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ARTICLE

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

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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.

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Key words: Aopamine, memory, methamphetamine, neurotoxicity, σ receptors.

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.* 2001*a*, *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

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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 abuser'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 σ_1 and σ_2 receptors at physiologically relevant concentrations (2 ± 0.3 and $47 \pm 10\,\mu$M, 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 σ_1/σ_2 receptor antagonists such as AC927 (*N*-phenethylpiperdine oxalate) or CM156 (3-(4-(4-cyclohexylpiperazin-1-yl)butyl)benzo[D]thiazole-2(3H)-thione) have been shown to attenuate methamphetamine-induced hyperthermia, dopaminergic neurotoxicity and serotonergic 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[D]thiazol-2(3H)-one), a mixed σ_1/σ_2 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.) + saline; (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.) + methamphetamine (5 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 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% 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 (MAB369, dilution 1:10 000; Chemicon International, USA) was performed for 36 h at 4 °C. The labelled sections were then washed twice in TBS and processed using Vectastain Elite ABC (Vector Laboratories, USA). Sections were then incubated with biotinylated secondary anti-rat antisera (ab6844, dilution 1:200; Abcam, USA) in TBS 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 gelatine-coated slides and dried. The sections were then dehydrated, cleared and coverslipped. The images were captured digitally using a Zeiss Axiovert 40 microscope (Carl Zeiss Microscopy, USA) and optical density readings were quantified in anterior regions of the striatum using ImageJ software (National Institutes of Health, USA). To obtain the data point for a given animal, at least two sections per mouse brain were processed and the optical density readings from the striatal region of each section were averaged.

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 σ_1 and σ_2 receptors in the nanomolar and subnanomolar range (Seminerio *et al.* 2012). Compared to its high affinity for σ receptors, AZ66 displayed a >100-fold preference relative to all 64 non-σ binding sites tested.

Neurotoxicity evaluations

Dopamine assays

Figure 1 shows the effects of the σ 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 ($F_{3,88}=118.70$, $p<0.0001$). *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 ($F_{3,15}=19.08$, $p<0.001$). ANOVA of body temperature measured following each treatment time-point revealed significant changes in all but the first-time point (BT1) (Fig. 3): BT1 ($F_{3,39}=9.67$, $p>0.05$); BT2 ($F_{3,39}=13.93$, $p<0.01$); BT3 ($F_{3,39}=14.02$, $p<0.01$); BT4 ($F_{3,39}=21.14$, $p<0.01$). *Post-hoc* Tukey's tests confirmed that methamphetamine significantly increased body temperature after the second injection onwards (BT2, $q=8.09$, $p<0.001$; 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

	Radioligand	Non-specific binding	Tissue or cell	K_i
Sigma (σ) receptors				
σ_1	5 nM [^{3}H](+)-pentazocine	10 μM haloperidol	Rat brain	2.4 ± 0.63
σ_2	3 nM [^{3}H]di-o-tolylguanidine	10 μM haloperidol	Rat brain	0.51 ± 0.15
Neurotransmitter related				
Adenosine	4.0 nM [^{3}H]NECA	1 μM NECA	Bovine striatum	> 10 000
Adrenergic, α1	0.3 nM [^{3}H]7-MeOxy-Prazosin	1 μM phentolamine mesylate	Rat forebrain	> 100
Adrenergic, α2	1 nM [^{3}H]Rx 821002	1 μM phentolamine mesylate	Rat cortex	> 100
Adrenergic, β1	0.04 nM [^{125}I](-) iodocyanopindolol	3 μM alprenolol	Human neuroepithelioma	> 10 000
Cannabinoid, CB1	0.5 nM [^{3}H]CP 55940	1 μM HU-210	Human recombinant HEK293 cells	> 10 000
Cannabinoid, CB2	0.5 nM [^{3}H]CP 55940	1 μM HU-210	Human recombinant CHO cells	> 10 000
Dopamine D4.2	0.15 nM [^{3}H]spiperone	1 μM haloperidol	Human recombinant CHO cells	> 100
GABA A, agonist site	5 nM [^{3}H]GABA	1 μM GABA	Bovine cerebellum	> 10 000
GABA A, BDZ α 1	1 nM [^{3}H]flunitrazepam	0.5 μM flumazenil	Bovine cortex	> 10 000
GABA-B	1 nM [^{3}H]CGP 54626A	100 μM baclofen	Rat cerebral cortex	> 10 000
Glutamate, AMPA	5 nM [^{3}H]AMPA	100 μM AMPA	Rat forebrain	> 10 000
Glutamate, kainate	10 nM [^{3}H]kainic acid	10 μM kainic acid	Rat forebrain	> 10 000
Glutamate, NMDA agonist	2 nM [^{3}H]CGP 39653	300 μM NMDA	Rat forebrain	> 10 000
Glutamate, NMDA glycine	4 nM [^{3}H]MDL-105,519	3 μm MDL-105,519	Rat cortex/hippocampus	> 10 000
Glutamate, NMDA/PCP	10 nM [^{3}H]TCP	100 μM (+)-MK801	Rat forebrain	> 10 000
	5 nM [^{3}H]TCP	10 μM cyclazocine	Rat brain	> 10 000
Glutamate, mGluR1	20 nM [^{3}H]quisqualic acid	1 mM L-glutamate	Rat cerebellum	> 10 000
Glutamate, mGluR5	10 nM [^{3}H]MPEP	10 μM MPEP	Rat brain	> 10 000
Glycine, strychnine	16 nM [^{3}H]strychnine	100 μM strychnine nitrate	Rat spinal cord	> 10 000
Histamine H_1	2 nM [^{3}H]pyrilamine	10 μM triprolidine	Bovine cerebellum	> 100
Histamine H_2	0.1 nM [^{125}I]aminopotentidine	3 μM tiotidine	Guinea pig striatum	> 100
Histamine H_3	0.2 nM [^{3}H]N-α-MeHistamine	100 nM R(-)α-methylhistamine	Rat forebrain	> 100
Muscarinic, central	0.15 nM [^{3}H]QNB	0.1 μM atropine	Rat cerebral cortex	> 100
Muscarinic, peripheral	0.3 nM [^{3}H]QNB	0.1 μM atropine	Guinea pig bladder	> 100
Muscarinic M_1	0.5 nM [^{3}H]N-methyl scopolamine	1 μM (−)scopolamine	Human recombinant CHO cells	> 100
Muscarinic M_2	0.5 nM [^{3}H]N-methyl scopolamine	1 μM methylscopolamine	Human recombinant CHO cells	> 100
Nicotinic, muscle	1 nM [^{125}I]α-bungarotoxin	10 μM nicotine	Human TE671 cells	> 10 000
Nicotinic, neuronal	0.05 nM [^{3}H]epibatidine	20 nM epibatidine	Human SK-N-F1 cells	> 10 000
Opioid, κ 1	0.75 nM [^{3}H]U-69593	1 μM U-69593	Guinea pig cerebellum	> 100
Opioid, μ	1 nM [^{3}H]DAMGO	1 μM naloxone	Rat forebrain	> 100
Angiotensin II, AT_1	0.06 nM [^{125}I](Sar^1-Ile^8)angiotensin	1 μM angiotensin II	Human KAN-TS cells	> 10 000
Angiotensin II, AT_2	0.1 nM [^{125}I]Tyr^4-angiotensin II	0.05 μM angiotensin II	Bovine cerebellum	> 10 000
Bradykinin, BK_2	0.2 nM [^{3}H]bradykinin	100 nM bradykinin TFA	Guinea pig ileum	> 10 000
CCK_1	0.02 nM [^{125}I]CCK-8	1 μM CCK-8	Mouse pancreas	> 10 000
CCK_2	0.02 nM [^{125}I]CCK-8	1 μM CCK-8	Mouse forebrain	> 10 000
CRF, non-selective	0.1 nM [^{125}I]Tyr^0-oCRF	1 μM Tyr^0-oCRF	Rat cerebral cortex	> 10 000
Endothelin, ET_A	0.033 nM [^{125}I]endothelin-1	0.1 μM endothelin-1	Human neuroblastoma	> 10 000
Endothelin, ET_B	0.025 nM [^{125}I]endothelin-1	0.1 μM endothelin-1	Human astrocytoma	> 10 000
Oestrogen	0.1 nM [^{125}I]3,7β-oestradiol	10 nM 17β-oestradiol	Human breast cancer	> 10 000
Galanin, non-selective	0.07 nM [^{125}I]galanin	100 nM galanin (porcine)	Rat brain	> 10 000
Glucocorticoid	1 nM [^{3}H]dexamethasone	10 μM triamcinolone	Human recombinant	> 10 000
Neurokinin, NK_1	1.4 nM [^{3}H]substance P	1 μM substance P	Rat submaxillary gland	> 10 000
Neurokinin, NK_2 (NKA)	0.1 nM [^{125}I]neurokinin A	1 μM neurokinin A	Human recombinan CHO cells	> 10 000
Neurokinin, NK_3 (NKB)	0.1 nM [^{125}I]eledoisin	1 μM eledoisin	Rat cerebral cortex	> 10 000
Oxytocin	1 nM [^{3}H]oxytocin	1 μM oxytocin	Rat uterus	> 10 000
Testosterone, cytosolic	0.5 nM [^{3}H]methyltrienolone	0.7 μM methyltrienolone	Human LnCAP cells	> 10 000
TRH	2 nM [^{3}H](3MeHis^2)TRH	10 μM TRH	Rat forebrain	> 10 000
VIP, non-selective	0.05 nM [^{125}I]VIP	1 μM VIP	Rat forebrain	> 10 000
Vasopressin 1	0.5 nM [^{3}H]phenylalanyl- 3,4,5-v	1 μM Arg^8-vasopressin	Rat liver	> 10 000

Table 1 (*cont.*)

	Radioligand	Non-specific binding	Tissue or cell	K_i
Ion channels:				
Calcium, type L (Benzothiazepine site)	5 nM [^{3}H]diltiazem, cis(+)	10 μM diltiazem	Rat cerebral cortex	>100
Calcium, type L (Dihydropyridine site)	0.2 nM [^{3}H]nitrendipine	1 μM nifedipine	Rat cerebral cortex	>10 000
Calcium, type N	0.01 nM [^{125}I]ω-conotoxin GVIA	0.1 μM ω-conotoxin GVIA	Rat cerebral cortex	>10 000
Potassium, ATP-sensitive	0.2 nM [^{3}H]glibenclamide	0.1 μM glibenclamide	Rat cerebral cortex	>10 000
Potassium, Ca^{2+} act VI	0.05 nM [^{125}I]apamin	100 nM apamin	Rat forebrain	>10 000
Sodium, site 2	2 nM [^{3}H]batrachotoxin	1 mM aconitine	Rat forbrain	>100
Enzymes and other miscellaneous				
Acetylcholine esterase	0.3 mM [^{3}H]acethylthiocholine	100 μM physostigmine	Human recombinant	>100
Choline acetyltransferase	0.2 nM [^{14}C]acetyl coenzyme	0.1 μM Ro 41-1049	Rat cerebral cortex	>10 000
Glutamic acid decarboxylase	4 μM [^{14}C]L-glutamic acid	100 μM aminooxy acetic acid	Rat striatum	>10 000
Leukotriene, LTB_4 (BLT)	0.48 nM [^{3}H]leukotriene B_4	500 nM leukotriene B_4	Guinea pig spleen	>10 000
Leukotriene, LTD_4 (CysLT1)	0.2 nM [^{3}H]leukotriene D_4	1 μM leukotriene D_4	Guinea pig lung	>10 000
MAOA oxidase, peripheral	50 μM [^{14}C]5-HT	1 μM Ro 41-1049	Rat liver mitochondria	>10 000
MAOB oxidase, peripheral	10 μM [^{14}C]phenylethylamine	10 μM Ro 16-6491	Rat liver mitochondria	>10 000
Nitric oxide, NOS (neuronal binding)	5 nM [^{3}H]NOARG	100 μM NOARG	Rat brain	>10 000
Platelet activating factor	1.7 nM [^{3}H]hexadecyl-acetyl-PAF	1 μM C_{16}-PAF	Rabbit platelets	>10 000
Thromboxane, TXA_2	2 nM [^{3}H]SQ 29,548	10 μM pinane-thromboxane	Human platelets	>10 000

GABA, γ-aminobutyric acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; NMDA, *N*-methyl-D-aspartate; PCP, phencyclidine; Ro 41,1049, *N*-(2-aminoethyl)-5-(3-fluorophenyl)-4-thiazolecarboxamide; Ro 16-6491, *N*-(2-aminoethyl)-4-chlorobenzamide; CCK, cholecystokinin; CRF, corticotrophin releasing factor; TRH, thyrotropin releasing hormone; VIP, vasoactive intestinal peptide; NECA, 5′-*N*-ethylcarboxamidoadenosine; Rx 821002, 2-(2,3-dihydro-2-methoxy-1,4-benzodioxin-2-yl)-4,5-dihydro-1*H*-imidazole; CP 55,940, 2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl) cyclohexyl]-5-(2-methyloctan-2-yl)phenol; HU-210, (6a*R*,10a*R*)-9-(hydroxymethyl)-6,6-dimethyl-3-(2-methyloctan-2-yl)-6a,7,10,10a-tetrahydrobenzo [c]chromen-1-ol; MAO, monoamine oxidase; CGP 54626A, cyclohexylmethyl-[(2S)-3-[[(1S)-1-(3,4-dichlorophenyl)ethyl]amino]-2-hydroxypropyl]phosphinic acid; CGP 39653, 2-amino-4-propyl-5-phosphono-3-pentenoic acid; MDL-105,519, (E)-4,6-dichloro-3-(2-phenyl-2-carboxyethenyl)indole-2-carboxylic acid; MK801, (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate dizocilpine hydrogen maleate; TCP,1-(1-(2-thienyl)cyclohexyl)piperidine; MPEP, 2-methyl-6-(phenylethynyl)pyridine; U-69593, (+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide; TFA, trifluoroacetic acid; QNB, quinuclidinyl benzilate; DAMGO, (2S)-2-[[2-[[(2R)-2-[[(2S)-2-amino-3-(4-hydroxyphenyl)propanoyl]amino]propanoyl]amino]acetyl]-methylamino]-*N*-(2-hydroxyethyl)-3-phenylpropanamide; GVIA, (3-iodotyrosyl22)ω-conotoxic; 5-HT, serotonin; NOARG, *L*-N^G-nitro-arginine; PAF, platelet activating factor; SQ 29,548, [1S-[1α, 2α(Z),3α,4α]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; Affinities (K_i in nanomolar, mean ± S.E.M.) were determined in tissue or cell homogenates. Values of >100 and >10 000 signify that there was <50% displacement of the radioligand at that concentration.

significant differences between the groups in the object recognition test ($F_{3,31}=9.01$, $p<0.001$). *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 ($q=2.50$, $p>0.05$).

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. 5*a*) and the 24 h latency to enter the dark compartment during testing (Fig. 5*b*) among any of the groups tested ($F_{3,31}=1.03$, $p>0.05$). 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 σ receptor antagonist, AZ66, has been optimized for metabolic stability and tested against the stimulant effects of methamphetamine in our previous study (Seminerio *et al.* 2012). The current study

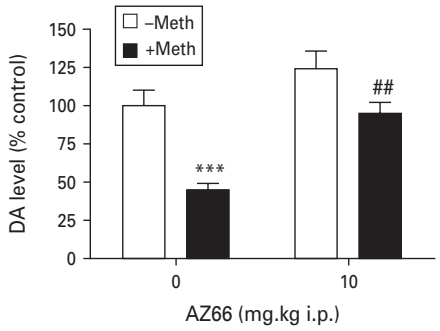

Fig. 1. Effects of methamphetamine (Meth) and AZ66 on dopamine (DA) levels in the mouse striatum. Mice were pretreated with saline (0 mg/kg i.p. AZ66) or AZ66 (10 mg/kg i.p.). After 15 min, the mice were then treated with saline (−Meth) or Meth (+Meth, 5 mg/kg i.p.). This treatment schedule was repeated 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.

demonstrates that AZ66 has protective effects against methamphetamine-induced dopaminergic neurotoxicity, hyperthermia and memory impairment. These findings are important as AZ66 retained its protective pharmacological profile and high selectivity for σ receptors, following optimization from its parent compound CM156, deeming it credible as a lead compound for future drug development.

Consistent with prior studies (Kaushal & Matsumoto, 2011), this work also demonstrates that targeting σ receptors can provide neuroprotective effects. While future studies will need to be conducted to delineate the exact mechanism of this interaction, a number of hypotheses can begin to explain the neuroprotective effects of σ receptor antagonists. Sigma receptors have been shown to modulate various neurotransmitter systems afflicted by methamphetamine (Bastianetto *et al.* 1995; Guitart *et al.* 2004; Mishina *et al.* 2005). Specifically, our results further emphasize a modulatory role of σ receptors in the dopaminergic system and regions of the brain responsible for dopamine transmission.

Dopamine depletions following methamphetamine administration appear primarily dependent on DAT function (Cadet & Krasnova, 2009; Krasnova & Cadet, 2009; Pu *et al.* 1994; Schmidt *et al.* 1985). Animals lacking DATs are protected against dopamine depletions (Fumagalli *et al.* 1998; Giros *et al.* 1996). In addition, striatal dopamine depletions are proportional to the degree of hyperthermia, which is linked to methamphetamine-induced lethality (Bowyer *et al.* 1994). The selective σ receptor antagonist, AZ66, was found to protect against methamphetamine-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 demonstrated 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 dopamingeric 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 methamphetamine 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.* 2001*a*, *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 function (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 repeated 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 involved in methamphetamine-induced memory impairment, including dopamine (Gough *et al.* 2002; Han & Gu, 2006; Kuczenski *et al.* 1995). Dopamine has been shown to modulate different cognitive functions, including memory, 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 dopamine 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 prefrontal 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 methamphetamine-induced dopamine release (Kaushal *et al.* 2012; Rodvelt *et al.* 2011), suggesting a role for σ receptors in the

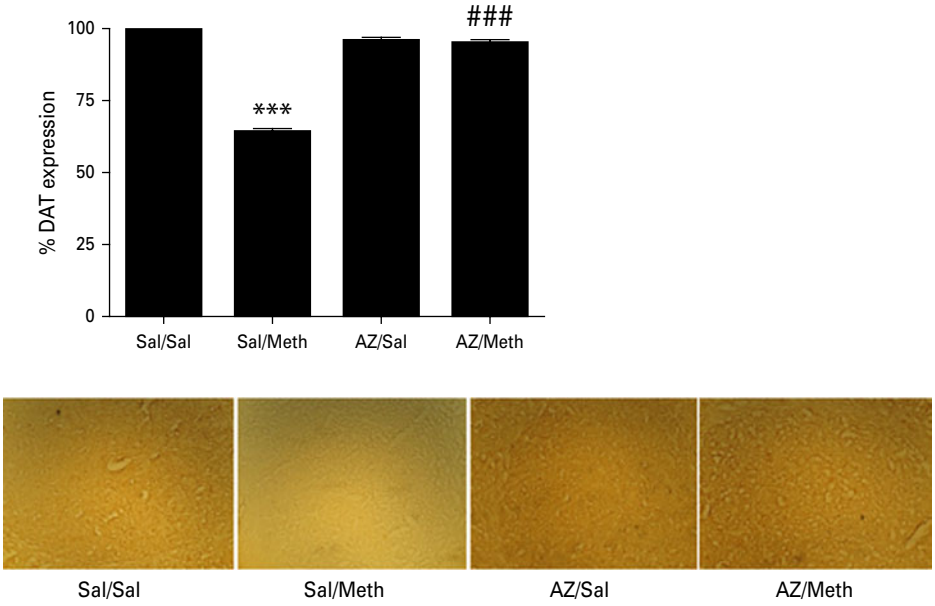

Fig. 2. Effects of methamphetamine (Meth) and AZ66 (AZ) on dopamine transporter (DAT) immunoreactivity in the mouse striatum. Mice were pretreated with saline (Sal) or AZ (10 mg/kg i.p.). After 15 min, the mice were then treated with Sal or Meth (5 mg/kg i.p.). This treatment schedule was repeated four times at 2 h intervals. One week later, the brains were removed and stained for DAT immunoreactivity. A representative section from each treatment group is shown, together with average optical density readings (mean ± S.E.M.). *** $p<0.001$ *vs.* saline, ### $p<0001$ *vs.* Meth; $n=4$ per group.

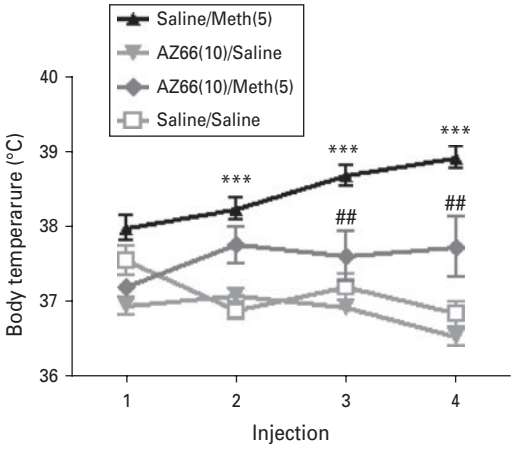

Fig. 3. Effects of AZ66 on methamphetamine (Meth)-induced hyperthermia. Mice were pretreated with saline or AZ66 (10 mg/kg i.p.) and after 15 min the mice were treated with saline or Meth (5 mg/kg i.p.). Core body temperature was measured 1 h after each injection combination. This regimen was repeated four times at 2 h intervals. Data are reported as mean ± S.E.M. *** $p<0.001$ *vs.* saline, ## $p<0.01$ *vs.* Meth; $n=6$–8 per group.

dopaminergic effects of methamphetamine. In addition, other σ 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 σ 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 σ 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

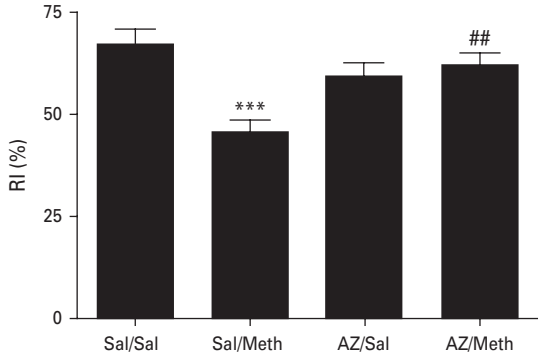

Fig. 4. Effects of AZ66 (AZ) on methamphetamine (Meth)-induced memory impairment in the object recognition test. Mice were pretreated with saline (Sal) or AZ (10 mg/kg i.p.). After 15 min, the mice were then treated with Sal or Meth (5 mg/kg i.p.). This treatment schedule was repeated four times at 2 h intervals. One week later, the animals underwent the object recognition test. RI, Recognition Index. Data are reported as mean ± S.E.M. *** $p < 0.001$ *vs.* Sal; ## $p < 0.01$ *vs.* Meth; $n = 10$ per group.

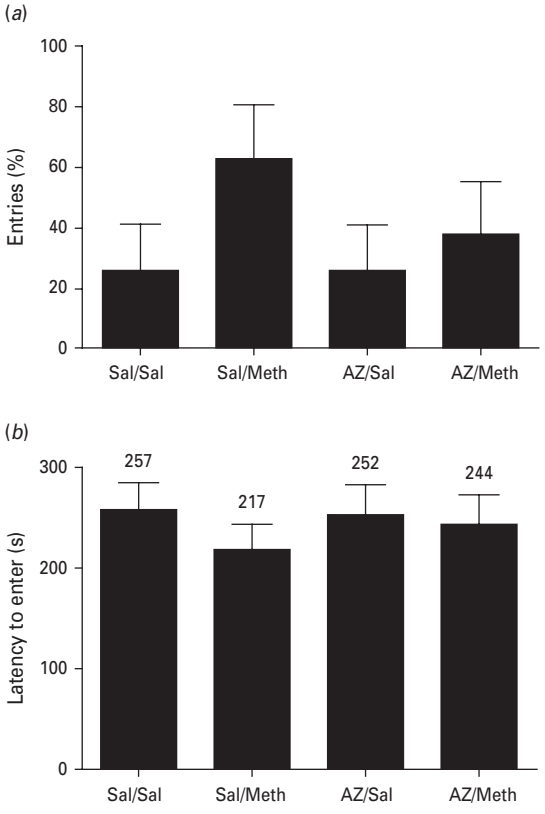

Fig. 5. Effects of AZ66 (AZ) on methamphetamine (Meth)-induced cognitive impairment in the step-through passive avoidance test. Mice were pretreated with saline (Sal) or AZ (10 mg/kg i.p.). After 15 min, the mice were then treated with Sal or Meth (5 mg/kg i.p.). This treatment schedule was repeated four times at 2 h intervals. One week later, the animals underwent the step-through passive avoidance test. Animals were observed for (*a*) % entries into the dark compartment during training and (*b*) latency to enter dark compartment. Data were reported as mean ± S.E.M., no significant changes were observed; $n = 10$ per group.

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, σ 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 σ receptors. While earlier studies have shown cognitive enhancement is associated with σ 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 σ 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 σ receptors in methamphetamine-induced neurotoxicity and cognitive impairment. However, our studies, taken with previous literature, suggest that σ 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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