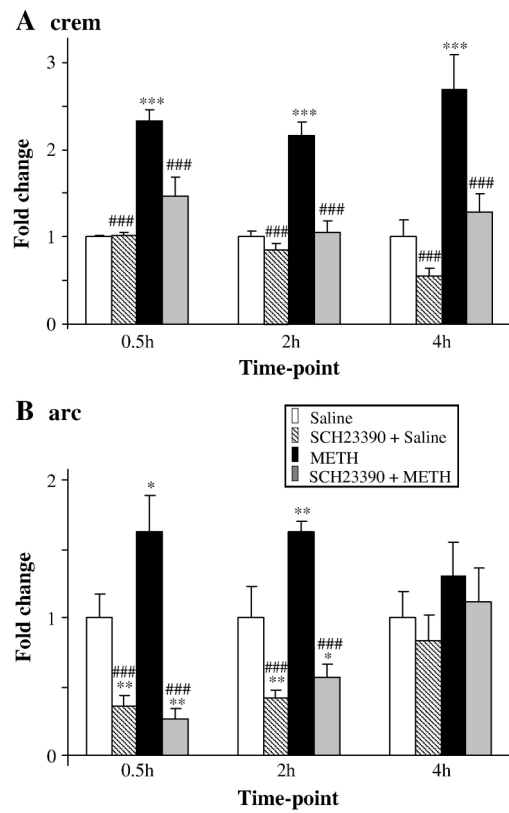
**Fig. 4. Effects of METH and SCH23390 on c-Jun and phospho-c-Jun protein levels**

(A and C) show representative photomicrographs of results showing the effects of METH and SCH23390 pre-treatment on the levels of c-Jun and phospho-c-Jun in rat striatal tissues at different time-points. (B and D) show the quantitative data of the western blots represent means  $\pm$  SEM ( $n = 3$ ). The experiments were repeated three times for each protein. For quantification, the signal intensity was normalized over the signal intensity of  $\alpha$ -tubulin. (C) METH-induced changes in c-Jun were seen only at the 2-hr time point and this was blocked by SCH23390 pre-treatment. (D) Phospho-c-Jun showed significant increases at 16 hr and 24 hr after METH treatment. Pretreatment of SCH23390 blocked METH-induced changes in phospho-c-Jun. Normalization, quantification, and statistics are as described in Fig. 1. Key to statistics: \*, \*\*, \*\*\* =  $p < 0.05, 0.01, 0.001$ , respectively in comparison to the control (C) group. #, ##, ### =  $p < 0.05, 0.01, 0.001$ , respectively in comparison to the METH (M) group.



**Fig. 5. METH caused induction of striatal egr mRNA levels**

(A, B, C) Time-dependent induction of egr-1, egr-2 and egr-3 was observed after METH injections. SCH23390 blocked METH-induced changes in these genes. Normalization, quantification, and statistics are as described in Fig. 1. Key to statistics \*, \*\*, \*\*\* =  $p < 0.05$ , 0.01, 0.001, respectively in comparison to the control (C) group. #, ##, ### =  $p < 0.05$ , 0.01, 0.001, respectively in comparison to the METH (M) group.



**Fig. 6. METH caused rapid induction of crem and arc mRNA levels**  
 (A) METH induced rapid increases in the crem transcript levels. (B) arc expression was also induced by METH. Normalization, quantification, and statistics are as described in Fig. 1. Key to statistics: \*, \*\*, \*\*\* =  $p < 0.05, 0.01, 0.001$ , respectively in comparison to the control (C) group. #, ##, ### =  $p < 0.05, 0.01, 0.001$ , respectively in comparison to the METH (M) group.



**Table 1**

List of primer sequences used for quantitative RT-PCR.

	Forward	Reverse
<i>c-fos</i>	TGA GGT GGT CTG AAT G	TTG ACA ATG TCT TGG AAC
<i>fosB</i>	GAA GCT GGA GTT CAT GC	ATG GGC TTG ATG ACA GA
<i>fra-1</i>	GAT GAG GAA GGT CAA AGT	TTT CCT TCA GCC TGT AGT
<i>fra-2</i>	AGC AAT GCT AAT GGG C	CTG TGT GCA CCC TCA GT
<i>c-jun</i>	TCT CAG GAG CGG ATC AA	TGT TAA CGT GGT TCA TGA C
<i>junB</i>	TCT TTC TCT TCA CGA CTA CA	CTA GCT TCA GAG ATG CG
<i>junD</i>	GTG TGT TTC CTT GTG TTG	TTT GGC GTA ACG AAG AC
<i>egr-1</i>	AGG TCT CCC TGT TGT TGT GG	TGC ACC CAC CTT TCC TAC TC
<i>egr-2</i>	CAG ATC CGA CAC TGG AA	CCT GAG ACC TCG AAA GTA
<i>egr-3</i>	AAA GTT CGC TTT CGA C	GCC GAT TGG TAA TCC T
<i>crem</i>	CCA ACT TAC CAG ATC CGA	TTC TTT AGC AGC TTC CCT
<i>arc</i>	CTG GGT GGA GTT CAA GA	CGT CCA CAT ACA GTG TC