

Single or Multiple Injections of Methamphetamine Increased Dopamine Turnover but Did Not Decrease Tyrosine Hydroxylase Levels or Cleave Caspase-3 in Caudate-Putamen

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ABSTRACT Methamphetamine (METH), leading to striatal dopamine (DA) nerve terminal toxicity in mammals, is also thought to induce apoptosis of striatal neurons in rodents. We investigated the acute effects induced by multiple injections of METH (4×5 mg/kg, i.p.) at 2-h intervals or a single injection of METH (20 mg/kg, i.p.) on terminal dopaminergic toxicity markers, including DA levels, DA turnover, and tyrosine hydroxylase (TH) immunoreactivity in rat caudate-putamen (CPu). We further investigated whether both treatment paradigms would change Bax and activate caspase-3 expression, thus triggering striatal apoptotic mitochondria-dependent biochemical cascades. The first injection of METH (5 mg/kg, i.p.) produced a significant release of DA that peaked 30 min and stayed above control levels up to 1.5 h within CPu. In another set of experiments, rats were killed 1 and 24 h following the last injection, for tissue DA and metabolite content measurement and Western blot analysis (24 h). Multiple doses induced DA depletion and increased turnover at both endpoints. Single-dose METH reproduced these effects at 24 h; however, turnover was significantly higher than that evoked by the multiple doses at 24 h. Although both paradigms evoked similar DA depletion, however, none of the dosing regimens induced changes in TH expression at 24 h. The former paradigm produced an increase in Bax expression in CPu not sufficient to induce cleavage of caspase-3 proenzyme at 24 h. This study suggests that both paradigm induced changes in striatal dopaminergic markers that are independent of terminal degeneration and striatal apoptotic mitochondria-dependent caspase-3 driven cascade within 24 h. **Synapse 60:185–193, 2006.** Published 2006 Wiley-Liss, Inc.[†]

INTRODUCTION

Methamphetamine (METH) is a potent, indirect acting, sympathomimetic amine that is abused worldwide by humans (Seiden and Sabol, 1996). Short- and long-term health effects of METH use include stroke, cardiac arrhythmia, anxiety, paranoia, hallucinations, and structural changes to the brain (Anglin et al., 2000). Acute METH intoxication might be due to an increase in the levels of synaptic striatal dopamine (DA) (Pereira et al., 2002, 2004), whereas long-term effects could be secondary to persistent perturbations

in monoaminergic systems (Wagner et al., 1980). METH-induced neurotoxicity in monoaminergic systems has been observed in rats given single large doses (in the range of 20–100 mg/kg) (Cappon et al.,

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2000; Fukumura et al., 1998; Imam and Ali, 2001; Seiden and Vosmer, 1984) or 5–10 mg/kg, given four times at intervals of 2 h (Chapman et al., 2001). Toxicity in dopaminergic systems was also reported in mice given either a single dose of METH (25 mg/kg) (Hayashi et al., 2001) or multiple doses, with the animals receiving four injections of METH varying from 5 to 10 mg/kg at 2-h intervals (Ali et al., 1994; Fornai et al., 2004; Thomas et al., 2004). Recently, *in vivo* studies have suggested that METH can also cause neuronal apoptosis in rodents by the concurrent activation of mitochondrial and endoplasmic reticulum death pathways in mice striatal cells (Jayanthi et al., 2004), and by the upregulation of Fas ligand/Fas death pathway in rat striatal cells (Jayanthi et al., 2005). An increasing number of proteins are implicated in neuronal apoptosis mitochondria dependent, including Bcl-2 family members Bax and the executioner caspase-3 (Mattson and Duan, 1999). There is increasing evidence that exposure to METH is associated with caspases and proapoptotic Bcl-2 family genes that are recruited during cell death (Itzhak and Achat-Mendes, 2004). Bax exists in a stimulus-dependent equilibrium between an inactive soluble cytosolic form and an active mitochondrial membrane-associated or inserted form that induces cytochrome *c* release and subsequent activation of execution phase of apoptosis through triggering the activation of terminal caspases such as caspase-3 (Benn and Woolf, 2004; Cao et al., 2001). Recent studies clearly pointed out that Bax could trigger cell death through mechanisms independent of the BH3 domain and independent of its interaction with Bcl-2 and Bcl-x-1 (Eskes et al., 1998; Jurgensmeier et al., 1998). Although the majority of the earlier papers were concerned with the long-term effects of the drug, the purpose of the present study was to investigate the acute changes on terminal dopaminergic toxicity markers, including DA levels, DA turnover, and tyrosine hydroxylase (TH) immunoreactivity in caudate-putamen (CPu) of adult male Sprague-Dawley rats within 24 h following the last injection using two METH-treatment paradigms. Furthermore, the effects of the two METH treatments on Bax immunoreactivity and caspase-3 cleavage in CPu within 24 h were also evaluated.

MATERIALS AND METHODS

Animals and METH treatments

Adult male Sprague-Dawley rats (Charles River Laboratories, Barcelona, Spain), weighing 250–380 g, were housed two per cage under controlled environmental conditions (12-h light/dark schedule, at room temperature of $21 \pm 1^\circ\text{C}$) with food and water supplied *ad libitum* and subsequently housed individually after surgery or dosing. All experiments were

performed under the rules of the European Convention on Animal Care.

In the first set of experiments, rats were submitted to *in vivo* microdialysis to test the effect of an injection of 5 mg/kg of METH on nigrostriatal dopaminergic transmission. In a second set of experiments, animals were injected with either four injections of METH (5 mg/kg, *i.p.* in a volume of 1.0 ml/kg) at 2-h intervals or a single dose of METH (20 mg/kg, *i.p.* in a volume of 1.0 ml/kg). Controls were injected with saline (SAL).

Single-day METH dosing regimens could be a good model of overdose in naive nontolerant abusers (Davidson et al., 2005). The single-day single-dose regimen was used to simplify the method of neurotoxicity induction (Fukumara et al., 1998). All animals survived these dosing regimens throughout the duration of the study, being killed by decapitation at either 1 or 24 h following last injection. Brains were quickly removed and CPu was dissected on ice and stored at -80°C . Unilateral CPu was used for DA and DA metabolites' tissue content measurement (1 and 24 h) and the contralateral hemisphere was used for Western-blot analyses (24 h).

METH HCl was synthesized in the Organic Chemistry Department, University of Porto, Portugal. METH HCl solutions were prepared in saline.

Surgery and *in vivo* microdialysis

Rats were anesthetized with sodium pentobarbital and placed into a stereotaxic frame. The dorsal skull was exposed and a small hole was drilled to allow implantation of intracerebral guide cannula (CMA/12, Carnegie Medicine, Sweden) into CPu (AP, +0.5; Lat, -3; DV, -3). The cannula was fixed to the skull with dental acrylic and two anchor screws. To avoid effects of surgery and anesthesia, the dialysis experiments were started 1 week after surgery. On the day of tests, animals were hand held and a $4.0 \times 0.5 \text{ mm}^2$ dialysis probe (CMA/12) was slowly inserted through the guide cannula into the CPu of awaken rats. The animals were then placed in clear Plexiglas test chambers and tethered to counterbalance arms that allowed relatively free movement. The probes were perfused at $1.3 \mu\text{l}/\text{min}$ with a microperfusion pump (CMA/100) with artificial cerebrospinal fluid (ACSF) containing (in mM): 140 NaCl, 2.4 KCl, 2.4 CaCl_2 , 1.0 MgCl_2 , 1.2 Na_2HPO_4 , and 0.27 NaH_2PO_4 , adjusted to pH 7.2. Dialysate samples were collected in 30-min intervals on ice and their DA and dihydroxyphenylacetic acid (DOPAC) content was analyzed by high-performance liquid chromatography (HPLC)-EC. A minimum of two samples were taken to establish baseline levels of extracellular DA and DOPAC before drug administration (5 mg/kg METH, *i.p.* in a volume of 1.0 ml/kg), which occurred ~ 5 h following probe

insertion. Controls were injected with SAL. After each experiment, animals were killed and each probe track was visualized.

Measurement of DA, DOPAC, and homovanillic acid (HVA) by HPLC-EC

Extracellular levels of DA and DOPAC

Extracellular levels of DA and DOPAC were separated on a reversed-phase Supelcosil LC-18 column (4.6 mm; 7.5 cm; 3 μ m particle size) with a mobile phase (pH = 3.0) consisting of 0.07 M KH_2PO_4 , 2.85 mM L-heptanesulfonic acid, 0.12 mM NaEDTA, and 7% methanol (v/v). A flow rate of 1.2 ml/min was maintained and detection of the compounds achieved using a glassy carbon working electrode set at 0.75 V. The settings of sensitivity used were 0.05 and 0.5 nA/V for DA and DOPAC, respectively. Thirty microliters were injected onto the column.

Postmortem tissue content of DA, DOPAC, and HVA

Animals were sacrificed by decapitation 1 and 24 h following the last injection. The concentrations of DA, DOPAC, and HVA were quantitated in CPU by a modified HPLC method combined with electrochemical detection (Ali et al., 1994). Contents of DA, DOPAC, and HVA were calculated as ng/mg of tissue weight.

Western blot analyses

Animals were killed by decapitation 24 h following the last injection. Western blotting was performed for TH, Bax, and caspase-3. Each CPU was homogenized in RIPA buffer (50 mM Tris \cdot HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.5) containing protease inhibitor cocktail (Sigma, St. Louis, MO), and samples were measured for protein concentration with BCA protein reagent (Pierce, Rockford, IL). Equal amounts of protein (3 μ g (TH and Bax analysis) and 20 μ g (caspase-3 analysis)) were loaded on each lane and the electrophoresis was performed in a NOVEX XCell Sure Lock electrophoresis unit (Invitrogen Corp/NOVEX, Carlsbad, CA) using NOVEX NuPage precast 10% Bis-Tris 15-lane gels. Gels were transferred overnight to a polyvinylidene difluoride (Invitrolon PVDF, Invitrogen Corp/NOVEX) membrane and blocked with 5% nonfat dry milk in Tris-buffered saline for 1 h. The blots were probed with rabbit anti-TH (1:1000, Chemicon, Temecula, CA), rabbit anti-Bax (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal caspase-3 (1:1000, BD PharMingen, San Diego, CA) for 2 h at RT. To confirm equal protein loading and sample transfer, blots were reprobed with mouse monoclonal anti- α -tubulin (1:3000, Sigma; 2 h at room tempera-

ture). Immunoreactive bands were visualized by the addition of horseradish peroxidase (HRP) conjugated antibodies directed against rabbit and mouse IgG (1:4000, Santa Cruz Biotechnology; 1:6000, Sigma) and a highly sensitive enhanced chemiluminescent substrate (SuperSignal[®] West Dura Extended Duration Substrate, Pierce). Analysis was performed using a BioRad Chemi Doc XRS imaging system (Richmond, CA) and densitometric analyses were performed using the BioRad Quantity One. The relative density of each band was normalized against that of α -tubulin.

Statistical analysis

Results were expressed as means \pm SEM. Microdialysis data were converted to percent-of-baseline, defined as the average of the last two values prior to drug administration. Normalized Western blot data were converted to percent-of-saline for each gel. This approach normalizes differences in the development of the chromagen solution between blots. Two to three gels were run for each protein. Each blot contained all experimental groups, allowing standardization across all blots and these data were averaged. The differences between groups were determined by ANOVA followed by post hoc comparison (Bonferroni's multiple comparison test). Significant differences were defined at $P < 0.05$.

RESULTS

In vivo microdialysis

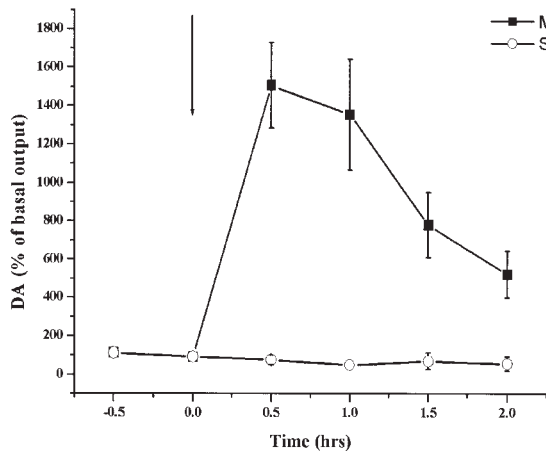
DA baseline extracellular levels for SAL and METH groups were pooled, being 15.5 ± 1.7 pg/30 μ l. Also, there appeared to be no significant difference between the DOPAC baseline levels from SAL and METH groups (5.98 ± 0.84 and 9.31 ± 1.55 ng/30 μ l, respectively, $P > 0.05$).

The first injection of METH (5 mg/kg, i.p.) elicited a 14-fold increase in the extracellular DA levels 30 min after dosing, and stayed significantly ($P < 0.05$) above control levels up to 1.5 h within CPU (Fig. 1A). Dialysate DOPAC levels showed a significant decrease that peaked within \sim 1 h and stayed significantly decreased up to 2 h following METH treatment (Fig. 1B). The extracellular levels of DA and DOPAC in the SAL-treated animals stayed constant throughout the experiment.

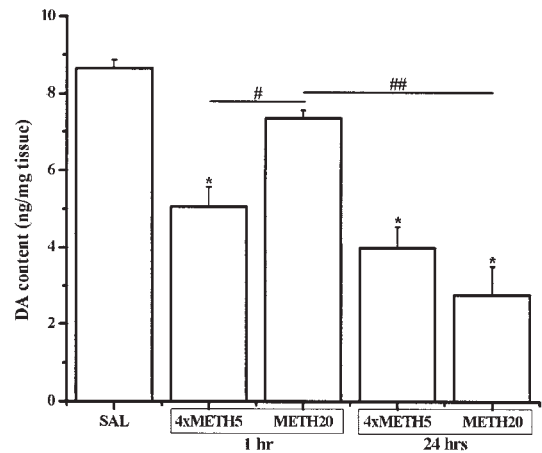
Caudate-putamen monoamine and metabolite tissue content

The effects of multiple injections (4×5 mg/kg, i.p.) and a single injection (20 mg/kg, i.p.) of METH on CPU DA and its metabolites' content were compared and are shown in Figs 2A, 2B, and 2C). The effect of both treatments on DA tissue content was significant ($P < 0.001$). The multiple-dose regimen produced a significant decrease (41%) in DA content in the CPU 1 h

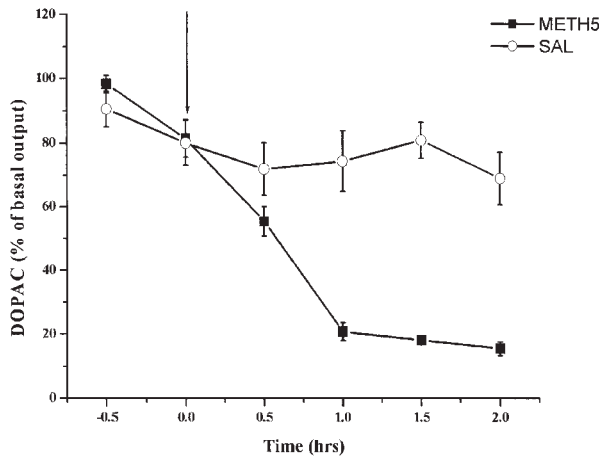
(A)



(A)



(B)



(B)

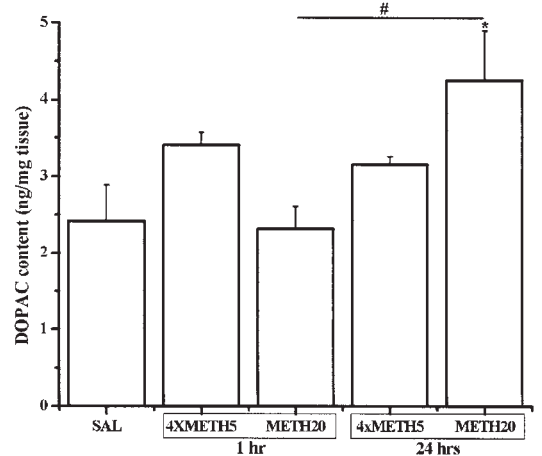


Fig. 1. Effects of METH on extracellular levels of DA (A) and DOPAC (B) in caudate-putamen (CPU). Extracellular levels of DA and DOPAC were measured in CPU from adult male SD rats by in vivo microdialysis following a single injection of METH (5 mg/kg, i.p.; METH5) or saline (SAL) at the time point indicated by the arrow. Each value is presented as mean \pm SEM ($n = 4$ for SAL and $n = 7$ for METH5).

after the last injection. Twenty-four hours following last METH injection, CPU DA remained depleted (54%). On the other hand, the single-dose regimen of METH had no significant effect on DA levels 1 h following treatment. In contrast, 24 h following single METH injection, CPU DA was significantly reduced compared with that of SAL-treated animals (68%).

(C)

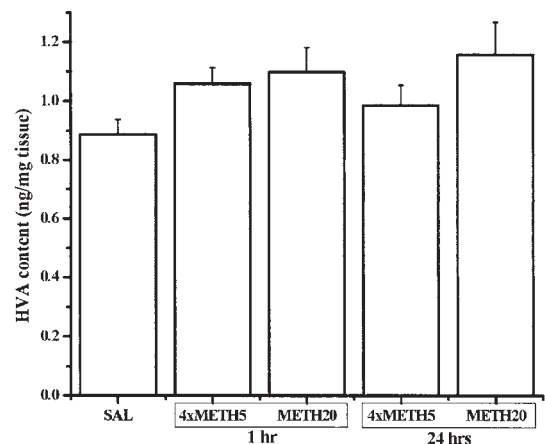


Fig. 2. Effects of METH on DA (A), DOPAC (B), and HVA (C) contents in CPU. Multiple injections of METH (4×5 mg/kg, i.p. at 2-h intervals; $4 \times$ METH5) or a single injection of METH (20 mg/kg, i.p.; METH20) was administered to adult, male SD rats. Animals were sacrificed at 1 and 24 h following treatment. Each value is presented as mean \pm SEM ($n = 4-5$). A: * $P < 0.001$ compared with saline group (SAL); # $P < 0.05$, ## $P < 0.001$ compared to METH20 at 1-h group. B: * $P < 0.05$ compared with saline group (SAL); # $P < 0.05$ compared with METH20 at 24-h group.

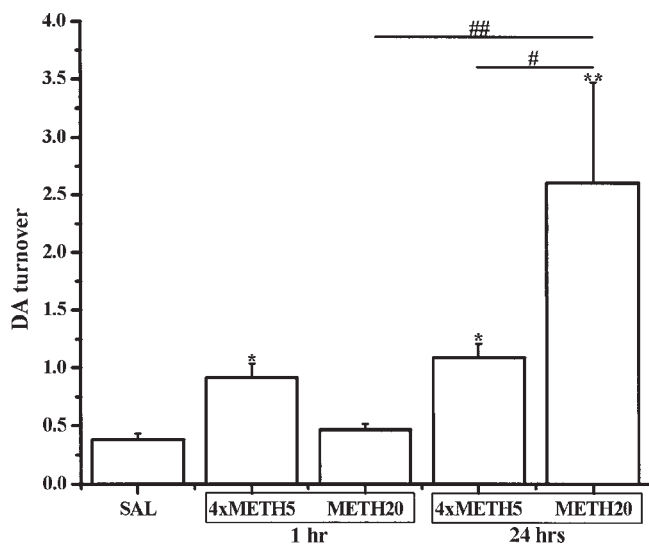


Fig. 3. Effects of METH on DA turnover ((DOPAC + HVA)/DA) in CPu. Multiple injections of METH (4 × METH5) or a single injection of METH (METH20) was administered to adult, male SD rats. Animals were sacrificed at 1 and 24 h following treatment. Each value is presented as mean ± SEM ($n = 4-5$). * $P < 0.05$, ** $P < 0.001$ compared with saline group (SAL); # $P < 0.05$, ## $P < 0.001$ compared with METH20 at 24-h group.

DA depletion induced by both treatments 24 h following the last injection was not significantly different from each other. However, simultaneous measurement of DOPAC and HVA contents in the CPu (Figs. 2B and 2C) did not show any significant alterations except for the single METH injection that increased DOPAC tissue levels at 24 h (175% of control, $P < 0.05$). Finally, both treatments induced significant alterations ($P < 0.001$) in DA turnover ((DOPAC + HVA)/DA) (Fig. 3). The multiple-dose regimen produced a similar increase in DA turnover in CPu at both endpoints (240 and 285% of control, $P < 0.05$). The single-dose regimen of METH had no significant effect on DA turnover 1 h following treatment. However, this regimen induced a robust increase (681% of control; $P < 0.001$) in DA turnover 24 h following METH injection. Moreover, the increment in DA turnover 24 h following single-dose regimen of METH was significantly higher than the one induced by the multiple-dose regimen ($P < 0.05$).

Caudate-putamen TH expression

Results from the densitometric quantitation of Western blots of CPu TH are shown in Figure 4. No significant changes were observed in TH expression in any of the dosing regimens 24 h following METH treatment.

Caudate-putamen apoptotic proteins expression

There is scarce information on the damaging effect of METH on neurons postsynaptic to the DA termi-

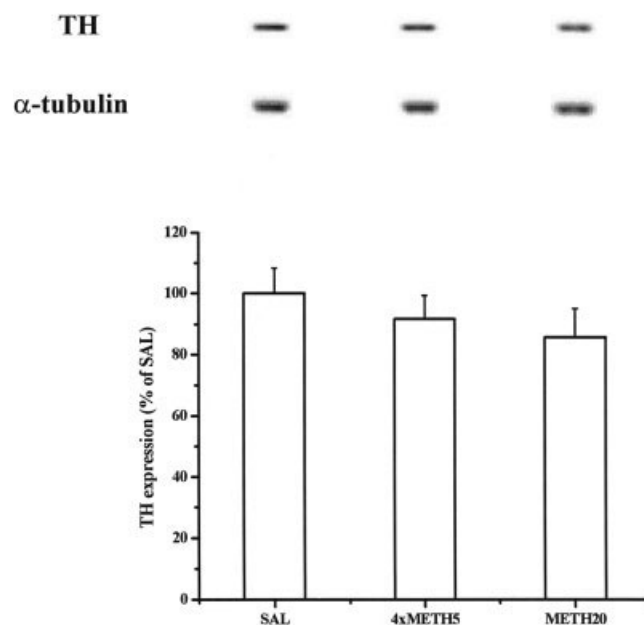


Fig. 4. Effects of METH on CPu TH expression as revealed by Western blot analysis. Multiple injections of METH (4 × METH5) or a single injection of METH (METH20) was administered to adult, male SD rats. Animals were sacrificed at 24 h following treatment. The bands shown represent the 62-kDa TH and the 50-kDa α-tubulin. Bands are taken from the same blot and are representative of three different experiments with similar results. The density of TH band/α-tubulin band determines the relative quantity of TH in each sample. Data are expressed as percentages of the control (SAL) and are presented as means ± SEM ($n = 4$ animals per group).

nals. To this end, we studied the expression of the apoptotic regulator protein Bax and the cleavage of the executioner caspase-3 under METH effect. Results from the densitometric quantitation of Western blots of CPu Bax are shown in Figure 5. Only the multiple injection of METH produced a significant ($P < 0.05$), although modest, increase in Bax expression in CPu 24 h following METH treatment (Fig. 5). However, no significant changes were observed in cleavage of the caspase-3 proenzyme (32 kDa) 24 h following METH treatment (Fig. 6).

DISCUSSION

This study showed that although both METH administration paradigms induced a significant loss of DA, with increased DA turnover in the rat CPu 24 h after treatment, however, similar dosing neither changed TH immunoreactivity nor cleaved pro-caspase-3. Typically, rodent methamphetamine research uses a single-day regimen of multiple 5–10 mg/kg i.p. injections, which result in loss of DA content in the early withdrawal period and eventually neurotoxicity (Davidson et al., 2005). In the present study, we used both multiple injections of METH (4 × 5 mg/kg, i.p.) at 2-h intervals and a single injection of METH (20 mg/kg, i.p.) to further evaluate the impact of METH

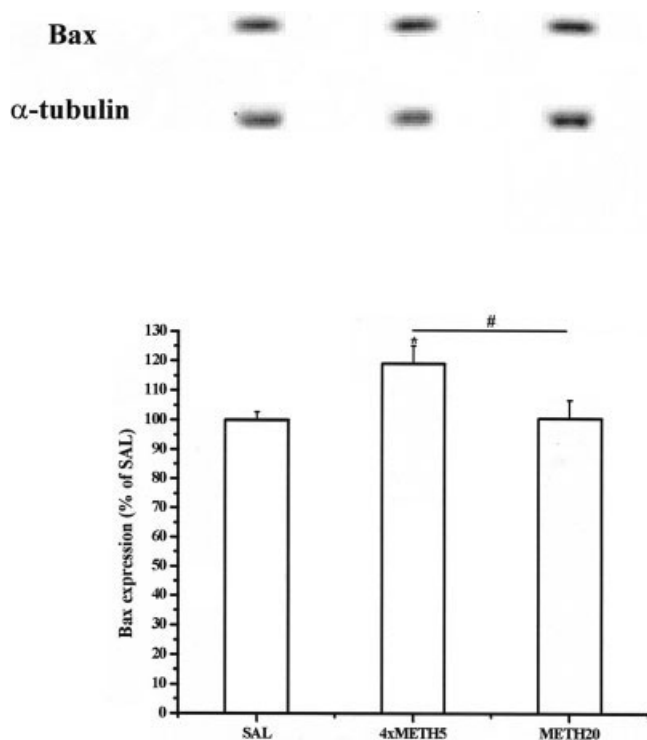


Fig. 5. Effects of METH on CPU Bax expression as revealed by Western blot analysis. Multiple injections of METH ($4 \times$ METH5) or a single injection of METH (METH20) was administered to adult, male SD rats. Animals were sacrificed at 24 h following treatment. The bands shown represent the 23-kDa Bax and the 50-kDa α -tubulin. Bands are taken from the same blot and are representative of three different experiments with similar results. The density of Bax band/ α -tubulin band determines the relative quantity of Bax in each sample. Data are expressed as percentages of the control (SAL) and are presented as means \pm SEM ($n = 4$ animals per group). * $P < 0.05$ compared with control group (SAL); # $P < 0.05$, compared with METH20 group.

on terminal dopaminergic toxicity markers, including DA levels, DA turnover, and TH immunoreactivity in CPU of rats within 24 h. Single-dose regimens may aid in uncovering the as yet unknown mechanisms of substituted amphetamine neurotoxicity, because they reduce the inherent complexity present in repeated dosage regimens (Miller and O'Callaghan, 2003). It is noteworthy that these METH doses are within the range of doses that are taken by human bingers (Davidson et al., 2005). We chose the time frame in which damage to DA nerve terminals is considered to be initiating and amplifying. For example, METH was shown to cause a significant increase in microglial activation, implicated in many forms of neuronal damage and disease 24–48 h after treatment (Thomas et al., 2004).

Most of the METH-induced neurotoxicity literatures have revolved around increased DA outflow (Cadet and Brannock, 1998). In the present study, it is shown that the first injection of METH (5 mg/kg, i.p.) produced a robust output of DA to about 14-fold

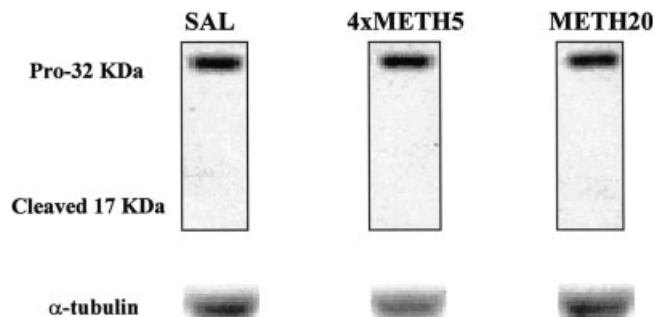


Fig. 6. Effects of METH on CPU caspase-3 cleavage as revealed by Western blot analysis. Multiple injections of METH ($4 \times$ METH5) or a single injection of METH (METH20) was administered to adult, male SD rats. Animals were sacrificed at 24 h following treatment. The bands shown represent the 32-kDa pro-caspase 3 and the 50-kDa α -tubulin. Bands are taken from the same blot and are representative of two different experiments with similar results. Each group comprises four animals.

above basal levels that stayed significantly above control levels up to 1.5 h within CPU. In recent studies, we showed that higher METH concentrations (10 and 20 mg/kg, i.p.) evoked about 20-fold increase in extracellular levels (Pereira et al., 2002, 2004). The main difference between the present and the former studies would be the duration of the dopaminergic signal: transient (5 mg/kg METH) vs. sustained (higher METH concentrations). Dopaminergic transmission is highly regulated presynaptically by auto- and heteroreceptors and DA transporters (DATs). The mutual interaction between receptors and DATs provides a complex regulatory system that shapes the DA signal (see Schmitz et al., 2003 for a recent review). Therefore, the interference of higher doses of METH with this regulation is apparently more important than the one exerted by 5 mg/kg METH. In addition, METH also inhibits DA uptake and intraneuronal oxidative metabolism of DA through MAO inhibition (Jones et al., 1998; Metzger et al., 2000). Accordingly, dialysate DOPAC levels showed a significant decrease that peaked within ~ 1 h and stayed significantly decreased up to 2 h following 5 mg/kg METH treatment. As most of striatal DA measured in postmortem tissue is intracellular rather than synaptic, our finding of tissue concentrations of DA suggests that both METH treatments lead to depletion of tissue stores in rat at 24 h. It is noteworthy that DA depletion induced by both treatments was not significantly different. DA depletion from striatum faithfully reflects other measures of DA nerve ending damage caused by METH, such as reduced TH immunoreactivity (Thomas et al., 2004). However, none of the dosing regimens induced changes in TH expression at 24 h. Striatal TH immunoreactivity is one of the markers used to assess the integrity of dopaminergic terminals. Therefore, our data suggest that DA depletion does not correspond to terminal degeneration within

this time frame. This is consistent with chronic exposure to METH inducing reduced striatal DA levels without causing permanent degeneration of striatal DA nerve terminals in young human subjects (Wilson et al., 1996). This DA depletion could reflect, instead, a compensatory downregulation of DA biosynthesis in response to prolonged dopaminergic stimulation caused by METH (Wilson et al., 1996). Interestingly, Wang et al. (2004, 2005) showed similar results with MDMA inducing 5-HT depletion, independent of terminal degeneration. Alternatively, Wang et al. (2004) showed that 7.5 mg/kg METH (four times at 2-h intervals) induced a decrease in TH immunoreactivity in rat CPu at 24 h. The dosing regimen and the technique used to measure TH immunoreactivity by this group are different from those used in our study. Therefore, caution should be taken while drawing a comparison between studies. However, our data does not rule out the possibility that METH treatment could induce TH depletion at later time points. In fact, Fukumara et al. (1998) found that a single dose of 20 mg/kg METH reduced TH immunoreactivity 3 days posttreatment. Also, Zhu et al. (2005) demonstrated that using a single bolus of METH (30 mg/kg) does not cause depletion of TH at 16 or 24 h, but induced TH depletion at 2 days, reaching the lowest point 3 days after exposure. These might suggest that down-regulation of TH and other events at the DA terminals (e.g., inhibition of vesicular DA uptake (Brown et al., 2000)) must precede the depletion of TH.

Increased DA turnover after METH treatment, and thus the potential for increased oxidative stress, is also widely used as an index of METH-induced toxicity to DA nerve endings. The DA turnover increase shown in the present study may reflect the combination of two factors: (1) DA depletion and (2) an increase in intraneuronal oxidative metabolism of DA through MAO, as indicated by either no changes or an increase in DOPAC tissue content (multiple and single injection, respectively). Taking into consideration that increased turnover elicited by single dose of METH was significantly higher than that evoked by the multiple doses at 24 h, this suggests that single injection is more important to dopaminergic terminals than the multiple-injection paradigm. This might be due to pharmacodynamics of a bulk injection of 20 mg/kg rather than 4×5 mg/kg of METH. Cohen (1984) pointed out that conditions that increase turnover of DA might also increase the formation of reactive oxygen species. Consistently, Pereira et al. (2004) showed that 10 mg/kg METH evoked a delayed $\cdot\text{OH}$ formation in rat CPu when compared with the fast DA peak. Drug-induced oxidative stress is an attractive construct that can account for many of the effects of METH on the DA nerve endings, such as inhibition of TH activity (Haughey et al., 1999). It could also be an early event that would eventually lead to METH-

induced apoptosis (Cadet et al., 2003). Recent animal studies have suggested that in addition to the involvement of pro-death genes of the Bcl-2 family (Bax), AMPH and METH toxicity are associated with increases in caspase-3 activity in striatal postsynaptic neurons (Cadet et al., 2003; Jayanthi et al., 2004; Krasnova et al., 2005). The present work also aimed to evaluate whether the typical neurotoxic regimen (4×5 mg/kg METH, i.p.; at 2-h intervals) and the single METH injection (20 mg/kg, i.p.) would also change Bax and activate caspase-3 expression, thus triggering striatal apoptotic mitochondria-dependent biochemical cascades in rat CPu 24 h posttreatment. Again, it is important to assess early time points because cells undergoing apoptosis might be removed by endogenous phagocytes and microglia, before the damaged cells can lyse and spill their contents into surrounding areas (Deng et al., 1999). Only the multiple-injection paradigm produced an increase in Bax expression in Cpu, although not sufficient to induce cleavage of the caspase-3 proenzyme at 24 h. This is the first study to show an increase in Bax expression in rat striatal cells in vivo induced by METH. Our observation is in line with METH inducing overexpression of Bax in an immortalized cell line (Deng et al., 2002). The lack of activation of caspase-3 is not surprising, considering that Bowyer et al. (1994) did not find any evidence of cell death in rat striatum following 4×5 mg/kg METH treatment. On the contrary, Jayanthi et al. (2004, 2005) showed that 40 mg/kg METH induced cleavage of pro-caspase-3 peaking between 8 and 24 h in mice striatum and caspase-3 activation at 24 h in rat striatum. However, it should be pointed out that in these studies, the doses of METH used were twice as much as was used in our study.

Finally, one should not neglect the hypothesis that there could be apoptotic processes that progress independent of caspase activity and that may possibly be mediated by release of the apoptosis inducing factor (AIF) from mitochondria and their translocation to the nucleus (Chu et al., 2005; Cregan et al. 2002; Joza et al. 2001). Interestingly, Jayanthi et al. (2004) reported that the release of AIF from mitochondria was detected 30 min after 40 mg/kg METH injection and in the nuclear fraction 4 h thereafter in mouse striatum.

As demonstrated in the present study, METH has greater impact on the presynaptic terminals than it does on the postsynaptic neurons, suggesting that presynaptic terminals are subjected to more damaging influences or that the nigrostriatal pathway is more vulnerable to toxic injury. We further suggest that METH-induced dopaminergic neurotoxicity, as seen by DA depletion and increased DA turnover, may occur independently of terminal degeneration. Bowyer et al. (1992, 1994) reported that METH-induced terminal degeneration generally occurs only with the use of

regimens producing a lethal hyperthermia ($\geq 41^\circ\text{C}$) that often requires an "ice-bath rescue." In the present study, all animals survived the dosing regimens. However, we did not measure body temperature, and there is a possibility that we had some transient hyperthermia that was not lethal. It is possible that alternative mechanisms, besides axonal degeneration, are operational in decreasing dopaminergic phenotypic markers in the METH-exposed brain, as was suggested by Guilarte et al. (2003). It is also proposed that METH-induced striatal neurotoxicity is not dependent on mitochondria-driven apoptotic cascades through caspase-3 activation. The extent of neurotoxicity to striatal postsynaptic neurons might be dependent upon several factors including: (a) animal species, (b) dosage and frequency of drug administration, (c) the ambient temperature during drug administration, (d) thermoregulation, and (e) seizures. This study further stresses that the use of a single dose model proves to be a valuable tool to further understand the mechanisms underlying METH-induced nigrostriatal dopaminergic neurotoxicity.

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