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CM156, a high affinity sigma ligand, attenuates the stimulant and neurotoxic effects of methamphetamine in mice

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Abstract

Methamphetamine (METH) is a highly addictive psychostimulant drug of abuse. Low and high dose administration of METH leads to locomotor stimulation, and dopaminergic and serotonergic neurotoxicity, respectively. The behavioral stimulant and neurotoxic effects of METH can contribute to addiction and other neuropsychiatric disorders, thus necessitating the identification of potential pharmacotherapeutics against these effects produced by METH. METH binds to σ receptors at physiologically relevant concentrations. Also, σ receptors are present on and can modulate dopaminergic and serotonergic neurons. Therefore, σ receptors provide a viable target for the development of pharmacotherapeutics against the adverse effects of METH. In the present study, CM156, a σ receptor ligand with high affinity and selectivity for σ receptors over 80 other non- σ binding sites, was evaluated against METH-induced stimulant, hyperthermic, and neurotoxic effects. Pretreatment of male, Swiss Webster mice with CM156 dose dependently attenuated the locomotor stimulation, hyperthermia, striatal dopamine and serotonin depletions, and striatal dopamine and serotonin transporter reductions produced by METH, without significant effects of CM156 on its own. These results demonstrate the ability of a highly selective σ ligand to mitigate the effects of METH.

1. Introduction

Methamphetamine (METH), a synthetic psychostimulant drug of abuse, has increased in popularity due to its ease of synthesis from over-the-counter drugs and its long duration of action (Krasnova and Cadet, 2009). The acute effects of METH, including enhanced locomotor activity, are caused by the release of dopamine in the mesolimbic brain region (Campbell et al., 1997). Moreover, the locomotor stimulant effects are also linked with the ability of psychostimulants to act as reinforcers and cause addiction (Wise and Bozarth, 1987). Chronic, high dose administration of METH results in hyperthermia, cardiac complications, neurotoxicity and several neurological disabilities including addiction (Cruickshank and Dyer, 2009). At high doses, neurotoxic cascades are activated due to

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excessive dopamine and serotonin (5-HT) release into the cytoplasm and synapse (Kuczenski et al., 1995; Baldwin et al., 1993; Gough et al., 2002). Neuroimaging and postmortem studies in human METH users, as well as studies performed using animal models, have shown damage to dopaminergic and serotonergic neurons measured as reductions in dopamine transporters (DAT), serotonin transporters (SERT), dopamine and 5-HT levels in several brain regions (Krasnova and Cadet, 2009). Recent studies have indicated an association between neurotoxicity and several neuropsychiatric disorders including psychosis, motor and cognitive deficits (Cruickshank and Dyer, 2009; Walsh and Wagner, 1992), conferring additional urgency to the development of effective pharmacotherapies against the effects of METH.

In the present study, the role of σ receptors in METH-induced locomotor stimulation and neurotoxicity is evaluated. We focus on σ receptors for several reasons. First, METH binds to σ receptors at physiologically relevant concentrations (Nguyen et al., 2005). Second, σ receptors are present in the basal ganglia and affect motor function (Bouchard and Quirion, 1997; Walker et al., 1993), indicating their potential involvement in the locomotor stimulant effects of METH. Third, σ receptors are present on dopamine and 5-HT neurons, which are damaged by neurotoxic doses of METH, and can modulate their function (Bastianetto et al., 1995; Derbez et al., 2002; Campbell et al., 1989). Fourth, σ receptors participate in cell death pathways in tumor cells (Bowen, 2000), suggesting their possible contribution in METH-induced neurotoxic cascades. Fifth, targeting σ receptors can attenuate chronic disabilities like cognitive deficits, psychosis, and depression, which are also caused by METH's neurotoxicity (Cruickshank and Dyer, 2009). Finally, a selective σ receptor ligand, AC927 (1-(2-phenethyl)piperidine oxalate), has been shown to prevent METH-induced locomotor activity, hyperthermia, and dopaminergic neurotoxicity (Matsumoto et al., 2008).

Recently, a novel σ receptor ligand, CM156 (3-(4-(4-cyclohexylpiperazin-1-yl)butyl)benzo[d]thiazole-2(3*H*)-thione), was developed and shown to have an even better nanomolar affinity for σ receptors than AC927 (K_i of CM156: $\sigma_1 = 1.3$ nM, $\sigma_2 = 0.6$ nM; K_i of AC927: $\sigma_1 = 30$ nM, $\sigma_2 = 138$ nM), and >1,000-fold selectivity over 80 other non- σ binding sites (Xu et al., 2010; Matsumoto et al., 2008). Additionally, a larger therapeutic window in comparison to AC927, makes CM156 more amenable as a potential drug candidate. In an earlier study, CM156 was shown to attenuate the toxic and behavioral effects of cocaine, a psychostimulant that acts as a putative agonist at σ receptors (Xu et al., 2010). Therefore, in the present study, CM156 was evaluated against the stimulant and neurotoxic effects of METH. Endpoints that were evaluated included locomotor stimulation, hyperthermia, as well as dopaminergic and serotonergic neurotoxicity.

2. Material and methods

2.1. Animals

Male, Swiss Webster mice (21–30 g, Harlan, Indianapolis, IN; Frederick, MD) were used. The rodents were housed 1–6 per cage with a 12:12-h light/dark cycle and *ad libitum* food and water. The animals were given one week to acclimate after their arrival before they were used in an experiment. All procedures were approved by the Institutional Animal Care and Use Committees at West Virginia University and the University of Mississippi. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques, if available.

2.2. Drugs and reagents

METH hydrochloride was obtained from Research Biochemicals International (Natick, MA). CM156 was synthesized as previously described (Mesangeau et al., 2008). Dopamine

and 5-HT immunoassay kits were purchased from Rocky Mountain Diagnostics (Colorado Springs, CO).

2.3. Locomotor activity

Locomotor activity was measured as an index of the stimulant effects of METH using an automated activity monitoring system (San Diego Instruments, San Diego, CA). The mice were given 30–60 min to acclimate to the testing room before being habituated to the testing chambers for an additional 30 min. Each testing chamber consisted of two 16 × 16 photobeam arrays to detect the movements of the animals. Ambulatory, fine, and rearing movements were quantified to give an overall activity score. To obtain a dose response for METH-induced locomotor activity, the mice ($n = 41$) were given a 15 min pretreatment with saline followed by saline or METH (0.1–5 mg/kg, i.p.). The animals were then returned to the testing chambers and their activity was quantified for the next 30 min.

To evaluate the effects of CM156 on the locomotor stimulant effects of METH, three types of experiments were performed. For all of the studies, the pretreatment time was 15 min, which is based on effective pretreatment times for earlier pharmacological studies involving σ receptor ligands (Nguyen et al., 2005; Matsumoto et al., 2008). In addition, this pretreatment time was previously successful for CM156 in attenuating the locomotor stimulant effects of cocaine (Xu et al., 2010).

First, the effects of CM156 prior to receiving the locomotor stimulant dose of METH were determined. The mice ($n = 80$) were pretreated with saline or CM156 (0.1–20 mg/kg, i.p.), followed 15 min later by either saline or a peak stimulant dose of METH (1 mg/kg, i.p.). The mice were then returned to the testing chambers and their activity was quantified for the next 30 min.

Second, to determine how CM156 affected the time course of METH-induced locomotor stimulation, the mice ($n = 78$) were given a pretreatment dose of saline or CM156 (20 mg/kg, i.p.), 15 min prior to saline or METH (1 mg/kg) administration. These doses of CM156 and METH were selected because they produced peak effects in the earlier parts of this study. The locomotor activity was recorded following the pretreatment injection of CM156 or saline for a total of 135 min (15 min pretreatment, 120 min post-treatment). A longer testing period was used in this part of the study to evaluate the effects of CM156 across the entire time window in which the locomotor stimulant effects of METH are apparent in the mice. Earlier preliminary studies confirmed the ability of σ receptor antagonists to attenuate the stimulant effects of METH after 30, 90 and 120 min; the 30 min time period was chosen for routine assessments to help increase the number of animals that could be tested in a given day.

Third, the effect of a single dose of CM156 on shifting the dose response curve for METH was determined. The mice ($n = 89$) were pretreated with saline or CM156 (20 mg/kg, i.p.), followed 15 min later by either saline or different doses of METH (0.1–50 mg/kg, i.p.). The 20 mg/kg dose of CM156 was used because it was the most effective at attenuating the peak effects of METH in the earlier dose response experiment. The treated mice were returned to the chambers and their locomotor activity quantified for the next 30 min.

2.4. Neurotoxicity studies

Male, Swiss Webster mice were randomly divided into groups that were injected with saline or CM156 (5–20 mg/kg, i.p.) 15 min prior to injection with saline or METH (1.25–10 mg/kg, i.p.). Each group of mice received their treatment a total of four times at two hour intervals. One hour after each treatment, the body temperature of the mice was recorded. To allow sufficient time for the METH-induced degeneration of nerve terminals to occur, the

animals were sacrificed and the brains removed one week following treatment (Cappon et al., 2000). One week after this neurotoxicity schedule, the striatal and cerebellar brain regions of the mice were collected on ice and evaluated for dopamine, DAT, 5-HT and SERT levels. The detailed procedure for each of the end points is provided below.

2.4.1. Dopamine assays—The mice ($n = 5$ –9/group) were randomly assigned to one of the following treatments: (1) Saline + Saline; (2) Saline + METH (1.25, 2.5, or 5 mg/kg, i.p.); (3) CM156 (5, 10, or 20 mg/kg, i.p.) + Saline; (4) CM156 (5, 10, or 20 mg/kg, i.p.) + METH (5 mg/kg, i.p.). The striatum and cerebellum were dissected from each treated mice and then frozen in liquid nitrogen. The tissues were stored at -80°C for later analysis of dopamine content.

Using a dopamine research enzyme immunoassay kit and protocols provided by the manufacturer (Rocky Mountain Diagnostics, Colorado Springs, CO), mouse brain striatal and cerebellar dopamine were quantified. Brain tissues were homogenized in 0.01 N HCl. Dopamine was extracted and then acylated to N-acyldopamine using the buffer and reagents provided by the ELISA kit. Acylated dopamine from the tissue samples was then incubated with solid phase bound dopamine, dopamine antiserum, and antiserum buffer to compete for a fixed number of antiserum binding sites. Free antigen and free antigen-antiserum complexes were removed via the wash buffer. The antibody bound to the solid phase dopamine was detected using an anti-rabbit IgG-peroxidase conjugate with TMB as the substrate. The amount of antibody bound to the solid phase dopamine was measured by monitoring the reaction at 450 nm. The solid phase dopamine measured was inversely proportional to the dopamine concentration of the tissue sample and was quantified relative to a standard curve of known concentrations.

2.4.2. DAT immunohistochemistry—Striatal sections were assessed for DAT expression. The mice ($n = 4$ /group) were randomly assigned to one of the following treatment groups: (1) Saline + Saline; (2) Saline + METH (5 mg/kg, i.p.); (3) CM156 (10 mg/kg, i.p.) + METH (5 mg/kg, i.p.); (4) CM156 (10 mg/kg, i.p.) + Saline. One week following treatment, the mice were perfused transcardially with 0.1 M phosphate buffered saline (pH 7.4), followed by 4% paraformaldehyde. The brains were further fixed overnight in 4% paraformaldehyde. Coronal sections (50 μm) of the fixed tissue were made throughout the rostral-caudal extent of the striatum using a cryostat, and processed in a free-floating state in 0.1 M Tris-HCl buffered saline (TBS, pH 7.5). The sections were treated with 0.3% in H_2O_2 in TBS for 30 min at room temperature. The sections were then treated with TBS containing 0.2% Triton X-100 and 1.5% normal goat serum for 30 min at room temperature. Incubation of the sections with rat anti-mouse DAT antibody (Chemicon International, Temecula, CA; MAB369, dilution 1:10,000) was performed for 36 h at 4°C . The labeled sections were then washed twice in TBS and processed using Vectastain Elite ABC (Vector Laboratories, Burlingame, CA). Sections were then incubated with biotinylated secondary anti-rat antisera (diluted 1:200) in TBS-NBS for 60 min. This was then followed by incubation of the sections with avidin-biotinylated peroxidase substrate in TBS for 60 min. The staining was then visualized by reacting 3,3'-diaminobendine containing 0.01% H_2O_2 for 5 min.

The stained sections were mounted onto gelatin-coated slides and dried. The sections were then dehydrated, cleared, and coverslipped. The images were captured digitally using a Leica DMIL microscope (Leica Microsystems, Bannockburn, IL) and optical density readings were quantified in anterior regions of the striatum using ImageJ software (National Institutes of Health, Bethesda, MD). To obtain the data point for a given animal, at least two sections per mouse brain were processed and the optical density readings from both the striatal regions of each section were averaged.

2.4.3. 5-HT assays—The mice ($n = 5$ –10/group) were randomly assigned to one of the following treatments: (1) Saline + Saline; (2) Saline + METH (1.25, 2.5, 5 or 10 mg/kg, i.p.); (3) CM156 (5, 10, or 20 mg/kg, i.p.) + Saline; (4) CM156 (5, 10, or 20 mg/kg, i.p.) + METH (10 mg/kg, i.p.). One week after the injections, the striatum and cerebellum were dissected from each treated mice and then frozen in liquid nitrogen. The tissues were stored at -80°C for later analysis of 5-HT content. The protocol was the same as described above for dopamine, with the exception that brain tissues were homogenized in 0.2 M perchloric acid, followed by centrifugation at 3,000 rpm for 10 min at 4°C . The supernatants were collected and evaluated for 5-HT levels using 5-HT research enzyme immunoassay kits (Rocky Mountain Diagnostics, Colorado Springs, CO).

2.4.4. SERT immunohistochemistry—Striatal sections were assessed for SERT expression. This experiment was performed after relocation of the lab to a different building, with a slightly higher ambient temperature (SERT expression: 23 – 23.5°C) than the earlier lab (5-HT levels: 21 – 22°C). Since serotonin neurotoxicity is sensitive to changes in ambient room temperature (Malberg and Seiden, 1998), a dose response validation for serotonin neurotoxicity was conducted. Moreover, because higher mortality was observed in the 10 mg/kg METH group, CM156 was evaluated against the 5 mg/kg dose of METH. The mice ($n = 4$ /group) were randomly assigned to one of the following treatment groups: (1) Saline + Saline; (2) Saline + METH (5, 10 mg/kg, i.p.); (3) CM156 (20 mg/kg, i.p.) + METH (5 mg/kg, i.p.); (4) CM156 (10 mg/kg, i.p.) + Saline. One week following treatment, striatal sections were collected, processed, and analyzed as for DAT immunohistochemistry, with the following modifications. SERT immunohistochemistry involved incubations with a rabbit anti-mouse SERT primary antibody (Millipore, Temecula, CA; AB9726, dilution 1:5,000) overnight at 4°C . The sections were then washed three times in TBS and incubated with a secondary anti-rabbit IgG-peroxidase (Sigma Aldrich, A0545; diluted 1:400) in TBS for 60 min. The sections were then washed three times in TBS and processed using Histostain-Plus kits (DAB, Broad Spectrum) (Invitrogen, Camarillo, CA). Briefly, the labeled sections were incubated in 100 μl of DAB substrate for 4 min. The sections were then washed in 5 ml of distilled water.

2.5. Body temperature

The mice ($n = 5$ –10/group) were randomly assigned to treatment groups, which were the same as those described for the dopamine and 5-HT assays. All of the combinations of drug treatments were given i.p. at two hour intervals a total of four times. Core body temperature was measured 1 h following each of the treatment combinations with a Thermalert TH-S monitor (Physitemp Instruments Inc., Clifton, NJ). During the temperature measurements, mice were gently held at the base of the tail and a probe (RET-3) was inserted approximately 2.5 cm past the rectum into the colon for 8–10 s until a rectal temperature was maintained for 3–4 s.

2.6. Data analysis

The data from the locomotor measurements, dopamine and 5-HT assays, immunohistochemical studies, and core body temperature readings were evaluated using one- or two-way analysis of variance. Post-hoc analyses were performed with Dunnett's tests or Bonferroni's tests for comparisons to controls and Tukey's tests for pairwise comparisons. For all analyses, $p < 0.05$ was considered statistically significant. ED_{50} calculations were performed using the ascending arm of the dose response curve for METH. GraphPad Prism (San Diego, CA) was used for all data analyses.

3. Results

3.1. Locomotor activity

Consistent with earlier investigations (Nguyen et al., 2005), the 1 mg/kg dose of METH produced peak increases in locomotor activity compared to saline controls (Fig. 1A). One-way ANOVA showed a significant effect of METH treatment ($F(4,34) = 8.80, p < 0.0001$). Post-hoc Dunnett's tests confirmed that 1 mg/kg and 5 mg/kg caused a significant increase in locomotor activity ($q = 4.78, p < 0.001, q = 3.06, p < 0.05$).

When administered alone, CM156 had no significant effects on locomotor activity (Fig. 1B; $F(4,37) = 1.53, n.s.$). However, when CM156 was administered prior to METH treatment, two-way ANOVA showed a significant effect of METH treatment ($F(1,68) = 29.66, p < 0.0001$), CM156 treatment ($F(4,68) = 5.33, p < 0.001$), and METH treatment $\times$ CM156 treatment interaction ($F(4,68) = 6.08, p < 0.0005$). Post-hoc Tukey's tests confirmed that METH (1 mg/kg) caused a significant increase in locomotor activity ($q = 6.08, p < 0.01$) and the following pretreatment doses of CM156 significantly attenuated METH-induced locomotor activity: 10 mg/kg ($q = 4.96, p < 0.05$) and 20 mg/kg ($q = 6.76, p < 0.001$).

Two-way ANOVA of the time course conducted starting with CM156 or saline pretreatment followed by METH (1 mg/kg) or saline treatment showed a significant effect of treatment ($F(3,513) = 181.9, p < 0.0001$), time ($F(26,513) = 1.62, p < 0.05$), and treatment $\times$ time interaction ($F(78,513) = 2.26, p < 0.0001$) as shown in Fig. 1C. Bonferroni's post hoc tests confirmed a significant effect of METH treatment when compared with saline at the following time points: 25 min ($t = 3.94, p < 0.01$), 30 min ($t = 4.39, p < 0.001$), 35 min ($t = 5.27, p < 0.001$), 40 min ($t = 5.56, p < 0.001$), 45 min ($t = 5.84, p < 0.001$), 50 min ($t = 5.03, p < 0.001$), 55 min ($t = 5.91, p < 0.001$), 60 min ($t = 5.97, p < 0.001$), 65 min ($t = 5.52, p < 0.001$), 70 min ($t = 4.05, p < 0.01$), 75 min ($t = 5.17, p < 0.001$), 80 min ($t = 5.32, p < 0.001$), 85 min ($t = 4.21, p < 0.001$), 95 min ($t = 3.90, p < 0.01$), 100 min ($t = 3.70, p < 0.01$), 105 min ($t = 3.75, p < 0.01$) and 110 min ($t = 3.54, p < 0.05$). None of the other treatments were significantly different from saline treatment.

When a single dose of CM156 (20 mg/kg) was tested against different doses of METH, it shifted the dose response curve 10 fold towards the right (Fig. 1D). The ED_{50} of METH treatment was 0.49 mg/kg in the absence and 4.96 mg/kg in the presence of CM156 (20 mg/kg) pretreatment. In addition to the rightward shift, the $\%E_{max}$ of the CM156+METH group decreased to $47 \pm 10.24\%$. Two-way analysis of variance showed a significant effect of CM156 pretreatment ($F(1,65) = 30.36, p < 0.0001$), METH treatment ($F(5,65) = 6.68, p < 0.0001$), and CM156 pretreatment $\times$ METH treatment interaction ($F(5,65) = 9.83, p < 0.0001$). Post hoc Bonferroni's tests showed a significant difference between the following doses of METH in the presence vs. absence of CM156: (0.5 mg/kg, $t = 3.67, p < 0.01$; 1 mg/kg, $t = 7.48, p < 0.001$; 5 mg/kg, $t = 3.33, p < 0.05$).

3.2. Neurotoxicity studies

3.2.1. Dopamine assays—Consistent with other investigations (Matsumoto et al., 2008), one-way ANOVA showed that METH produced a dose dependent reduction of dopamine levels in the striatum (Fig. 2A; $F(3,30) = 7.26, p < 0.005$). Post-hoc Dunnett's tests revealed that the striatal changes produced by the following doses of METH differed significantly from the saline control group: 2.5 mg/kg ($q = 2.55, p < 0.05$), 5 mg/kg ($q = 4.61, p < 0.01$). However, no significant changes in dopamine concentration following doses of METH, were observed in the cerebellum ($F(3,30) = 0.20, n.s.$).

When the selective σ receptor ligand CM156 was administered in the absence of METH, one-way ANOVA showed that there were no significant changes in striatal and cerebellar

dopamine levels (Fig. 2B; $F(3,16) = 0.36$, n.s.; $F(3,16) = 0.37$, n.s. respectively). In Fig. 2B, two-way ANOVA showed that pretreatment of CM156 prior to METH led to a significant effect of METH treatment ($F(1,32) = 18.39$, $p < 0.0005$), CM156 pretreatment ($F(3,32) = 8.03$, $p < 0.0005$), and METH treatment $\times$ CM156 pretreatment interaction ($F(3,32) = 5.15$, $p < 0.01$). Post-hoc Tukey's tests verified that METH (5 mg/kg) treatment caused a significant decrease in dopamine levels ($q = 7.16$, $p < 0.001$) and that pretreatment with the following doses of CM156 differed significantly from saline in protecting against METH (5 mg/kg)-induced striatal dopamine depletions: 10 mg/kg ($q = 7.41$, $p < 0.001$) and 20 mg/kg ($q = 7.02$, $p < 0.001$).

3.2.2. DAT immunohistochemistry—Fig. 3 depicts the effects of METH and CM156 on DAT immunoreactivity in the mouse striatum, with a significant difference between the treatment groups ($F(3,34) = 14.89$, $p < 0.0001$). Post-hoc Tukey's multiple comparisons tests confirmed that METH caused a significant reduction in DAT immunoreactivity relative to treatment with saline alone ($q = 7.70$, $p < 0.001$). Pretreatment with CM156 (10 mg/kg) significantly attenuated METH-induced neurotoxicity ($q = 7.9$, $p < 0.001$), whereas treatment with CM156 alone had no significant effects on DAT expression compared to saline alone ($q = 0.75$, n.s.).

3.2.3. 5-HT assays—Fig. 4A illustrates striatal 5-HT levels following administration of METH. A one-way ANOVA showed a significant reduction in 5-HT levels in the mouse striatum ($F(4,40) = 9.87$, $p < 0.0001$). Post-hoc Dunnett's tests confirmed that 5-HT levels were significantly reduced relative to the saline control following injection of 10 mg/kg, i.p. of METH ($q = 5.98$, $p < 0.01$). However, in the cerebellum, METH did not produce significant 5-HT depletions ($F(4,35) = 2.33$, n.s.).

One-way ANOVA also showed that administration of CM156 alone produced no significant changes in 5-HT levels in the striatum ($F(3,21) = 2.37$, n.s.) or cerebellum ($F(3,21) = 0.49$, n.s.). However, similar to that seen with dopamine, pretreatment with CM156 protected against METH-induced 5-HT depletions in the striatum. A two-way ANOVA showed a significant effect of METH treatment ($F(1,42) = 42.54$, $p < 0.0001$, Fig. 4B). Post-hoc Tukey's tests confirmed that METH (10 mg/kg) treatment caused a significant decrease in 5-HT levels ($q = 9.34$, $p < 0.001$) and that pretreatment with CM156 (20 mg/kg) significantly attenuated METH (10 mg/kg)-induced striatal 5-HT depletions ($q = 5.15$, $p < 0.05$) when compared to pretreatment with saline.

3.2.4. SERT immunohistochemistry—Immunohistochemical analysis revealed that METH induced reductions in striatal SERT expression. One-way ANOVA showed that METH caused a significant decrease in SERT expression in the striatum of the mice (Fig 5A; $F(2,22) = 56.67$, $p < 0.0001$). Post-hoc Dunnett's tests showed that both the 5 and 10 mg/kg doses of METH significantly reduced striatal SERT expression ($q = 9.27$, $p < 0.01$ and $q = 8.41$, $p < 0.01$, respectively). However, the 10 mg/kg METH group had higher mortality and therefore, CM156 was evaluated against the 5 mg/kg dose of METH. Fig. 5B depicts the effects of METH and CM156 on SERT immunoreactivity in the mouse striatum, with a significant difference between the treatment groups ($F(3,37) = 69.21$, $p < 0.0001$). Post-hoc Tukey's multiple comparisons tests confirmed that METH (5 mg/kg, i.p.) caused a significant reduction in SERT immunoreactivity relative to treatment with saline alone ($q = 17.63$, $p < 0.001$). Pretreatment with CM156 (20 mg/kg, i.p.) significantly attenuated METH-induced neurotoxicity compared to pretreatment with saline ($q = 15.40$, $p < 0.001$), whereas treatment with CM156 alone had no significant effects on SERT expression compared to treatment with saline alone ($q = 0.50$, n.s.).

3.3. Hyperthermia

The hyperthermic effects of METH are represented in Fig. 6A. One-way ANOVA demonstrated that METH produced a dose dependent increase in core body temperature after the first injection ($F(4,96) = 11.99, p < 0.0001$), second injection ($F(4,96) = 13.80, p < 0.0001$), third injection ($F(4,96) = 36.16, p < 0.0001$), and fourth injection ($F(4,96) = 35.23, p < 0.0001$). Post-hoc Dunnett's tests confirmed that compared to saline, the following doses of METH significantly increased body temperature under the specified conditions: after the first injection with METH 2.5 mg/kg ($q = 2.81, p < 0.05$), 5 mg/kg ($q = 4.62, p < 0.01$), and 10 mg/kg ($q = 6.46, p < 0.01$); after the second injection with METH 1.25 mg/kg ($q = 4.51, p < 0.01$), 2.5 mg/kg ($q = 4.07, p < 0.01$), 5 mg/kg ($q = 6.27, p < 0.01$), and 10 mg/kg ($q = 5.84, p < 0.01$); after the third injection with METH 1.25 mg/kg ($q = 4.66, p < 0.01$), 2.5 mg/kg ($q = 5.03, p < 0.01$), 5 mg/kg ($q = 9.73, p < 0.01$), and 10 mg/kg ($q = 10.33, p < 0.01$); and after the fourth injection with METH 1.25 mg/kg ($q = 3.18, p < 0.01$), 2.5 mg/kg ($q = 4.34, p < 0.01$), 5 mg/kg ($q = 7.35, p < 0.01$) and 10 mg/kg ($q = 11.35, p < 0.01$).

The effects of CM156 alone on body temperature are depicted in Fig. 6B. One-way ANOVA showed that CM156 alone had no significant effects on body temperature after the first ($F(3,39) = 0.05, n.s.$), second ($F(3,39) = 1.81, n.s.$), third ($F(3,39) = 1.37, n.s.$), or fourth ($F(3,39) = 1.68, n.s.$) injection.

Pretreatment with CM156 attenuated METH (5 mg/kg, i.p.)-induced hyperthermia after all but the first injection (Fig. 7A): first ($F(3,62) = 1.81, n.s.$); second ($F(3,62) = 6.04, p < 0.005$); third ($F(3,62) = 6.34, p < 0.001$); fourth ($F(3,62) = 4.53, p < 0.01$). Post-hoc Dunnett's tests showed that the following changes were significant from the second injection onwards: after the second injection, CM156 (20 mg/kg, i.p.), when compared to saline-pretreatment, significantly mitigated the hyperthermia effects of METH ($q = 3.95, p < 0.01$); after third injection, CM156 (10 and 20 mg/kg, i.p.) attenuated the hyperthermic effects of METH ($q = 3.37, p < 0.01$ and $q = 3.54, p < 0.01$ respectively); after the fourth injection, CM156 (20 mg/kg, i.p.) attenuated the hyperthermic effects of METH ($q = 3.45, p < 0.01$ respectively).

Fig. 7B shows the effects of pretreatment with the σ receptor ligand CM156 against the hyperthermia produced by a high (10 mg/kg, i.p.) dose of METH. One-way ANOVA showed a significant attenuation of METH-induced hyperthermia after the fourth injection (first: $F(3,28) = 1.20, n.s.$; second: $F(3,28) = 1.29, n.s.$; third: $F(3,28) = 3.37, p < 0.05$; fourth: $F(3,28) = 10.83, p < 0.0001$). Post-hoc Dunnett's tests revealed that pretreatment with CM156 (10 and 20 mg/kg, i.p.) attenuated the hyperthermic effects of METH after the fourth injection ($q = 2.63, p < 0.05$; $q = 5.58, p < 0.01$ respectively).

4. Discussion

The current study demonstrated that pretreatment with CM156, a novel substituted piperazine with high affinity and selectivity for σ receptors (Xu et al., 2010), mitigated METH-induced locomotor stimulation, hyperthermia, and striatal dopamine and 5-HT neurotoxicity. These protective effects likely involve modulation through both σ_1 and σ_2 receptors.

In the first part of the study, CM156 attenuated the acute locomotor stimulant effects of METH. The locomotor stimulant effects of METH are due to increased dopamine release in extrapyramidal and mesolimbic brain regions (Kelly and Iversen, 1976). σ Receptors are present in motor areas of the brain (Bouchard and Quirion, 1997) and can modulate the synthesis and release of dopamine (Bastianetto et al., 1995; Booth and Baldessarini, 1991; Derbez et al., 2002; Walker et al., 1993). The subtypes, σ_1 and σ_2 , have both been

implicated in locomotor activating effects in earlier studies (Walker et al., 1993; Nguyen et al., 2005). With regard to METH, antisense and antagonists for σ_1 receptors, including BD1063 and BD1047, attenuate locomotor stimulation, reducing the $E_{\max}$ for METH (Nguyen et al., 2005). This suggests that σ_1 antagonists are modulating the effects of METH downstream from the receptor, an interpretation that is consistent with the known ligand-gated chaperone function of σ_1 receptors, which allows them to undergo translocation between subcellular organelles, participate in protein-protein interactions, and modulate other receptor systems (Su et al., 2010). In the presence of CM156, the dose response curve for METH was shifted toward the right along with a decreased $E_{\max}$. Therefore, it is likely that interactions of CM156 with σ_2 receptors contribute to the rightward shift in the METH dose response curve. However, confirmation of the individual roles contributed by each σ receptor subtype must await the development of additional experimental tools, particularly selective σ_2 receptor ligands. Nevertheless, an overall important role of σ receptors in METH's locomotor stimulant effect is emphasized by the fact that σ receptor ligands not only attenuate the acute locomotor stimulation but also the chronic locomotor sensitization response to METH (Ujike et al., 1996).

In addition to producing acute stimulant effects at low doses, high subchronic dose administration of METH leads to dopaminergic and serotonergic neurotoxicity along with hyperthermia (Krasnova and Cadet, 2009). Consistent with earlier reports that METH preferentially targets dopamine over 5-HT (Kuczenski et al., 1995), neurotoxicity in the present study was more pronounced in endpoints (neurotransmitter levels and transporter expression) related to dopamine compared to 5-HT. The damage to 5-HT neurons was observed at comparatively higher doses than dopamine neurons, most likely because METH has higher affinity for DAT than SERT (Han and Gu, 2006). In addition, METH causes greater release of dopamine than 5-HT in the striatum (Gough et al., 2002), providing an opportunity for increased oxidative damage to dopaminergic neurons. Moreover, the longer retention time for METH in the striatum as compared to the cerebellum (Fowler et al., 2007) may account for the significantly higher neurotoxicity observed in the striatum.

The mechanisms of METH-induced neurotoxicity are complex and involve multiple interacting and cascading processes: excessive release of monoamines like dopamine and 5-HT; autooxidation of monoamines leading to reactive oxygen species (ROS) generation; activation of endoplasmic reticulum (ER) stress pathways, mitochondrial death cascades, and glutamatergic/N-methyl-D-aspartate (NMDA)-mediated excitotoxic mechanisms (Krasnova and Cadet, 2009). Modulation of σ_1 and σ_2 receptors can alter similar processes in the other studied systems: release of neurotransmitters such as dopamine and 5-HT (Bastianetto et al., 1995; Campbell et al., 1989; Derbez et al., 2002); protective effects against ROS-related dysfunctions such as brain ischemia (Schetz et al., 2007); regulation of ER stress and mitochondrial death cascades (Tsai et al., 2009; Su et al., 2010); and modulation of NMDA receptor function, including excitotoxic mechanisms (DeCoster et al., 1995; Shen et al., 2008; Shimazu et al., 2000). Therefore, CM156 most likely provides neuroprotection by intervening at multiple points in the neurotoxic cascade of METH through both σ receptor subtypes via a combination of competitive and non-competitive interactions; additional details of mechanisms through which σ ligands may mitigate the neurotoxicity of METH are discussed in a recent review (Kaushal and Matsumoto, 2011).

In addition to the aforementioned mechanisms related to METH, targeting σ receptors has been reported to effectively attenuate neurotoxicity in several other models including β -amyloid, carbon monoxide, and ischemic stroke (Marrazzo et al., 2005; Meunier et al., 2006; Shen et al., 2008). Furthermore, σ receptors appear to play a role in animal models of neuropsychiatric complications like depression, psychosis, cognitive and motor deficits (Mishina et al., 2005; Guitart et al., 2004), which are also observed in METH users and have

been associated with the neurotoxic effects of METH (Cruickshank and Dyer, 2009). Therefore, the ability of CM156 to mitigate METH-induced neurotoxicity may have broader implications for other neurotoxic and neuropsychiatric conditions.

In addition to stimulant and neurotoxic effects, METH also produces hyperthermia. The hyperthermic effects of METH have been linked to its production of ROS (Fukami et al., 2004; Yamashita et al., 2000; Riedel and Maulik, 1999; Numachi et al., 2007; Imam et al., 1999; Halladay et al., 2003; Dascombe et al., 1989; Bowyer et al., 1994), as well as activation of dopaminergic and serotonergic systems (Numachi, et al., 2007). It is therefore conceivable that σ receptor ligands can attenuate hyperthermia by mitigating early events that lead to increases in body temperature, such as release of monoamines and generation of ROS (Schetz et al., 2007; Bastianetto et al., 1995; Campbell et al., 1989; Derbez et al., 2002). Additionally, σ_1 and σ_2 receptors are located in the hypothalamus (Bouchard and Quirion, 1997) and σ ligands have previously been shown to be involved in the modulation of body temperature (Rawls et al., 2002). A combination of these factors may explain how CM156 can prevent METH-induced hyperthermia.

It is important to note that hyperthermia on its own does not cause neurotoxicity, but can exacerbate the neurotoxic potential of METH (Bowyer et al., 1994). The ability of CM156 to attenuate the hyperthermic effects of METH in vivo is most likely associated with its neuroprotective potential. However, correlation does not equal causation, and it has been demonstrated with AC927, another $\sigma_{1/2}$ receptor ligand, that while a significant correlation exists between its ability to mitigate METH-induced hyperthermia and neurotoxicity in vivo (Seminerio et al., 2011), when temperature is controlled for in an in vitro model, neuroprotection is still observed (Matsumoto et al., 2008). Thus, it is likely that the effects of CM156 on METH-induced hyperthermia contribute to its neuroprotection, but is not dependent on it.

In conclusion, pretreatment with CM156 significantly protects against METH's behavioral stimulant effects, hyperthermia, and dopamine and 5-HT neurotoxicity. The manner in which CM156 antagonizes the effects of METH appears to involve both competitive and non-competitive interactions at σ receptors. Together with other studies, the data suggest an important role of σ receptors in various METH-induced complications, and suggest a mechanistic-based strategy for mitigating these adverse effects. Future studies to elucidate the role of σ receptor subtypes in the effects of METH using more subtype selective ligands will provide a more complete understanding of the underlying mechanisms through which σ ligands convey neuroprotective actions.

Acknowledgments

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List of non-standard abbreviations

AC927	1-(2-phenethyl)piperidine oxalate
ANOVA	analysis of variance
BD1063	1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine
CM156	3-(4-(4-cyclohexylpiperazin-1-yl)butyl)benzo[d]thiazole-2(3 <i>H</i>)-thione
DAT	dopamine transporters
ER	endoplasmic reticulum

MAM	mitochondria associated membrane
METH	methamphetamine
NMDA	N-methyl-D-aspartate
ROS	reactive oxygen species
SERT	serotonin transporters

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Research highlights

- CM156 attenuates methamphetamine (METH)-induced locomotor stimulation.
- CM156 attenuates METH-induced reductions in striatal dopamine and serotonin levels.
- CM156 attenuates METH-induced deficits in striatal DAT and SERT expression.
- CM156 attenuates METH-induced hyperthermia.
- CM156 (without METH) has no significant effects on the endpoints investigated.

Figure 1.

(A) Dose dependent effects of METH on locomotor activity. Male, Swiss Webster mice ($n = 6-11$ per group) were injected with saline or METH (0.1, 0.5, 1.0, 5 mg/kg, i.p.). (B) Effects of CM156 on locomotor activity following METH treatment. Male, Swiss Webster mice ($n = 6-8$ per group) were pretreated with saline or CM156 (0.1, 1, 10, 20 mg/kg, i.p.) prior to saline or METH (1 mg/kg, i.p.). (C) Effect of CM156 on the time course of METH treatment. Male, Swiss Webster mice ($n = 6$ per group) were pretreated with saline or CM156 (20 mg/kg, i.p.) followed 15 min later by saline or METH (1 mg/kg, i.p.). Locomotor activity was recorded for a total of 135 min starting at the pretreatment injection. (D) Effect of CM156 pretreatment on the shift in the dose response curve for METH. Male,

Swiss Webster mice ($n = 6-11$ per group) were pretreated with saline or CM156 (20 mg/kg, i.p.) followed by saline or METH (0.1, 0.5, 1, 5, 10, 30, 50 mg/kg, i.p.). Locomotor activity was recorded for 30 min after saline or METH treatment. Data are reported as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. saline; # $p < 0.05$, ### $p < 0.001$ vs. METH.

Figure 2.

Dose response effects of METH on dopamine (DA) levels in the striatum. Male, Swiss Webster mice ($n = 7-9$ per group) were injected (i.p.) with METH (1.25– 5.0 mg/kg, i.p.) or saline (0 mg/kg, i.p.) at 2 h intervals for a total of four times. DA levels in the striatum were measured one week later. (B) Effects of CM156 on METH-induced depletion of dopamine (DA) levels in striatum of mouse brain tissue. Male, Swiss Webster mice ($n = 5$ per group) were pretreated with saline or CM156 (5, 10, 20 mg/kg, i.p.). Mice were then treated with saline (–METH 0 mg/kg, i.p.) or METH (+METH 5 mg/kg, i.p.) after 15min. This treatment regimen was repeated at 2 h intervals for a total of four times. Tissue samples from mouse striatum were collected and DA concentration was measured one week later. Data was

reported as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. saline, ### $p < 0.001$ vs. METH.

Saline/Saline

Saline/METH(5)

CM156(10)/METH(5)

CM156(10)/Saline

Figure 3.

Effects of METH and CM156 on DAT immunoreactivity in the mouse striatum. Male, Swiss Webster mice ($n = 4$ per group) were pretreated with saline (SAL) or CM156 (CM, 20 mg/kg, i.p.). After 15 min, the mice were then treated with saline (SAL) or METH (5 mg/kg, i.p.). This treatment schedule was repeated 4 times at 2 h intervals. One week later, the brain was removed and stained for DAT immunoreactivity. A representative section from each treatment group is shown, along with average optical density readings (mean $\pm$ SEM). *** $p < 0.001$ vs. saline, ### $p < 0.001$ vs. METH.

Figure 4.

(A) Dose response effects of METH on 5-HT levels in the striatum. Male, Swiss Webster mice ($n = 5-10$ per group) were injected with saline or METH (1.25–10.0 mg/kg, i.p.) at 2 h intervals for a total of four times. Striatal tissue samples were collected one week later and measured for 5-HT concentration. (B) Effect of CM156 pretreatment on METH-induced alteration of 5-HT levels in the striatum of mouse brain. Male, Swiss Webster mice ($n = 5-10$ per group) were pretreated with saline or CM156 (5, 10, 20 mg/kg, i.p.), and 15 min later, the mice were treated with saline (–METH 0 mg/kg, i.p.) or METH (+METH 10 mg/kg, i.p.). These treatment combinations were repeated at 2 h intervals a total of four times. One

week later, mouse striatum were collected and 5-HT concentration was measured. Data was reported as mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$ vs. saline, # $p < 0.05$ vs. METH.

Figure 5.

(A) Dose response effects of METH on SERT immunoreactivity in striatum. Male, Swiss Webster mice ($n = 4$ per group) were injected with METH (5, 10 mg/kg, i.p.) or saline at 2 h intervals for a total of four times. One week later, the brain was removed and stained for SERT immunoreactivity. (B) Effect of pretreatment of CM156 on METH-induced decreases in SERT immunoreactivity. Male, Swiss Webster mice ($n = 4$ per group) were pretreated with saline (SAL) or CM156 (CM, 20 mg/kg, i.p.), and 15 min later, injected (i.p.) with saline or METH (5 mg/kg, i.p.). These treatment combinations were repeated at 2 h intervals for a total of four times. One week later, the brain was removed and evaluated for SERT immunoreactivity. A representative section from each treatment group is shown, along with

average optical density readings (mean $\pm$ SEM). *** $p < 0.001$ vs. saline, ### $p < 0.001$ vs. METH.

Figure 6.

Dose response effects of (A) METH and (B) CM156 on core body temperature. Male, Swiss Webster mice ($n = 5-10$ per group) were injected with saline or CM156 (5–10 mg/kg, i.p.) followed by saline or METH (1.25–10 mg/kg, i.p.) at 2 h intervals for a total of four times. Core body temperature was measured via rectal thermometer 1 h after each injection and data was reported as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ vs. saline. No significant difference was observed with CM156.

Figure 7.

Effects of CM156 on (A) METH (5 mg/kg, i.p.) or (B) METH (10 mg/kg, i.p.) induced hyperthermia. Male, Swiss Webster mice ($n = 5-10$ per group) were pretreated with saline or CM156 (5, 10, 20 mg/kg, i.p.), and after 15 min, the mice were treated with saline or METH (5, 10 mg/kg, i.p.). Core body temperature was measured 1 h after each injection combination. This regimen was repeated four times at 2 h intervals. Data was reported as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ vs. saline.