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The evaluation of AZ66, an optimized sigma receptor antagonist, against methamphetamine-induced dopaminergic neurotoxicity and memory impairment in mice

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3 Department of Pharmacology and Department of Medicinal Chemistry, School of Pharmacy, University of Mississippi, University, MS, USA

Abstract

Sigma (σ) receptors have recently been identified as potential targets for the development of novel therapeutics aimed at mitigating the effects of methamphetamine. Particularly, σ receptors are believed to mitigate some of the neurotoxic effects of methamphetamine through modulation of dopamine, dopamine transporters and body temperature. Furthermore, recent evidence suggests that targeting σ receptors may prevent cognitive impairments produced by methamphetamine. In the present study, an optimized σ receptor antagonist, AZ66, was evaluated against methamphetamine-induced neurotoxicity and cognitive dysfunction. AZ66 was found to be highly selective for σ receptors compared to 64 other sites tested. Pretreatment of male, Swiss Webster mice with i.p. dosing of AZ66 significantly attenuated methamphetamine-induced striatal dopamine depletions, striatal dopamine transporter reductions and hyperthermia. Additionally, neurotoxic dosing with methamphetamine caused significant memory impairment in the object recognition test, which was attenuated when animals were pretreated with AZ66; similar trends were observed in the step-through passive avoidance test. Taken together, these results suggest that targeting σ receptors may provide neuroprotection against the neurotoxicity and cognitive impairments produced by methamphetamine.

Introduction

Methamphetamine is an addictive psychostimulant and currently listed as the second most abused illicit substance in the world (United Nations, 2007). Methamphetamine abuse can result in several negative consequences, including significant neurotoxicity at high or repeated doses (Cadet & Krasnova, 2009; Kita et al. 2003). Chronic use results in long-lasting nerve terminal degeneration in specific regions of the brain (Cadet & Krasnova, 2009). Methamphetamine is believed to exert these effects through its interaction with monoamine transporters, primarily in the dopaminergic system (Krasnova & Cadet, 2009; Schep et al. 2010; Sora et al. 2009). This results in the release of dopamine from synaptic vesicles within the nerve terminal and a resulting release of excess dopamine into the synapse by inhibition of reuptake and reversal of flow through dopamine transporters (DATs; Krasnova & Cadet, 2009; Schep et al. 2010). This is believed to lead to nerve terminal degeneration through the formation of reactive oxygen species and reactive nitrogen species (Kita et al. 2003).

The neurotoxic effects of methamphetamine appear to have significant clinical implications, as neurological deficits have been found in human clinical populations of chronic methamphetamine abusers (McCann et al. 1998; Schep et al. 2010; Volkow et al. 2001a, b; Wilson et al. 1996). In addition, it has been documented that significant and long-lasting nerve terminal degeneration can occur in these patients (McCann et al. 1998; Wilson et al. 1996), potentially resulting in cognitive impairments (Hart et al. 2012). The role of methamphetamine abuse in cognitive-related decline has remained controversial. However, current studies suggest that, while acute use of...
methamphetamine may actually improve memory and attention, chronic use results in decreases in memory and reaction speed (Hart et al. 2012). Methamphetamine-induced cognitive impairment has been observed both in humans via clinical tests such as the Wisconsin Card Sorting Test and animal studies, which evaluate maze sequential learning (Chapman et al. 2001), motor performance (Walsh & Wagner, 1992), spatial impairment (Friedman et al. 1998) and object recognition (Belcher et al. 2008; Bisagno et al. 2002; Kamei et al. 2006; O’Dell et al. 2011; Reichel et al. 2012). It is hypothesized that methamphetamine use may increase an user’s risk of neurodegenerative diseases such as Parkinson’s disease (Callaghan et al. 2010; Kuehn, 2011; Morrow et al. 2011). While these cognitive effects of methamphetamine have primarily been studied in the hippocampal regions of the brain, recent evidence has shown that the striatum plays an important role in memory (Sadeh et al. 2011) and striatal dopaminergic deficits are evident in patients with Parkinson’s disease (Altgassen et al. 2007; Beste et al. 2009).

Previous work has demonstrated that sigma (σ) receptors may be a viable target to attenuate some of the effects of methamphetamine. Methamphetamine interacts with both σ1 and σ2 receptors at physiologically relevant concentrations (2 ± 0.3 and 47 ± 10 μM, respectively; Nguyen et al. 2005) and σ receptors have been shown to be involved in many of the behavioural and physiological effects of methamphetamine (Kaushal & Matsumoto, 2011; Kaushal et al. 2011, 2012; Matsumoto et al. 2008; Seminerio et al. 2011, 2012). Pretreatment with selective σ1/σ2 receptor antagonists such as AC927 (N-phenethylpiperdine oxalate) or CM156 (3-(4-(4-cyclohexylpiperazin-1-yl)butyl)benzo[σ]thiazole-2(3H)-thione) have been shown to attenuate methamphetamine-induced hyperthermia, dopaminergic neurotoxicity and serotoninergic neurotoxicity, in addition to mitigating some of the stimulant effects of methamphetamine, such as increases in locomotor activity (Kaushal et al. 2011; Matsumoto et al. 2008). Other reports have shown that activation of σ receptors can provide anti-amnesic and neuroprotective effects in various models of cognitive dysfunction (van Waarde et al. 2011) and σ receptors are thought to have a functional role in Parkinson’s disease (Mishina et al. 2005).

The current study utilized AZ66 (3-(4-(4-cyclohexylpiperazin-1-yl)pentyl)-6-flourobenzo[σ]thiazole-2(3H)-one), a mixed σ1/σ2 antagonist derived from CM156 and optimized for metabolic stability (Seminerio et al. 2011), to determine its effects as a pretreatment against methamphetamine-induced hyperthermia, striatal dopaminergic neurotoxicity and cognitive dysfunction. AZ66 has previously been shown to mitigate many of the behavioural effects of methamphetamine, including the development and expression of behavioural sensitization (Seminerio et al. 2012), suggesting its potential importance toward future drug development studies. This study is the first to evaluate a selective σ receptor antagonist for its ability to attenuate cognitive impairment following repeated methamphetamine administration.

Method

Receptor binding studies

To evaluate the overall selectivity of AZ66 for σ receptors, the compound was subject to NOVAScreen (Caliper Life Sciences, USA) at targets not previously reported (Seminerio et al. 2012). Further details of each assay condition can be accessed through their website (www.caliperls.com).

Animals

Male, Swiss Webster mice (21–30 g; Harlan, USA) were used for all experiments. Animals were housed 1–5 per cage with a 12:12 h light/dark cycle (lights on 06:00 hours) and ad libitum food and water. They were allowed 1 wk to acclimatize following their arrival before being used in an experiment. All procedures were approved by the Institutional Animal Care and Use Committee at West Virginia University.

Drugs and treatment

(+)-Methamphetamine hydrochloride was purchased from Sigma-Aldrich (USA) and sterile saline solution was purchased from Teknova (USA). The σ receptor antagonist, AZ66, was synthesized as previously described (Seminerio et al. 2012). All drug solutions were made with saline and the solution volumes were administered relative to body weight (0.1 ml/10 g).

Mice were randomly divided into groups that were injected with saline (0.1 ml/10 g i.p.) or AZ66 (10 mg/kg i.p.) 15 min prior to injection with saline or methamphetamine (5 mg/kg i.p.). The dose of AZ66 was chosen based on previous studies, which demonstrated significant effects against methamphetamine while exerting no effects on its own (Seminerio et al. 2012). Similarly, previous work in our lab has shown neurotoxic dosing with methamphetamine produces a dose-dependent depletion of dopamine levels in the mouse striatum, with 5 mg/kg being the lowest dose producing statistically significant effects (Kaushal et al. 2011). Therefore, 10 mg/kg AZ66 and 5 mg/kg methamphetamine were used.

Each group of mice received their treatment a total of four times at 2 h intervals. One hour after each treatment, the body temperatures of the mice were recorded. To allow sufficient time for the methamphetamine-induced degeneration of nerve terminals to occur, the animals were killed and the brains removed 1 wk later (Cappon et al. 2000). The striata of the mice were then collected on ice and evaluated for dopamine levels and DAT expression. The detailed procedure for each of the endpoints is provided below.
**Dopamine assays**

Mice (n=6–8/group) were randomly assigned to one of the following treatments: (1) saline + saline; (2) saline + methamphetamine (5 mg/kg i.p.); (3) AZ66 (10 mg/kg i.p.); (4) AZ66 (10 mg/kg i.p.) + methamphetamine (5 mg/kg i.p.). The mice received their designated treatments a total of four times at 2 h intervals. One week later, the striatum was dissected from the 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, USA), mouse brain striatal dopamine was 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 enzyme-linked immunosorbent assay (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 3,3',5,5'-tetramethylbenzidine 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.

**DAT immunohistochemistry**

Striatal sections were assessed for DAT expression. Mice (n=4/group) were randomly assigned to one of the following treatment groups: (1) saline + saline; (2) saline + methamphetamine (5 mg/kg i.p.); (3) AZ66 (10 mg/kg i.p.); (4) AZ66 (10 mg/kg i.p.) + methamphetamine (5 mg/kg i.p.); (4) AZ66 (10 mg/kg i.p.); (4) AZ66 (10 mg/kg i.p.) + saline. The mice received their treatments at 2 h intervals, a total of four times. One week later, the striatum was dissected from the 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, USA), mouse brain striatal dopamine was 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 enzyme-linked immunosorbent assay (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 3,3',5,5'-tetramethylbenzidine 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.

**Body temperature**

Mice (n=6–8/group) were randomly assigned to treatment groups, which were the same as those described for the dopamine assays. All of the combinations of drug treatments were given i.p. at 2 h 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., USA). 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.

**Memory measurements**

Mice (n=10/group) were randomly assigned to treatment groups, which were the same as those described for the neurotoxicity studies. All of the combinations of drug treatments were given i.p. at 2 h intervals a total of four times. Following 1 wk, animals were evaluated for memory in the object recognition test and step-through passive avoidance test. The detailed procedure for each of the tests is provided below.

**Object recognition test**

The test was carried out as described previously (Li et al. 2011). Each mouse was allowed to move freely in an open-field box for 5 min as habituation. Twenty-four hours later, mice were individually placed in the centre of the box containing two identical objects (Lego blocks) located in the two diagonal corners. The cumulative time spent exploring each object was recorded during a 5 min period. Exploration was defined as actively touching or facing (within 2 cm toward) the object. One day later (24 h after training), mice were tested for memory using the same procedure except that one of the familiar objects
was replaced with a novel object. The time of exploration of each object \( T_f \) and \( T_n \) for familiar \( f \) and novel \( n \) objects, respectively, was recorded for determination of the recognition index (RI) = \( T_n / (T_f + T_n) \).

**Step-through passive avoidance test**

The test was performed as described previously (Zhang et al. 2005) with some modifications. The apparatus (Model E10–16SC; Coulbourn Instruments, USA) consisted of a two-compartment chamber with an illuminated compartment connected to a darkened compartment by a guillotine door. The experiment consisted of single training and testing sessions.

On the first day, the animal was placed in the chamber and allowed to roam freely between the illuminated and darkened side for 5 min. During training (24 h later), the mouse was placed in the illuminated compartment, facing away from the closed guillotine door, for 1 min before the door was raised. The latency to enter the darkened compartment was recorded. After the mouse entered the darkened compartment, the door was closed and an electric shock (0.4 mA, 5 s) was delivered from the steel-rod floor. This was repeated until the latency for the animal to enter the dark compartment exceeded 60 s once the door was open. The number of shocks the animal received before meeting the >60 s criterion was also recorded.

Twenty-four hours later, mice began the testing session. To begin the test, the mouse was again placed in the illuminated compartment, with the guillotine door closed for 1 min. After 1 min, the door was opened and the retention latency to enter the darkened compartment was recorded for up to 300 s, at which time the test was terminated. No shocks were delivered to mice that entered the darkened compartment during the test trial.

**Data analysis**

The data from the dopamine assays, immunohistochemical studies, core body temperature readings, object recognition test and step through passive avoidance test were evaluated using one-way analysis of variance (ANOVA). Post-hoc analyses were performed with Tukey’s tests for pairwise comparisons. For all analyses, \( p < 0.05 \) was considered statistically significant. GraphPad Prism (USA) was used for all data analyses.

**Results**

**Radioligand binding assays**

Table 1 summarizes the affinities of AZ66 for radioligand binding sites. Previous reports showed that AZ66 had high affinity for both \( \alpha_1 \) and \( \alpha_2 \) receptors in the nanomolar and subnanomolar range (Seminero et al. 2012). Compared to its high affinity for \( \alpha \) receptors, AZ66 displayed a \( >100 \)-fold preference relative to all 64 non-\( \alpha \) binding sites tested.

**Neurotoxicity evaluations**

**Dopamine assays**

Figure 1 shows the effects of the \( \alpha \) receptor antagonist AZ66 on methamphetamine-induced dopamine depletions in the mouse striatum. ANOVA confirmed significant differences between groups \( (F_{3,36} = 13.67, p < 0.001) \). Post-hoc Tukey’s tests confirmed that methamphetamine produced significant decreases in striatal dopamine levels compared to saline-treated animals \( (q = 6.17, p < 0.001) \) and pretreatment with AZ66 significantly attenuated methamphetamine-induced dopamine depletions \( (q = 8.88, p < 0.001) \). When AZ66 was administered alone, the striatal dopamine levels were not significantly changed compared to saline-treated animals \( (p > 0.05) \).

**DAT immunohistochemistry**

To test the effects of AZ66 on methamphetamine-induced DAT reductions, immunohistochemical analyses were conducted. Figure 2 depicts the effects of methamphetamine and AZ66 on DAT immunoreactivity in the mouse striatum, with a significant difference between the treatment groups \( (F_{3,38} = 118.70, p < 0.0001) \). Post-hoc Tukey’s multiple comparisons test confirmed that methamphetamine caused a significant reduction in DAT immunoreactivity relative to treatment with saline alone \( (q = 25.55, p < 0.001) \). Pretreatment with AZ66 significantly attenuated methamphetamine-induced neurotoxicity \( (q = 20.97, p < 0.001) \), whereas treatment with AZ66 alone had no significant effects on DAT expression compared to saline alone \( (q = 3.65, p > 0.05) \).

**Hyperthermia**

Methamphetamine produced a significant increase in body temperature, which was attenuated by AZ66. One-way ANOVA showed significant differences between all groups \( (F_{3,14} = 19.08, p < 0.001) \). ANOVA of body temperature measured following each treatment time-point revealed significant changes in all but the first-time point (BT1) \((F_{3,38} = 9.67, p > 0.05)\). BT2 \((F_{3,38} = 13.93, p < 0.01)\), BT3 \((F_{3,38} = 14.02, p < 0.01)\), BT4 \((F_{3,38} = 21.14, p < 0.01)\), Post-hoc Tukey’s tests confirmed that methamphetamine significantly increased body temperature after the second injection onwards \((BT2, q = 7.72, p < 0.001; BT3, q = 7.72, p < 0.001; BT4, q = 8.36, p < 0.001)\). AZ66 significantly mitigated the hyperthermic effects of methamphetamine \((BT3, q = 5.20, p < 0.01; BT4, q = 5.11, p < 0.01)\). When AZ66 was administered in the absence of methamphetamine, ANOVA showed that there were no significant changes in basal body temperature compared to saline-treated animals \((q = 1.56, p > 0.05)\).

**Memory measurements**

**Object recognition**

The effects of methamphetamine and AZ66 on recognition memory were evaluated in Fig. 4. ANOVA showed
Table 1. Binding affinities of AZ66

<table>
<thead>
<tr>
<th>Radioligand</th>
<th>Non-specific binding</th>
<th>Tissue or cell</th>
<th>$K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sigma ($\sigma$) receptors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\sigma_1$</td>
<td>5 nM [3H] (+)-pentazocine</td>
<td>10 µM haloperidol</td>
<td>Rat brain</td>
</tr>
<tr>
<td>$\sigma_2$</td>
<td>3 µM [3H]di-o-tolyguanidine</td>
<td>10 µM haloperidol</td>
<td>Rat brain</td>
</tr>
<tr>
<td><strong>Neurotransmitter related</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenosine</td>
<td>4.0 nM [3H]NECA</td>
<td>1 µM NECA</td>
<td>Bovine striatum</td>
</tr>
<tr>
<td>Adrenergic, a1</td>
<td>0.3 nM [3H]β-Adrenergic-Prazosin</td>
<td>1 µM phenotamine mesylate</td>
<td>Rat forebrain</td>
</tr>
<tr>
<td>Adrenergic, a2</td>
<td>1 nM [3H]RS 2112002</td>
<td>1 µM phenotamine mesylate</td>
<td>Rat cortex</td>
</tr>
<tr>
<td>Adrenergic, β1</td>
<td>0.04 µM [3H]-(+)-iodocyanopindolol</td>
<td>3 µM alprenol</td>
<td>Human neuroepithelioma</td>
</tr>
<tr>
<td>Cannabinoid, CB1</td>
<td>0.5 nM [3H]CP 59540</td>
<td>1 µM HU-210</td>
<td>Human recombinant</td>
</tr>
<tr>
<td>Cannabinoid, CB2</td>
<td>0.5 nM [3H]CP 59540</td>
<td>1 µM HU-210</td>
<td>Human recombinant</td>
</tr>
<tr>
<td><strong>Dopamine D4.2</strong></td>
<td>0.15 nM [3H]piperperone</td>
<td>1 µM haloperidol</td>
<td>Human recombinant</td>
</tr>
<tr>
<td>GABA A, agonist site</td>
<td>5 nM [3H]GABA</td>
<td>1 µM GABA</td>
<td>Bovine cerebellum</td>
</tr>
<tr>
<td>GABA A, BDZ α 1</td>
<td>1 nM [3H]flunitrazepam</td>
<td>0.5 µM flumazenil</td>
<td>Bovine cortex</td>
</tr>
<tr>
<td>GABA-B</td>
<td>1 nM [3H]CPP 45462A</td>
<td>100 µM bacofoxen</td>
<td>Rat cerebral cortex</td>
</tr>
<tr>
<td>Glutamate, AMPA</td>
<td>5 nM [3H]AMPA</td>
<td>100 µM AMPA</td>
<td>Rat forebrain</td>
</tr>
<tr>
<td>Glutamate, kainate</td>
<td>10 nM [3H]kainic acid</td>
<td>10 µM kainic acid</td>
<td>Rat forebrain</td>
</tr>
<tr>
<td>Glutamate, NMDA agonist</td>
<td>2 nM [3H]CP 39653</td>
<td>300 µM NMDA</td>
<td>Rat forebrain</td>
</tr>
<tr>
<td>Glutamate, NMDA glycine</td>
<td>4 nM [3H]MDL-105,519</td>
<td>3 µM MDL-105,519</td>
<td>Rat cortex/hippocampus</td>
</tr>
<tr>
<td>Glutamate, NMDA/PCP</td>
<td>10 nM [3H]TCP</td>
<td>100 µM (+) MK801</td>
<td>Rat forebrain</td>
</tr>
<tr>
<td>Glutamate, mGluR1</td>
<td>20 nM [3H]quisqualic acid</td>
<td>1 µM t-glutamate</td>
<td>Rat cerebellum</td>
</tr>
<tr>
<td>Glutamate, mGluR5</td>
<td>10 nM [3H]MPEP</td>
<td>10 µM MPEP</td>
<td>Rat brain</td>
</tr>
<tr>
<td>Glycine, strychnine</td>
<td>16 nM [3H]strychnine</td>
<td>100 µM strychnine nitrate</td>
<td>Rat spinal cord</td>
</tr>
<tr>
<td>Histamine H$_1$</td>
<td>2 nM [3H]pyrilamine</td>
<td>10 µM triprolidine</td>
<td>Bovine cerebellum</td>
</tr>
<tr>
<td>Histamine H$_2$</td>
<td>0.1 nM [3H]L-aminohippuric acid</td>
<td>3 µM tiotidine</td>
<td>Guinea pig striatum</td>
</tr>
<tr>
<td>Histamine H$_3$</td>
<td>0.2 nM [3H]H$_3$-Histamine</td>
<td>100 µM R(+)-methylhistamine</td>
<td>Rat forebrain</td>
</tr>
<tr>
<td>Muscarinic, central</td>
<td>0.15 nM [3H]QNB</td>
<td>0.1 µM atropine</td>
<td>Rat cerebral cortex</td>
</tr>
<tr>
<td>Muscarinic, peripheral</td>
<td>0.3 nM [3H]QNB</td>
<td>0.1 µM atropine</td>
<td>Guinea pig bladder</td>
</tr>
<tr>
<td>Muscarinic M$_1$</td>
<td>0.5 nM [3H]4-methylscopolamine</td>
<td>1 µM (–)scopolamine</td>
<td>Human recombinant</td>
</tr>
<tr>
<td>Muscarinic M$_2$</td>
<td>0.5 nM [3H]4-methylscopolamine</td>
<td>1 µM methylscopolamine</td>
<td>Human recombinant</td>
</tr>
<tr>
<td>Nicotinic, muscle</td>
<td>1 nM [3H]β-bungarotoxin</td>
<td>10 µM nicotine</td>
<td>Human TE671 cells</td>
</tr>
<tr>
<td>Nicotinic, neuronal</td>
<td>0.05 nM [3H]epibatidine</td>
<td>20 µM epibatidine</td>
<td>Human SK-N-F1 cells</td>
</tr>
<tr>
<td>Opioid, κ 1</td>
<td>0.05 µM [3H]U-69593</td>
<td>1 µM U-69593</td>
<td>Guinea pig cerebellum</td>
</tr>
<tr>
<td>Opioid, µ</td>
<td>1 nM [3H]DAMGO</td>
<td>1 µM naloxone</td>
<td>Rat forebrain</td>
</tr>
<tr>
<td>Angiotensin II, AT$_1$</td>
<td>0.06 µM [3H]((Sar$_8$-Ile$_9$)angiotensin</td>
<td>1 µM angiotensin II</td>
<td>Human KAN-TS cells</td>
</tr>
<tr>
<td>Angiotensin II, AT$_2$</td>
<td>0.1 µM [3H]Tyr$_8$-angiotensin II</td>
<td>0.05 µM angiotensin II</td>
<td>Bovine cerebellum</td>
</tr>
<tr>
<td>Bradykinin, BK$_A$</td>
<td>0.2 µM [3H]bradykinin</td>
<td>100 µM bradykinin TFA</td>
<td>Guinea pig ileum</td>
</tr>
<tr>
<td>CCK$_1$</td>
<td>0.02 µM [3H]CCK-8</td>
<td>1 µM CCK-8</td>
<td>Mouse pancreas</td>
</tr>
<tr>
<td>CCK$_2$</td>
<td>0.02 µM [3H]CCK-8</td>
<td>1 µM CCK-8</td>
<td>Mouse forebrain</td>
</tr>
<tr>
<td>CRF, non-selective</td>
<td>0.1 µM [3H]Tyr$_8$-oCRF</td>
<td>1 µM Tyr$_8$-oCRF</td>
<td>Rat cerebral cortex</td>
</tr>
<tr>
<td>Endothelin, ET$_\lambda$</td>
<td>0.033 µM [3H]endothelin-1</td>
<td>0.1 µM endothelin-1</td>
<td>Human neuroblastoma</td>
</tr>
<tr>
<td>Endothelin, ET$_\beta$</td>
<td>0.025 µM [3H]endothelin-1</td>
<td>0.1 µM endothelin-1</td>
<td>Human astrocytoma</td>
</tr>
<tr>
<td>Oestrogen</td>
<td>0.1 µM [3H]17β-oestradiol</td>
<td>10 µM 17β-oestradiol</td>
<td>Human breast cancer</td>
</tr>
<tr>
<td>Galanin, non-selective</td>
<td>0.07 µM [3H]galanin</td>
<td>100 µM galanin (porcine)</td>
<td>Rat brain</td>
</tr>
<tr>
<td>Glucocorticoid</td>
<td>1 nM [3H]dexamethasone</td>
<td>10 µM triamcinolone</td>
<td>Human recombinant</td>
</tr>
<tr>
<td>Neurokinin, NK$_A$</td>
<td>1.4 µM [3H] Substance P</td>
<td>1 µM substance P</td>
<td>Rat submaxillary gland</td>
</tr>
<tr>
<td>Neurokinin, NK$_A$ (NKA)</td>
<td>0.1 nM [3H]Neurokinin A</td>
<td>1 µM neurokinin A</td>
<td>Human recombinant</td>
</tr>
<tr>
<td>Neurokinin, NK$_B$ (NKB)</td>
<td>0.1 nM [3H]Leedoisin</td>
<td>1 µM Leedoisin</td>
<td>Rat cerebral cortex</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>1 nM [3H]oxytocin</td>
<td>1 µM oxytocin</td>
<td>Rat uterus</td>
</tr>
<tr>
<td>Testosterone, cytosolic</td>
<td>0.5 nM [3H]methyltrienolone</td>
<td>0.7 µM methyltrienolone</td>
<td>Human LnCAP cells</td>
</tr>
<tr>
<td>TRH</td>
<td>2 nM [3H]3(MeHis)TRH</td>
<td>10 µM TRH</td>
<td>Rat forebrain</td>
</tr>
<tr>
<td>VIP, non-selective</td>
<td>0.05 µM [3H]VIP</td>
<td>1 µM VIP</td>
<td>Rat forebrain</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>0.5 nM [3H]phenylalanyl-3,4,5-ν</td>
<td>1 µM Arg$^8$-vasopressin</td>
<td>Rat liver</td>
</tr>
</tbody>
</table>
significant differences between the groups in the object recognition test ($F_{3,21} = 4.01, p < 0.001$). Post-hoc Tukey's tests confirmed that methamphetamine produced significant impairment of recognition memory when compared to the saline control ($q = 7.04, p < 0.001$); this was prevented by pretreatment with AZ66 ($q = 5.31, p < 0.01$). Animals treated with AZ66 alone showed no significant difference from saline-treated animals ($q = 2.60, p > 0.05$).

**Step-through passive avoidance test**

Figure 5 depicts the effects of methamphetamine and AZ66 on memory using the step-through passive avoidance test. No significant effects were observed on the % entries required for acquisition of the passive avoidance task during training (Fig. 5a) and the 24 h latency to enter the dark compartment during testing (Fig. 5b) among any of the groups tested ($F_{3,21} = 1.03, p > 0.05$). However, while not significant, these results demonstrate a similar trend to that seen with the object recognition, in which animals treated with methamphetamine alone showed increased entries into the dark compartment during the testing period and decreased latency during the testing period. These tendencies were reduced by pretreatment with AZ66.

**Discussion**

The selective $\alpha$ receptor antagonist, AZ66, has been optimized for metabolic stability and tested against the stimulant effects of methamphetamine in our previous study (Seminero et al. 2012). The current study
demonstrates that AZ66 has protective effects against
methamphetamine-induced dopaminergic neurotoxicity,
hyperthermia and memory impairment. These findings
are important as AZ66 retained its protective pharmaco-
hyperthermia and memory impairment. These findings
demonstrates that AZ66 has protective effects against
methamphetamine-induced dopaminergic neurotoxicity,
hypermethamphetamine-induced striatal dopamine and DAT
reductions as well as increases in body temperature.
These neuroprotective properties of AZ66 are likely due
to its ability to modulate body temperature following
methamphetamine exposure. Previous work has demonstrat-
strated that hypothermia can provide neuroprotection
against methamphetamine-induced dopamine deficits
(Bowyer et al. 1994). In addition, earlier studies in our lab
have shown a strong correlation between the ability of σ
receptor ligands to mitigate methamphetamine-induced
hyperthermia and dopaminergic neurotoxicity (Kaushal
et al. 2011). These findings support previous studies that
also demonstrate neuroprotective properties of σ receptor
antagonists against methamphetamine (Kaushal et al.
2011; Matsumoto et al. 2008; Seminerio et al. 2011).

With evidence linking methamphetamine exposure to
an increased risk for the development of Parkinson’s
disease, dopaminergic neurotoxicity remains a central
theme. A myriad of studies have shown that metham-
phetamine produces significant depletions of dopamine
levels and DATs in both humans and animal models
(Cadet & Krasnova, 2009; Kita et al. 2003; Morrow et
al. 2011; Schmidt et al. 1985; Volkow et al. 2001a, b; Wilson
et al. 1996). Furthermore, striatal neurotoxicity, which is
the focus of this study, has been implicated in Parkinson’s
disease and can impact cognitive function (Altgassen
et al. 2007; Beste et al. 2009; Callaghan et al. 2010).

While the majority of research has been dedicated to
the acute effects of methamphetamine on cognitive func-
tion (some showing an increase in cognitive function
following low to moderate doses; Hart et al. 2012), less
is known regarding the long-term effects of repeated
methamphetamine abuse on cognition. The following
paragraphs will discuss the relationship between re-
peated methamphetamine administration and its effects
on the dopaminergic system and cognitive functioning.
In addition, the functional importance of targeting σ
receptors to prevent methamphetamine-induced cognitive
impairments will also be discussed.

A number of neurotransmitter systems are likely in-
volved in methamphetamine-induced memory impair-
ment, including dopamine (Gough et al. 2002; Han & Gu,
2006; Kuczenski et al. 1995). Dopamine has been shown to
modulate different cognitive functions, including mem-
ory, attention, task switching and response inhibition
(Cohen & Servan-Schreiber, 1993; Nordahl et al. 2003).
Dopamine deficits in the striatum have been shown to
reduce reaction time and simple task performance
(Baunez & Robbins, 1999; Nordahl et al. 2003) while do-
pamine deficits in the prefrontal cortex also contribute to
cognitive dysfunction (Baunez & Robbins, 1999; Roberts
et al. 1994; Rogers et al. 1999). Methamphetamine is
known to produce effects in both the striatum and pre-
frontal cortex (Cadet & Krasnova, 2009; Kita et al. 2003),
in which σ receptors are expressed (Guitart et al. 2004;
Hayashi et al. 2010). In addition, σ receptors are thought
to modulate the dopaminergic system (Bastianetto et
al. 1995). The σ receptor ligands, SA 4503 and AC927, have
both been recently reported to modulate methamphet-
mine-induced dopamine release (Kaushal et al. 2012;
Rodvilt et al. 2011), suggesting a role for σ receptors in the

Fig. 1. Effects of methamphetamine (Meth) and AZ66 on
dopamine (DA) levels in the mouse striatum. Mice were
pretreated with saline (0 mg/kg i.p. AZ66) or AZ66 (10 mg/kg
i.p.). After 15 min, the mice were then treated with saline
(– Meth) or Meth (+ Meth, 5 mg/kg i.p.). This treatment
schedule was repeated four times at 2 h intervals. One week
later, the brain was removed and DA levels were measured
using enzyme-linked immunosorbent assay. Data are reported
as mean ± S.E.M. *** p < 0.001 vs. saline, ** p < 0.001 vs. Meth;
N = 6–8 per group.
Sera receptor ligands including CM156, SN79 and AC927, have all been shown to prevent striatal DAT reductions following methamphetamine exposure while having no significant effect on striatal DAT expression on its own (Kaushal et al. 2011; Matsumoto et al. 2008).

The striatum plays an important though often forgotten role in cognition. It has been shown that the striatum cooperates with the hippocampus in the formation of episodic memories (Sadeh et al. 2011), which are often impaired in patients with Parkinson’s disease, and subsequent dopaminergic striatal deficiencies (Altgassen et al. 2007; Beste et al. 2009). Dopamine plays a strong role in the formation of episodic memories similar to those seen in the object recognition test (Hotte et al. 2005). Therefore, it should come as no surprise that neurotoxic doses of methamphetamine, which significantly lower striatal dopamine levels, impair object recognition memory (Belcher et al. 2008; Bisagno et al. 2002; O’Dell et al. 2011; Reichel et al. 2012; Schroder et al. 2003). In the present study, pretreatment with AZ66 significantly attenuated the amnesic effect of neurotoxic methamphetamine in object recognition, which appears mediated at least in part through Sera receptors, as pretreatment with AZ66 also attenuated methamphetamine-induced dopaminergic neurotoxicity in the striatum. It is also possible that protection of these necessary striatal dopamine stores resulted in enhanced object recognition memory via an indirect modulatory role on glutamatergic transmission, given that striatal dopamine plays a role in modulating glutamatergic signalling (Marti et al. 2002; Yamamoto & Davy, 1992), which is important in mediating object recognition memory (Roullet et al. 2001; Sargolini et al. 2003) and Sera receptors can regulate glutamatergic transmission by functionally modulating the NMDA receptor complex (Guitart et al. 2004).

The pharmacology and neuroanatomy of object recognition memory is very complex and can rely upon many brain regions and neurotransmitters. However, recent
research has implicated the perirhinal cortex as an important brain region responsible for object recognition memory (Reichel et al. 2012; Wan et al. 1999; Warburton & Brown, 2010). While projections between the prefrontal cortex and hippocampus are thought to contribute to cognitive memory formation (Hirai et al. 2012; Miyashita & Chang, 1988), the prefrontal cortex does not directly project to the hippocampus, but rather to the perirhinal cortex and amygdala (Burwell, 2001; Furtak et al. 2007; Hirai et al. 2012). In addition to being expressed in the prefrontal cortex, $\alpha$ receptors are also located in the amygdala (Hayashi et al. 2010) and may play a modulatory role on cognition in these areas (Wang et al. 2007). The role of the hippocampus in object recognition remains controversial; however, the putative role is believed to be evoked when spatial cues or landmarks present in the room are used by animals while in the testing chamber (Morris & Frey, 1997). Since our object recognition testing chamber was enclosed within curtains, the effects of distal landmarks and the role of the hippocampus in our behavioural protocol can therefore be minimized.

Consistent with the result in object recognition, treatment with methamphetamine in the absence or presence of AZ66 produced a similar trend to memory changes in the step-through passive avoidance test, although the data were not statistically significant. Dopamine plays a strong role in different brain regions involved with the regulation of inhibitory avoidance memory. In the striatum, pharmacological blockade of dopamine receptors impairs step-through passive avoidance memory (Manago et al. 2009). Dopamine infused into the amygdala post-training enhances memory, while dopamine receptor antagonists impair memory retention in the passive avoidance test (Lalumiere et al. 2004). In addition, the dopamine uptake inhibitor GBR 12783 injected before training significantly improves passive avoidance memory in rats (Nail-Boucherie et al. 1998). One reason for the lack of significant results may be due to the strength of emotional memory created by our behavioural paradigm. Emotional or fear memory is one of the strongest forms of memory and also the easiest to learn. In our behavioural protocol, the mice received a moderately large intensity and duration of footshocks (0.4 mA/5 s). While this makes the training portion of the protocol easier, it can also have an effect of making the memory stronger in all of the treatments, resulting in a response that can mask the promnesic effects of the experimental variable or treatment (Nail-Boucherie et al. 1998; Rossato et al. 2009). It is possible that training animals with lower intensity and shorter duration of shocks (e.g. 0.3 mA/2–3 s) may cause less ‘extreme’ memory and thus allow more significance to be observed 24 h later during our testing paradigm. Nevertheless, our results, taken with the object...
recognition data, suggest that methamphetamine may produce memory impairment in part through α receptors. While earlier studies have shown cognitive enhancement is associated with α receptor agonists after an insult has occurred (van Waarde et al. 2011), we believe AZ66 is working by preventing or minimizing cognitive insult produced by methamphetamine.

In conclusion, the optimized selective α receptor antagonist AZ66 was found to significantly attenuate dopaminergic neurotoxicity and memory impairment produced by repeated exposure to methamphetamine. Future studies will need to be conducted to further characterize the role of α receptors in methamphetamine-induced neurotoxicity and cognitive impairment. However, our studies, taken with previous literature, suggest that α receptors represent a promising target for the development of novel therapeutics aimed at alleviating a multitude of effects produced by methamphetamine.

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Statement of Interest

None.

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