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## Chronic co-administration of nicotine and methamphetamine causes differential expression of immediate early genes in the dorsal striatum and nucleus accumbens

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### Abstract

Nicotine and methamphetamine (METH) cause addiction by triggering neuroplastic changes in brain reward pathways though they each engage distinct molecular targets (nicotine receptors and dopamine transporters respectively). Addiction to both drugs is very prevalent, with the vast majority of METH users being also smokers of cigarettes. This co-morbid occurrence thus raised questions about potential synergistic rewarding effects of the drugs. However, few studies have investigated the chronic neurobiological changes associated with co-morbid nicotine and METH addiction. Here we investigated the effects of these two drugs alone and in combination on the expression of several immediate early genes (IEGs) that are sensitive to drug exposures. Chronic exposure to either nicotine or METH caused significant decreases in the expression of *fosb*, *fra1*, and *fra2* in the nucleus accumbens (NAc) but not in the dorsal striatum whereas the drug combination increased *fra2* expression in both structures. Except for *junB* mRNA levels that were decreased by the three drug treatments in the NAc, there were no significant changes in the Jun family members. Of the Egr family members, NAc *egr2* expression was decreased after nicotine and the drug combination whereas NAc *egr3* was decreased after METH and the drug combination. The drug combination also increased striatal *egr3* expression. The Nr4a family member, *nr4a2/nurr1*, showed increased striatal expression after all 3 drug treatments, while striatal *nr4a3/nor-1* expression was increased by the drug combination whereas NAc *nr4a1/nurr77* was decreased by nicotine and the drug combination. These observations suggest that, when given in combination, the two drugs exert distinct effects on the expression of IEGs in dopaminergic projection areas from those elicited by each drug alone. The significance of these changes in IEG expression and in other molecular markers in fostering co-morbid METH and nicotine abuse needs to be further evaluated.

### Keywords

nicotine; methamphetamine; striatum; nucleus accumbens; immediate early genes

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## Introduction

Illicit and licit drug use are major public health problems associated with serious adverse medical consequences and with criminal behaviors. Methamphetamine (METH) is an illicit psychostimulant drug (Meredith et al., 2005; Krasnova and Cadet, 2009) that exerts its rewarding effects via dopamine transporters through which it releases dopamine (DA) into the synaptic terminal causing molecular changes in post-synaptic cells (Cho and Melega, 2008; Cadet et al., 2010). Nicotine is a licit but highly addictive substance (Benowitz, 2010) that exerts its effects by interacting with nicotinic acetylcholine receptors (nAChR) that are abundantly found on midbrain dopaminergic cell bodies that project to the dorsal striatum and nucleus accumbens (NAc) (Livingstone and Wonnacott, 2009). Stimulation of midbrain nicotinic receptors causes DA release in projection areas (Livingstone and Wonnacott, 2009).

The co-abuse of nicotine and METH is very prevalent (Goldsamt et al., 2005; Yen and Chong, 2006). This phenomenon might reflect possible augmentation of the rewarding effects of METH by nicotine (Suemaru et al., 1993; Kuribara, 1999) or conditioning of one drug to the other, as exemplified by METH re-instatement after nicotine exposure (Neugebauer et al., 2010). Of interest, behavioral differences have been reported in animals co-exposed to nicotine and METH when compared to animals exposed to either drug alone (Gatch et al., 2008; Neugebauer et al., 2010). These findings suggest that distinct consequences can emerge with the drug combination. As a first step towards investigating the molecular effects of the co-administration of nicotine and METH, we compared mRNA expression of several immediate early genes (IEGs) in the dorsal striatum and the NAc as consequences of the administration of these drugs being given alone or in combination. We sought to investigate the changes in IEG expression because IEGs encode transcription factors that regulate neuronal responses to stimuli (Hughes and Dragunow, 1995; Hergden and Leah, 1998) and because they have been implicated in the neuronal responses that contribute to drug addiction and abuse (McClung et al., 2004; Nestler, 2008). We chose the dorsolateral striatum because it participates in automaticity in drug taking behaviors and reinstatement of drug seeking and the NAc because it is thought to be involved in many aspects of drug addiction including drug reward and conditioning (Fuchs et al., 2006; Bossert et al., 2009; Pickens et al., 2011).

## Experimental Procedures

### Animals

Male Sprague-Dawley rats ~76–100g (Taconic Farms Hudson, NY) were used in this study. Treatment began one week after arrival at 5 weeks of age. Rats were single housed in clear plexi-glass cages in an environmental controlled room (temperature  $72 \pm 2^\circ$  F and humidity 40–60% with a 12 h light/dark cycle: lights off at 8 AM and lights on at 8 PM). Food and water was provided ad-lib. Body weight and food intake was measured daily.

### Drug Administration

Rats were subcutaneously implanted with an osmotic mini-pump supplying nicotine (1.3 mg/kg/day) or an equivalent volume saline at a rate of 2.5  $\mu$ l/hr. Subsequently, the rats received daily intraperitoneal (IP) injections of either (+)-methamphetamine (METH) hydrochloride (8mg/kg/day) or an equivalent volume of saline for 30 days. We chose to use the IP route for METH injections because, as reviewed previously, the most common route of METH administration, regardless of doses, is the IP injection (Krasnova and Cadet, 2009). METH was not delivered via the osmotic pumps as this would mean injecting METH via the subcutaneous route. Because of nicotine's shorter half life, 20 minutes in the plasma

of rats (Sastry et al., 1995; Ghosheh et al., 1999), the SC route is often the route of choice in these kinds of experiments (Benwell et al., 1995; Matta et al., 2007). The osmotic pump delivers a nicotine dose SC, at a controlled rate, the continuous flow of nicotine also keeps blood plasma concentrations of nicotine at a constant rate, similar to chronic human smokers. We did not mix the two drugs together as the pH of the nicotine solution needs to be adjusted to pH 7.4 and the addition of METH to a nicotine solution might have introduced additional confounds.

Four treatment groups were used: saline+saline (SS), nicotine+saline (NS), saline+METH (SM) or nicotine+METH (NM). Rats were euthanized 24 hours after the last drug injection. Striatal and NAc tissues were dissected out and analyzed separately. All experiments were conducted in conformity with the National Academy of Sciences Guide for the Care and Use of Laboratory Animals and Brookhaven National Laboratory Institutional Animal Care and Use Committee Protocols.

### qRT-PCR

Total RNA was isolated from the striatal and NAc tissues by using RNeasy Micro Kit and Mini Kit, respectively (QIAGEN, Valencia, CA). The RNA concentration was determined using NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). Unpooled total striatal RNA (1 $\mu$ g) obtained from 8 rats per group was reverse transcribed with oligo dT primers using Advantage RT for PCR kit (Takara Bio Inc., Mountain View, CA) while NAc RNA (50ng) was reverse transcribed with oligo dT primers using iScript Reverse Transcription Supermix for RT-qPCR (BioRad, Hercules, CA). PCR experiments were performed using Light Cycler RT-PCR machine (Roche, Indianapolis, IN) and iQ SYBR Green Supermix (BioRad). Primers were synthesized and HPLC-purified at John Hopkins University (Baltimore, MD) and were the same sequences used previously (see McCoy et al., 2011 for sequence data). Clathrin and 18S rRNA were used as an internal control for striatum and NAc, respectively.

### Statistical Analysis

Data were analyzed with the statistical software StatView (version 4.02, SAS Institute, Cary, NC). Statistical analysis was done using ANOVA followed by Fisher's protected least significant differences (PLSD). Differences were considered significant if the probability of error was less than 5%. Data are reported as fold changes (Means  $\pm$  SEM).

### Results

Figure 1 shows the effects of chronic METH and nicotine, separately and in combination, on the mRNA expression of the Fos family in the dorsal striatum. Nicotine or METH given alone caused no significant changes in their mRNAs (Fig. 1A–1D). However, co-administration of the two drugs increased striatal fosb (~1.6-fold,  $p < 0.05$ ; Fig. 1B) and fra2 (~2-fold,  $p < 0.05$ ; Fig. 1D) mRNA expression. In contrast, in the NAc, chronic nicotine decreased c-fos (–80%,  $p < 0.05$ ; Fig. 2A), fosb (–80%,  $p < 0.01$ ; Fig. 2B), fra1 (–40%,  $p < 0.05$ ; Fig. 2C), and fra2 (–80%,  $p < 0.01$ ; Fig. 2D) and chronic METH also decreased fosb (–50%,  $p < 0.05$ ; Fig. 2B), fra1 (–50%,  $p < 0.01$ ; Fig. 2C), and fra2 (–40%,  $p < 0.01$ ; Fig. 2D). However, there were no changes in these IEGs in the NAc with the drug combination (Fig. 2).

The various treatments did not change mRNA expression of Jun family members in the dorsal striatum (Fig. 3A–3C), although they all decreased junB expression in the NAc (Fig. 4B), without affecting c-jun (Fig. 4A) nor junD (Fig. 4C).

Nicotine or METH alone did not affect the expression of Egr family members in the striatum (Fig. 5) but the drug combination increased *egr3* mRNA levels (1.5-fold,  $p < 0.01$ ; Fig. 5C). In the NAc, *egr1* was not influenced by any of the treatments (Fig. 6A); *egr2* was decreased by nicotine ( $-90\%$ ,  $p < 0.01$ ; Fig. 6B) and the drug combination ( $-60\%$ ,  $p < 0.01$ ; Fig. 6B) and *egr3* was decreased by METH ( $-80\%$ ,  $p < 0.001$ ) and the drug combination ( $-60\%$ ,  $p < 0.01$ ) (Fig. 6C).

In the striatum, expression of *nr4a1/nurr77* was not affected by any of the treatments (Fig. 7A); *nr4a2/nurr1* was increased by nicotine (6.6-fold,  $p < 0.01$ ), METH (8.8-fold,  $p < 0.001$ ), and the drug combination (8.9-fold,  $p < 0.001$ ) (Fig. 7B); and *nr4a3/nor-1* was not affected by nicotine or METH but was increased by the drug combination (1.8-fold,  $p < 0.05$ ; Fig. 7C). In the NAc, *nr4a1/nurr77* mRNA expression was decreased by nicotine ( $-80\%$ ,  $p < 0.001$ ) and the drug combination ( $-50\%$ ,  $p < 0.01$ ) (Fig. 8A) but *nr4a2/nurr1* (Fig. 8B) and *nr4a3/nor-1* were not affected by any of the treatments (Fig. 8C).

## Discussion

The major findings in this study are that chronic exposures to: (1) nicotine and METH, given alone, increased the striatal expression of *nr4a2/nurr1*; (2) nicotine decreased the expression of all members of the Fos family in the NAc; (3) METH also suppressed the expression of the Fos family members (except for *cfos*) in the NAc; and (4) the co-administration of the two drugs increased the expression of several IEGs in the dorsal striatum. The potential implications for these differential changes in gene expression are discussed below.

### Chronic nicotine

Nicotine is thought to exert its addictive properties by enhancing dopamine (DA) release into the striatum and nucleus accumbens (NAc) through stimulation of nAChRs (Picciotto et al., 1998; Perez et al., 2009; Livingstone and Wonnacott, 2009). This is followed by activation of DA-related intracellular molecular signaling cascades (Hamada et al., 2004, 2005; Janhunen and Ahtee, 2007). In the current study, we show that chronic nicotine results in significant decreases in the expression of several IEGs in the NAc but not in the dorsal striatum. These observations are consistent with those of other studies that have reported that chronic administration of cocaine can suppress the expression of some IEGs in the NAc (Hope et al., 1992). These results are also consistent with the report that long-term nicotine exposure depresses dopamine release in the NAc (Perez et al., 2012) and decreases levels of phosphorylated CREB (pCREB) in the NAc (Brunzell et al., 2003). pCREB is known to control IEG expression in dopaminergic brain regions (Konradi et al., 1996; Hyman and Malenka, 2001). The lack of similar changes in the dorsal striatum might be related to the observations that nicotine preferentially affects DA release in the NAc in contrast to the dorsal striatum (Benwell and Balfour, 1997; Zhang et al., 2009).

### Chronic METH

Previously, we have examined the effects of increasing METH doses on striatal IEG expression (McCoy et al., 2011). We found that METH caused no changes or decreased the expression of several IEGs in the dorsal striatum (McCoy et al., 2011). For example, using that paradigm, chronic METH was shown to cause significant decreases in the striatal expression of AP-1, Egr and Nr4a IEGs (McCoy et al., 2011) in contrast to acute METH that causes increased IEG expression (Cadet et al., 2010; McCoy et al., 2011). When taken together with the present findings, the consensus suggests that chronic METH administration might have caused tolerance to the acute effects of the drug. This argument is relevant for all the IEGs, except *nr4a2/nurr1* that showed increased gene expression in the

dorsal striatum after chronic METH. In contrast to the observations in the striatum, chronic METH caused decreased expression of several IEGs in the NAc including fosB that has been implicated in the neurobiological processes of drug addiction (Nestler et al., 2001). These differential changes in the two structures further support the idea that the dorsal and ventral striatum play distinct roles in the long term effects of abused drugs. Our dichotomous findings also stress the fact that schedules of drug administration can result in differential molecular consequences. Our laboratory is actively investigating these issues further by using different models of METH intake.

### Chronic nicotine and METH

To our knowledge, our study is the first investigation of the effects of nicotine and METH co-administration on IEG expression in the rodent forebrain. Previous studies have already examined the acute effects of nicotine (Kiba and Jayaraman, 1994; Pich et al., 1997) or METH (McCoy et al., 2011) on the expression of IEG's in the striatum, with a general trend of increased IEG expression. In the current study, the expression of the majority of striatal and NAc IEG was not affected by chronic METH or nicotine given alone. These observations suggest that some degree of tolerance might have developed to the individual molecular effects of nicotine and METH. However, this tolerance might have been circumvented when the two drugs were given in combination. For example, co-administration of the two drugs increased fosB, fra2, egr3 and nr4a3/nor-1 expression in the striatum. Of note, when either nicotine or METH alone decreased IEG expression, the co-administration of the drugs either restored IEG expression to normal (i.e. c-fos, fosB and fra1) or increased their expression (fra2) in the NAc. These observations suggest that these two drugs, when given in combination, caused molecular changes in the brain that render it more permissible for the expression of certain genes. This idea is consistent with a recent report that suggested that administration of nicotine facilitated cocaine-induced molecular and behavioral effects in the brain, priming it to cocaine's rewarding effects (Levine et al., 2011). This priming appears to depend on increased binding of acetylated histones on the fosB promoter region (Levine et al., 2011). Nevertheless, it is important to point out that the co-administration of METH and nicotine suppressed the expression of egr2, egr3 and nr4a1/nurr77 in the NAc. However, with regards to these IEGs, either nicotine (egr2, nr4a1/nurr77) or METH (egr3) was able to suppress their expression, suggesting that the suppression that we observed with the drug combination might be reflecting, in part, the effects of one of the drugs.

### Summary

In summary, this is the first report to identify cross-talks between nicotine and METH on the transcriptional regulation of IEGs in the NAc and dorsal striatum, regions implicated in the neuroadaptive and reinforcing properties of drugs. The fact that the two drugs caused increased mRNA levels of certain IEGs that were not affected when the drugs were given alone supports the hypothesis that they might induce molecular alterations that lead to changes in gene expression and to the potential alteration of the rewarding effects of the individual drugs. It is also to be noted that the combination of METH and nicotine also caused additional changes in body weight, food intake, and locomotor activity in comparison to METH and nicotine alone (*Thanos et al, in preparation*). These observations from our behavioral studies suggest that the drug combination can potentiate decreased body weight and food intake while increasing locomotor activity caused by either drug alone (*Thanos et al, in preparation*). When taken together with these behavioral results, our molecular observations suggest a potential neurobiological substrate for the high prevalence of co-morbid METH and nicotine abuse and addiction.

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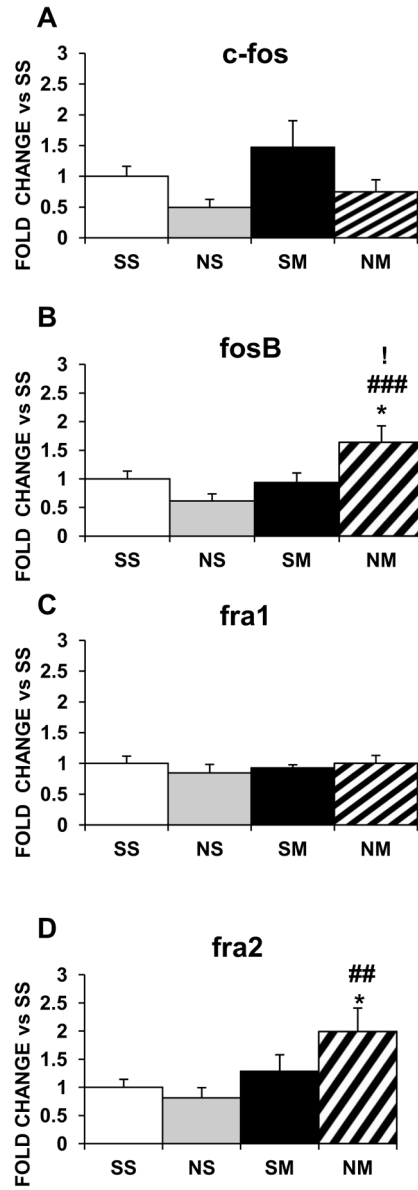
### Highlights

Chronic nicotine and METH, alone, increased striatal expression of nr4a2/nurr1

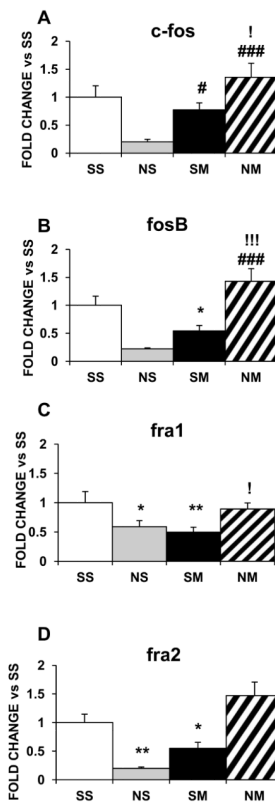
Chronic nicotine decreased the expression of all members of the Fos family in the NAc

Chronic METH suppressed expression of Fos family members (except cfos) in the NAc

Co-administration increased the expression of several IEGs in the dorsal striatum

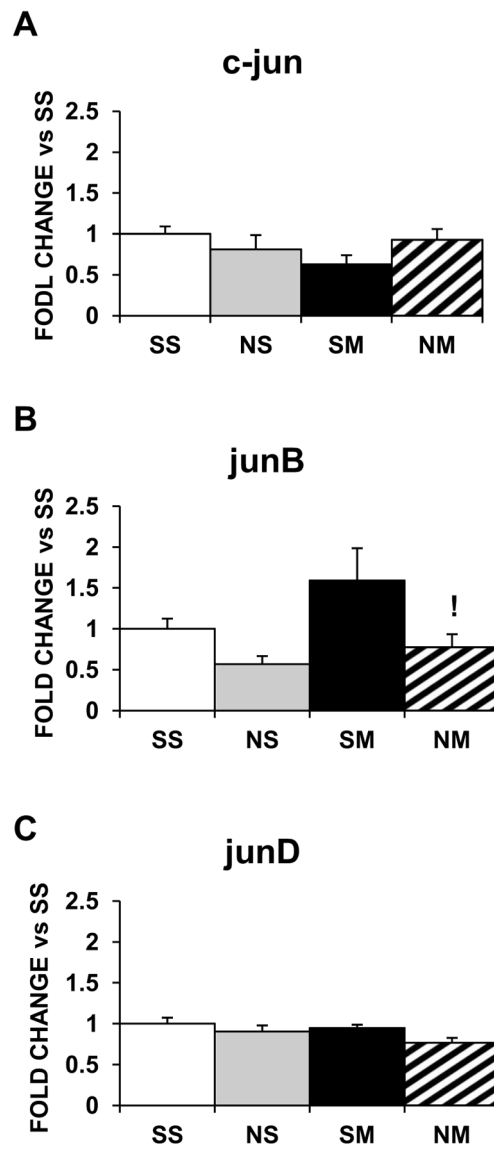


**Figure 1.** Effects of chronic nicotine, chronic METH and their combination on mRNA levels of Fos IEG family in the rat dorsal striatum. Rats were treated as described in the text. Results are presented as fold-changes SEM. The data were analyzed by one-way ANOVA followed by Fisher’s protected least significant difference (PLSD) post-hoc test. **Key to statistics:** \* compared to SS; # compared to NS; ! compared to SM. **Symbol guide:** single symbol,  $p < 0.05$ ; double symbol,  $p < 0.01$ , and triple symbol,  $p < 0.001$ .  $n = 7-8$  rats per groups.

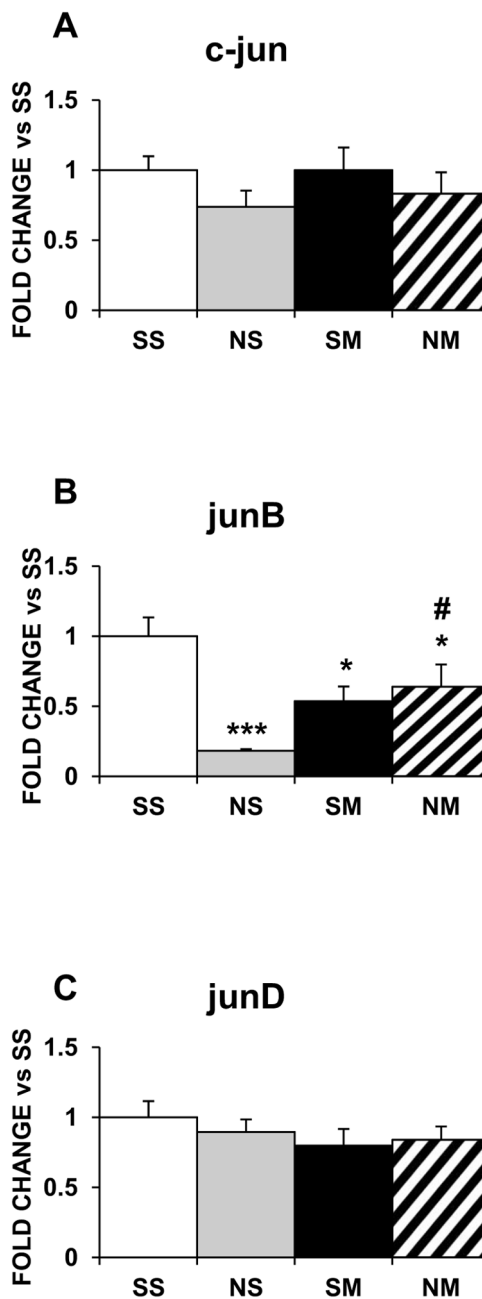


**Figure 2.**

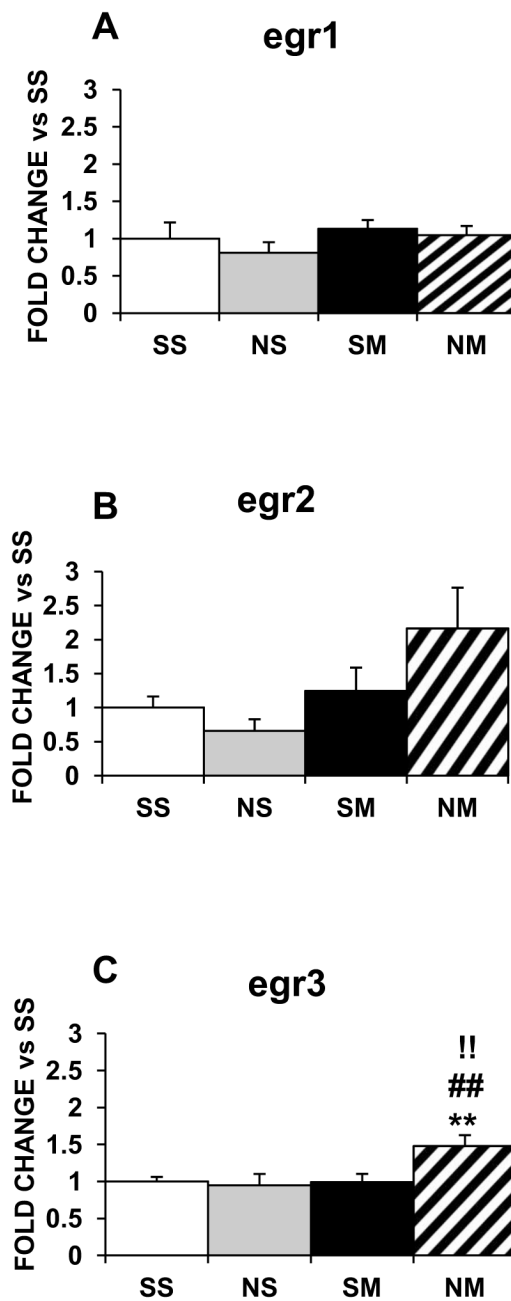
Effects of chronic nicotine, chronic METH and their combination on mRNA levels of Fos IEG family in the rat NAc. Treatment is as described in the text. Results are presented as fold-changes  $\pm$  SEM. The results were analyzed by one-way ANOVA followed by Fisher's PLSD post-hoc test. **Key to statistics:** \* compared to SS; # compared to NS; ! compared to SM. **Symbol guide:** single symbol,  $p < 0.05$ ; double symbol,  $p < 0.01$ , and triple symbol,  $p < 0.001$ .  $n = 4-7$  rats per groups.



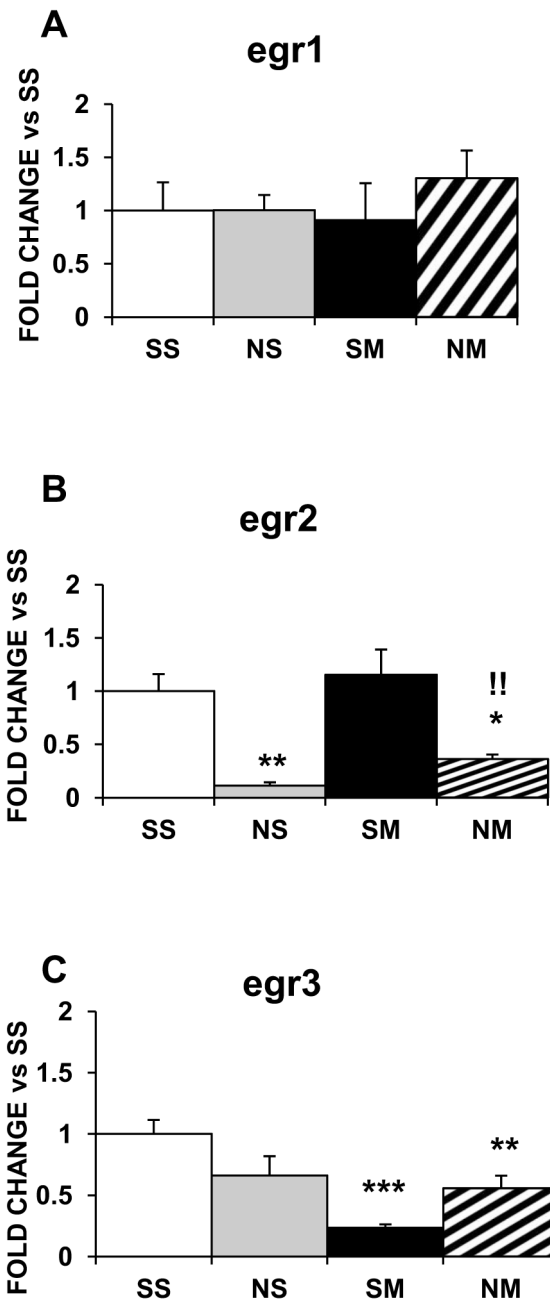
**Figure 3.** Effects of chronic nicotine, METH, and their combination on mRNA levels of mRNA levels of Jun IEG family members in the rat dorsal striatum. Treatment is as described in the text. Results are presented as described in figure 1. **Key to statistics:** !,  $p < 0.05$ , in comparison to the SM group.  $n=6-8$  rats per groups



**Figure 4.** Effects of chronic nicotine, METH and their combination on the expression of Jun IEG family in the rat NAc. Results are presented as described in figure 1. **Key to statistics:** \* compared to SS; # compared to NS. **Symbol guide:** single symbol,  $p < 0.05$  and triple symbol,  $p < 0.001$ .  $n = 4-8$  rats per groups.

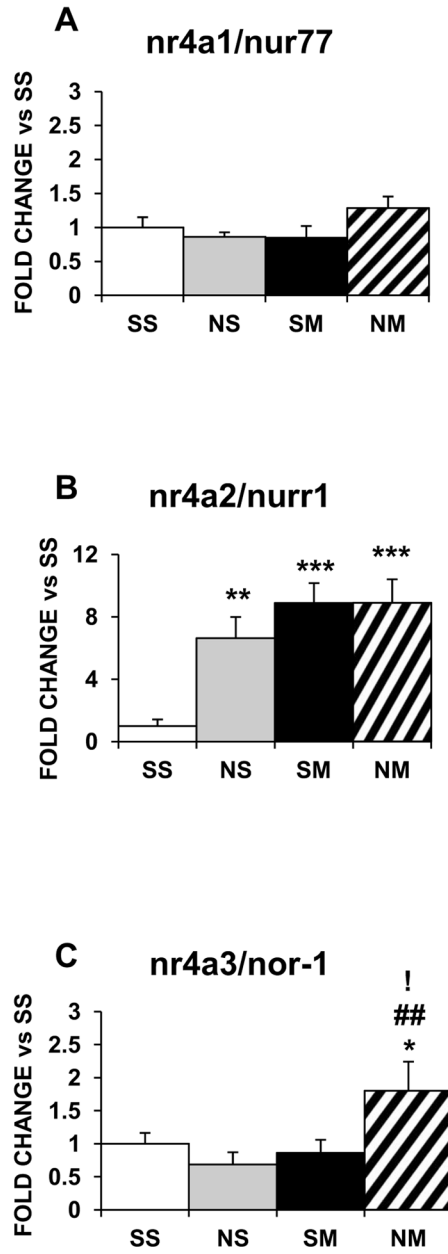


**Figure 5.** Effects of chronic nicotine, METH and their combination on mRNA levels of Egr IEG family members in the rat dorsal striatum. Results are presented as in figure 1. **Key to statistics:** \* compared to SS; # compared to NS; ! compared to SM. **Symbol guide:** double symbol,  $p < 0.01$ .  $n=6-8$  rats per groups.

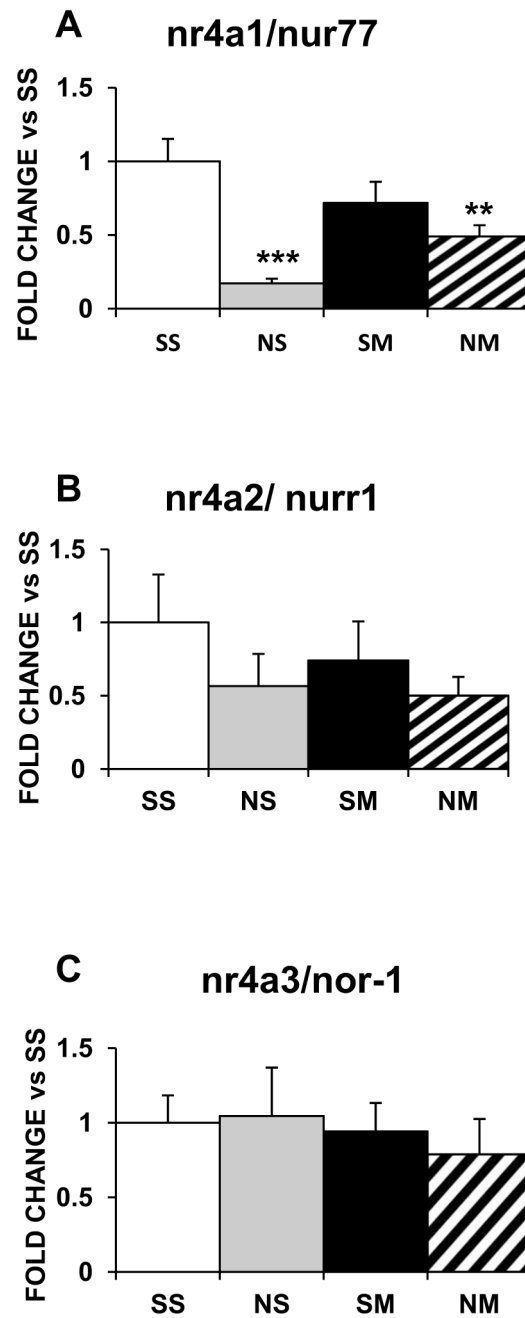


**Figure 6.** Effects of chronic nicotine, METH and their combination on the NAc expression of Egr IEG family members. Treatment is as described in the text. **Key to statistics:** \* compared to SS; # compared to NS; ! compared to SM. **Symbol guide:** single symbol,  $p < 0.05$ ; double symbol,  $p < 0.01$ , and triple symbol,  $p < 0.001$ .  $n=4-8$  rats per groups.





**Figure 7.** Effects of chronic nicotine, METH and their combination on mRNA levels of Nr4a IEG family members in the rat dorsal striatum. Treatment is as described in the text. Results are presented as above. **Key to statistics:** \* compared to SS; # compared to NS; ! compared to SM. **Symbol guide:** single symbol,  $p < 0.05$ ; double symbol,  $p < 0.01$ , and triple symbol,  $p < 0.001$ .  $n = 6-8$  rats per groups.



**Figure 8.** Effects of chronic nicotine, METH and their combination on the NAc expression of Nr4a IEG family members. **Key to statistics:** \* compared to SS; # compared to NS. **Symbol guide:** double symbol,  $p < 0.01$ , and triple symbol,  $p < 0.001$ .  $n=4-8$  rats per groups.