Methamphetamine-Induced Dopamine Transporter Complex Formation and Dopaminergic Deficits: The Role of D2 Receptor Activation

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Abbreviations: DAT, dopamine transporter; eticlopride, S-(−)-3-Chloro-5-ethyl-N-[(1-ethyl-2-pyrrolidinyl)methyl]-6-hydroxy-2-methoxybenzamide hydrochloride; GFAP, glial fibrillary acidic protein; METH, methamphetamine; SCH23390, R(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride; VMAT2, vesicular monoamine transporter 2.

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ABSTRACT

Methamphetamine (METH) abuse is a serious public health issue. Particularly concerning are findings that repeated, high-dose administrations of METH cause persistent dopaminergic deficits in rodents, non-human primates, and humans. Previous studies have also revealed that METH-treatment causes alterations in the dopamine transporter (DAT), including the formation of higher molecular weight DAT-associated complexes. The current study extends these findings by examining mechanisms underlying DAT complex formation. The association among DAT complex formation and other METH-induced phenomena, including alterations in vesicular monoamine transporter 2 (VMAT2) immunoreactivity, astrocytic activation (as assessed by increased glial fibrillary acidic protein (GFAP) immunoreactivity), and persistent dopaminergic deficits was also explored. Results revealed that METH-induced DAT complex formation and reductions in VMAT2 immunoreactivity precede increases in GFAP immunoreactivity. Further, and as reported previously for DAT complexes, pretreatment with the D2 receptor antagonist, eticlopride, attenuated the decrease in VMAT2 immunoreactivity as assessed 24 h after METH treatment. DAT complexes distinct from those present 24 h after METH treatment, decreases in VMAT2 immunoreactivity, and increased GFAP immunoreactivity were present 48 - 72 h after METH-treatment. Pretreatment with eticlopride attenuated these each of these phenomena. Finally, DAT complexes were present 7 d after METH-treatment, a time-point at which VMAT2 and DAT monomer immunoreactivity were also reduced. Eticlopride pretreatment attenuated each of these phenomena. These findings provide novel insight into not only receptor mediated mechanisms underlying the effects of METH but also the interaction among factors that are likely associated with the persistent dopaminergic deficits caused by the stimulant.
INTRODUCTION

Methamphetamine (METH) is a highly addictive psychostimulant whose abuse has significant individual and societal costs. One concern associated with METH abuse is the potential for long-term dopaminergic deficits. For example, METH abusers have reduced striatal dopamine (DA) transporter (DAT) densities (McCann et al., 1998; Wilson et al., 1996); an effect that has been associated with motor slowing and memory impairment (Volkow et al., 2001) and may be related to psychiatric symptoms (Sekine et al., 2001).

Studies involving rodents indicate there are many effects caused by repeated, high-dose administrations of METH including, but not limited to, oxidative stress (for review, see Brown and Yamamoto, 2003; Krasnova and Cadet, 2009), astrocytic/microglial activation (LaVoie et al., 2004; Thomas et al., 2004; O’Callaghan and Miller, 1994), DAT complex formation (Baucum et al., 2004; Hadlock et al., 2009), and alterations in vesicular monoamine transporter 2 (VMAT2) function (Brown et al., 2000; Eyerman and Yamamoto, 2007; Guillot et al., 2008). However, the relationship among these factors has not been elucidated fully. Additionally, an association between METH-induced DAT complex formation and persistent dopaminergic deficits has been suggested (Baucum et al., 2004; Hadlock et al., 2009) but has not been studied specifically. Accordingly, the purpose of this study was to investigate possible associations among three of these phenomena and METH-induced persistent dopaminergic deficits, in particular: METH-induced DAT complex formation, alterations in VMAT2 immunoreactivity, and astrocytic activation. Results revealed that alterations in VMAT2 and DAT immunoreactivity precede increases in glial fibrillary acidic protein (GFAP) immunoreactivity, a marker of astrocytic activation and neuronal damage (Eng et al., 2000). Further, high molecular weight DAT complexes distinct from those apparent 24 h after METH treatment were present 48 – 72 h, and also 7 d after METH-treatment. Finally, D2 receptor activation contributes to each of these phenomena.
METHODS

Animals. Male Sprague-Dawley rats (290-400 g; Charles River Laboratories, Inc., Raleigh, NC) were maintained under controlled lighting and temperature with constant access to food and water. Rats were housed 3-4 animals per cage during the experiments. METH-treated rats were maintained at warmer temperatures to ensure METH-induced hyperthermia. Rectal temperatures were assessed at 1-h intervals beginning 30 min prior to the first saline or METH injection. For experiments involving pretreatment with D1 or D2 receptor antagonists, rats received intraperitoneal injections of the drug treatment or saline vehicle 30 min prior to each saline or METH injection. Mean temperatures over the course of the experiments were determined. For METH-treated rats, only rats which achieved mean rectal temperatures greater than 38 °C over the course of the experiment were used for analysis. Rats were sacrificed by decapitation. All procedures were approved by the University of Utah Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Drugs and Chemicals. S-(-)-3-Chloro-5-ethyl-N-[(1-ethyl-2-pyrrolidinyl)methyl]-6-hydroxy-2-methoxybenzamide (eticlopride) hydrochloride and R(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine (SCH23390) hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). (±) METH hydrochloride was supplied by Research Triangle Institute (Research Triangle Park, NC). Drugs doses were calculated as the free base. Drugs were dissolved in 0.9% saline vehicle.

Tissue Preparation and Western Blot Analysis. Synaptosomal tissues were prepared as previously described (Baucum et al., 2004). Briefly, striata were dissected, homogenized in ice-cold 0.32 M sucrose, pH 7.4, and centrifuged (800 x g, 12 min; 4°C). Supernatants were centrifuged (22,000 x g, 15 min; 4°C) and the resultant pellets were resuspended in ice-cold
double distilled H$_2$O at concentrations of 45-55 mg/ml original wet weight. Total protein concentrations were determined as described by Bradford (1976). The samples were then diluted with a non-reducing loading buffer (final concentration: 2.25% SDS, 18% glycerol, 180 mM Tris base, pH 6.8, and bromophenol blue) and frozen at -80 °C until Western blot analysis. Equal quantities of total protein (4-10 µg) were loaded onto a 4-12% NuPAGE Novex Bis-Tris Midi gradient gel (Invitrogen, Carlsbad, CA) and electrophoresed using a XCell4 Surelock Midi-Cell (Invitrogen). Samples were then transferred to a polyvinylidene difluoride hybridization transfer membrane (PerkinElmer Life and Analytical Sciences, Waltham, MA). Western blot analysis was performed as described previously (Hadlock et al., 2009). Overall DAT complex immunoreactivity was defined as immunoreactivity greater than ~120 kDa and was determined for data presented in Fig. 1. As the majority of DAT complex immunoreactivity shifts to higher molecular weights at 72 h and remains at 7 d following METH-treatment, only the highest molecular weight regions of Western blots were used for determining DAT immunoreactivity as presented in Figs. 4 and 6, approximately the top third of the overall DAT complex range, spanning from ~120 kDa to the top of the gel. DAT was detected using a rabbit polyclonal N-terminal DAT antibody (generously provided by Dr. Roxanne Vaughan, University of North Dakota). VMAT2 was detected using a rabbit polyclonal antibody (AB1767; Millipore, Billerica, MA). GFAP was detected using a mouse monoclonal antibody (556329; BD Bioscience, San Jose, CA).

Data Analysis. Analysis of variance, followed by Newman-Keuls post hoc test, was performed to determine significant differences among experimental groups. Differences were considered significant if the probability of error was less than or equal to 5%. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA).
RESULTS

Results presented in Fig. 1 demonstrate that overall DAT complex immunoreactivity was increased (A and B) and DAT monomer immunoreactivity was decreased (A and C), as assessed 24, 48, and 72 h after METH-treatment. Noteworthy, differences in complex immunoreactivity became apparent over time, with the majority of DAT complex immunoreactivity concentrated at the highest molecular weights 48 and 72 h following METH-treatment (Fig. 1A). VMAT2 immunoreactivity was decreased 24, 48, and 72 h after METH treatment (Fig. 2A). GFAP immunoreactivity was increased at 48 and 72, but not 24 h, after METH treatment (Fig. 2B).

As demonstrated for METH-induced DAT complex formation (Hadlock et al., 2009), pretreatment with the D2 receptor antagonist, eticlopride (Fig. 3A), but not the D1 receptor antagonist, SCH23390 (Fig. 3B), attenuated the METH-induced decrease in VMAT2 immunoreactivity, as assessed 24 h after treatment. In both experiments, elevated body temperatures were maintained over the course of METH treatment in all METH-treated rats, including those pretreated with eticlopride (mean temperature of 39.4°C ± 0.2 and 39.6°C ± 0.1 for saline/METH- and eticlopride/METH-treated rats, respectively) or SCH23390 (mean temperature of 39.3°C ± 0.1 and 39.3°C ± 0.2 for saline/METH- and SCH23390/METH-treated rats, respectively). Fig. 4 demonstrates that eticlopride pretreatment also attenuated METH-induced DAT complex formation (Fig. 4A) and decreases in DAT monomer immunoreactivity (Fig. 4B), as assessed 72 h after treatment. At this time-point, eticlopride pretreatment also attenuated METH-induced decreases in VMAT2 immunoreactivity (Fig. 5A) and increases GFAP immunoreactivity (Fig. 5B). In this experiment, elevated body temperatures were maintained in all METH-treated rats (mean temperature of 39.6 ± 0.1 °C and 39.4 ± 0.1 °C for saline/METH and eticlopride/METH-treated rats, respectively).

Results presented in Fig. 6A and B reveal that DAT complexes were present 7 d following METH-treatment and their formation was attenuated by eticlopride pretreatment.
Noteworthy, and as after 48 and 72 h (Fig. 1A), the majority of overall DAT complex immunoreactivity was concentrated in the highest molecular weight regions of the Western blots. At this time-point, there were also decreases in DAT monomer immunoreactivity (Fig. 6C), decreases in VMAT2 immunoreactivity (Fig. 7A), and increases in GFAP immunoreactivity (Fig. 7B). All of these effects were attenuated by eticlopride pretreatment. In this experiment, elevated body temperatures were maintained in all METH-treated rats (mean temperature of 39.1 ± 0.2 °C and 38.9 ± 0.1 °C for saline/METH and eticlopride/METH-treated rats, respectively).
DISCUSSION

Previous studies have revealed that METH treatment causes alterations in monoaminergic transporters. For example, multiple, high-dose administrations of METH rapidly (within 1 h) decrease VMAT2 activity (Brown et al., 2000), an effect that may be caused by a rapid redistribution of VMAT2 to a location that is not retained in the preparation of synaptosomes (Riddle et al., 2002) and oxidation of VMAT2 (Eyerman and Yamamoto, 2007). The decrease in VMAT2 function, as well as a loss of VMAT2 immunoreactivity, persists 24 h following treatment (Chu et al., 2009; Eyerman and Yamamoto, 2005). The present studies extend this work by demonstrating that the loss of VMAT2 immunoreactivity after 24 h is attenuated by pretreatment with the D2 receptor antagonist, eticlopride. Similarly, pretreatment with eticlopride attenuates the reduction in VMAT2 immunoreactivity after 72 h. These results are of significance as several studies have indicated that aberrant VMAT2 function contributes to the monoaminergic deficits caused by METH. For example, pretreatment with the VMAT2 inhibitor, reserpine, worsens the dopaminergic deficits caused by METH (Wagner et al., 1983; Thomas et al., 2008). Additionally, heterozygous VMAT2 knock-out mice exhibit increased METH-induced dopaminergic deficits (Fumagalli et al., 1999). Noteworthy, treatment of mice with pituitary adenylyl cyclase activating polypeptide, 38 amino acids increases the expression and function of VMAT2 and attenuates METH-associated astrocytic activation (Guillot et al., 2008a).

The involvement of D2 receptor activation in the effects of METH is not restricted to VMAT2. In particular, both the METH-induced increase in DAT complex formation and decrease in DAT activity observed after 24 (Hadlock et al., 2009) and 72 h (Fig. 4) are attenuated by D2 antagonist pre-treatment. Thus, D2 receptor-mediated mechanisms underlie both the METH-induced alterations in DAT and VMAT2 24 - 72 h after treatment.

As oxidative stress likely contributes to DAT complex formation (Baucum et al., 2004; Hadlock et al., 2009) and METH-induced alterations in VMAT2 may contribute to oxidative
stress (for review, see Fleckenstein et al., 2009), it is reasonable to speculate that the alterations in VMAT2 contribute to DAT complex formation. This may occur since METH redistributes vesicular DA into the cytosol and causes DA-related oxidative stress (Cubells et al., 1994; for review, see Brown and Yamamoto, 2003; Krasnova and Cadet, 2009). A reduction in VMAT2 function/protein would promote this oxidative stress, as less DA would be sequestered. Indeed, reduced vesicular DA sequestration exacerbates METH-induced dopaminergic deficits (Wagner et al., 1983; Fumagalli et al., 1999; Guillot et al., 2008b; Thomas et al., 2008).

Astrocytes and microglia are activated after neuronal insults (Whitney et al., 2009), including METH treatment (LaVoie et al., 2004; Thomas et al., 2004; O’Callaghan and Miller, 1994). Consistent with these findings, results revealed that METH treatment increased GFAP immunoreactivity as assessed 48 and 72 h after treatment. Interestingly, the onset of this increase occurred after METH-induced DAT complex formation and loss of VMAT2 immunoreactivity. Like the effects on DAT and VMAT2, pretreatment with eticlopride attenuated this phenomenon as assessed 72 h after treatment. These results permit speculation that the earlier D2 receptor-mediated alterations and deficits in VMAT2 and DAT, described above, may contribute to astrocytic activation.

DAT complexes are present within 24 h and remain 7 d following METH treatment. However, a novel finding of the present study is that the nature of the DAT complexes change over time. Specifically, the DAT complexes present at 48 and 72 h are different than the DAT complexes at 24 h as evidenced by findings that the majority of overall immunoreactivity is concentrated in the highest molecular weight regions of the western blots. Further, DAT complexes present at 7 d also have a much higher molecular weight than those observed 24 h following METH-treatment. There are a number of possible explanations for these phenomena. For instance, the lower molecular weight DAT complexes present at 24 h may be preferentially degraded such that the higher molecular weight DAT complexes predominate at 48 – 72 h, and
at 7 d. Alternatively, the lower molecular weight DAT complexes present at 24 h may continue to increase in molecular weight to create the high molecular weight DAT complexes observed at 48 – 72 h and 7 d. Noteworthy, DAT monomer immunoreactivity levels do not change significantly between 24 - 72 h, suggesting that alterations in DAT complex immunoreactivity may be due primarily to DAT protein that has already formed complexes. Previous studies have suggested that DAT complex formation occurs through an oxidative mechanism (Baucum et al., 2004; Hadlock et al., 2009). The broad molecular weight range of the complexes observed in this and previous studies may be caused by numerous additional modifications and/or protein-protein interactions. Although further studies are needed to determine the precise composition of the DAT complexes, it is reasonable to postulate that an oxidative mechanism also contributes to the increase in molecular weight of DAT complexes seen at 48 h, 72 h, and 7 d following METH-treatment. This prolonged oxidative stress may be caused by numerous mechanisms involved in METH-induced neurotoxicity that cause oxidative stress including, but not limited to, microglial activation, astrocytic activation, and excitotoxicity (for review, see Krasnova and Cadet, 2009; Quinton and Yamamoto, 2006).

Previous studies have suggested that DAT complex formation may be associated with persistent dopaminergic deficits (Baucum et al., 2004; Hadlock et al., 2009). This is supported by evidence that prevention of METH-induced hyperthermia or prior treatment with the DA-depleting agent, α-methyl-p-tyrosine, attenuates both DAT complex formation (Baucum et al., 2004) and the persistent dopaminergic deficits caused by the stimulant (Schmidt et al., 1985; Bowyer et al., 1992). Additionally, METH-induced DAT complex formation does not occur in the nucleus accumbens (Hadlock et al., 2009), a brain region that is refractory to the METH-induced persistent dopaminergic deficits (Thomas et al., 2009; Eisch et al., 1992; Cass, 1997; Haughey et al., 1999; but see also Broening et al., 1997). The present findings further suggest an association between DAT complex formation and persistent METH-induced dopaminergic deficits since both phenomena are attenuated by D2 receptor antagonist pretreatment, even
when hyperthermia was maintained in the eticlopride pretreated rats (see also Broening et al., 2005).

In conclusion, the present study suggests an association between METH-induced DAT complex formation, decreases in VMAT2 immunoreactivity, astrocytic activation, and persistent dopaminergic deficits as each is prevented by D2 antagonist pretreatment. While these results do not prove causal relationships among these events, they allow speculation that early (within 24 h) alterations in DAT and/or VMAT2 contribute to astrocytic activation, and that each contributes to the persistent dopaminergic deficits caused by the stimulant. These findings provide novel insight into not only receptor mediated mechanisms underlying the effects of METH, but also the interaction among factors that are likely associated with the persistent dopaminergic deficits caused by the stimulant.
REFERENCES


FOOTNOTES

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FIGURE LEGENDS

**Fig. 1.** Time-course of METH-induced DAT complex formation (A and B) and loss of DAT monomer immunoreactivity (A and C). Rats received 4 injections of METH (7.5 mg/kg/injection; s.c.; 2-h intervals) or saline vehicle (1 mg/kg/injection; s.c.; 2-h intervals) and were sacrificed 24, 48, or 72 h later. Panel A presents a representative blot of samples from saline-treated rats (lane 1) and METH-treated rats 24 h (lane 2), 48 h (lane 3), and 72 h (lane 4) following treatment. Molecular weights (kDa) are indicated adjacent to the representative blot. Columns represent the means and vertical lines 1 SEM determinations in 5-10 rats. *, values different from saline-treated controls (p \( \leq \) 0.05).

**Fig. 2.** Time-course of METH-induced alterations in VMAT2 (A) and GFAP immunoreactivity (B). Rats received 4 injections of METH (7.5 mg/kg/injection; s.c.; 2-h intervals) or saline vehicle (1 mg/kg/injection; s.c.; 2-h intervals) and were sacrificed 24, 48, or 72 h later. Molecular weights (kDa) are indicated adjacent to the representative blot. Columns represent the means and vertical lines 1 SEM determinations in 5-10 rats. *, values different from saline-treated controls (p \( \leq \) 0.05).

**Fig. 3.** Eticlopride pretreatment (A), but not SCH23390 pretreatment (B), attenuates METH-induced loss of VMAT2 as assessed 24 h after METH-treatment. Rats received 4 injections of METH (7.5 mg/kg/injection; s.c.; 2-h intervals) or saline vehicle (1 ml/kg/injection; s.c.; 2-h intervals) and were sacrificed 24 h later. Thirty min prior to each injection, rats were pretreated with eticlopride (A; 0.5 mg/kg/injection; i.p.), SCH23390 (B; 0.5 mg/kg/injection; i.p.), or saline vehicle (1 ml/kg/injection; i.p.). Columns represent the means and vertical lines 1 SEM determinations in 6-9 rats. *, values different from saline-treated controls (p \( \leq \) 0.05).
Fig. 4. Eticlopride pretreatment attenuates METH-induced DAT complex formation (A) and loss of DAT monomer immunoreactivity (B), as assessed 72 h following METH-treatment. Rats received 4 injections of METH (7.5 mg/kg/injection; s.c.; 2-h intervals) or saline vehicle (1 ml/kg/injection; s.c.; 2-h intervals) and were sacrificed 72 h later. Thirty min prior to each injection, rats were pretreated with eticlopride (0.5 mg/kg/injection; i.p.) or saline vehicle (1 ml/kg/injection; i.p.). Columns represent the means and vertical lines 1 SEM determinations in 6-10 rats. *, values different from saline-treated controls (p ≤ 0.05). †, values different from saline/METH-treated group (p ≤ 0.05).

Fig. 5. Eticlopride pretreatment attenuates METH-induced loss of VMAT2 (A) and increased GFAP immunoreactivity (B), as assessed 72 h following METH-treatment. Rats received 4 injections of METH (7.5 mg/kg/injection; s.c.; 2-h intervals) or saline vehicle (1 ml/kg/injection; s.c.; 2-h intervals) and were sacrificed 72 h later. Thirty min prior to each injection, rats were pretreated with eticlopride (0.5 mg/kg/injection; i.p.) or saline vehicle (1 ml/kg/injection; i.p.). Columns represent the means and vertical lines 1 SEM determinations in 6-10 rats. *, values different from saline-treated controls (p ≤ 0.05). †, values different from saline/METH-treated group (p ≤ 0.05).

Fig. 6. Eticlopride pretreatment attenuates METH-induced DAT complex formation (A and B) and loss of DAT monomer immunoreactivity (A and C), as assessed 7 d after METH-treatment. Rats received 4 injections of METH (7.5 mg/kg/injection; s.c.; 2-h intervals) or saline vehicle (1 ml/kg/injection; s.c.; 2-h intervals) and were sacrificed 7 d later. Thirty min prior to each injection, rats were pretreated with eticlopride (0.5 mg/kg/injection; i.p.) or saline vehicle (1 ml/kg/injection; i.p.). Panel A presents a representative blot of saline/saline- (lane 1), saline/METH- (lane 2), eticlopride/saline- (lane 3), and eticlopride/METH- (lane 4) treated samples. Molecular weight (kDa) is shown adjacent to the representative blot. Columns
represent the means and vertical lines 1 SEM determinations in 6-8 rats. *, values different from saline-treated controls (p ≤ 0.05).

**Fig. 7.** Eticlopride pretreatment attenuates METH-induced loss of VMAT2 (A) and increase in GFAP immunoreactivity (B), as assessed 7 d after METH-treatment. Rats received 4 injections of METH (7.5 mg/kg/injection; s.c.; 2-h intervals) or saline vehicle (1 ml/kg/injection; s.c.; 2-h intervals) and were sacrificed 7 days later. Thirty min prior to each injection, rats were pretreated with eticlopride (0.5 mg/kg/injection; i.p.) or saline vehicle (1 ml/kg/injection; i.p.). Columns represent the means and vertical lines 1 SEM determinations in 6-8 rats. *, values different from saline-treated controls (p ≤ 0.05).
Figure 1
Figure 2

A

VMAT2 immunoreactivity (arbitrary units)

saline  24  48  72

B

GFAP immunoreactivity (arbitrary units)

saline  24  48  72

kDa  64  51

kDa  51
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7

A

**VMAT2 immunoreactivity** (arbitrary units)

- saline/saline
- saline/METH
- eticlopride/saline
- eticlopride/METH

B

**GFAP immunoreactivity** (arbitrary units)

- saline/saline
- saline/METH
- eticlopride/saline
- eticlopride/METH