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Evaluation of novel sigma receptor antagonists against METH-induced neurotoxicity: in vivo and in vitro studies

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Evaluation of novel sigma receptor antagonists against METH-induced neurotoxicity: *in vivo* and *in vitro* studies

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**A dissertation submitted to the School of Pharmacy at
West Virginia University in partial fulfillment of the requirements for the degree of**

Doctor of Philosophy

In

Pharmaceutical and Pharmacological Sciences

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2012**

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ABSTRACT

Evaluation of novel sigma receptor antagonists against METH-induced neurotoxicity:

***in vivo* and *in vitro* studies**

NIDHI KAUSHAL

Methamphetamine (METH) is a highly addictive psychostimulant drug of abuse, causing hyperthermia and neurotoxicity at high doses. Currently, there is no clinically proven pharmacotherapy to treat these effects of METH, necessitating identification of potential novel therapeutic targets. Earlier studies showed that METH binds to sigma receptors in the brain at physiologically relevant concentrations, where it acts in part as an agonist. AC927 (1-(2-phenethyl)piperidine oxalate), CM156 (3-(4-(4-cyclohexylpiperazin-1-yl)butyl)benzo[d]thiazole-2(3*H*)-thione) and SN79 (6-acetyl-3-(4-(4-(4-florophenyl)piperazin-1-yl)butyl)benzo[d]oxazol-2(3*H*)-one) were synthesized as putative sigma receptor antagonists with nanomolar affinity and selectivity for sigma receptors over 29-80 other binding sites. AC927, CM156 and SN79 pretreatment afforded protection against METH-induced hyperthermia and striatal dopaminergic and serotonergic neurotoxicity in male Swiss Webster mice (measured as depletions in striatal dopamine and serotonin levels, and reductions in striatal dopamine and serotonin transporter expression levels). To elucidate the mechanism of neuroprotection provided by sigma receptor antagonists, differentiated NG108-15 cells were used as a cell culture model. METH, at physiologically relevant micromolar concentrations, caused apoptosis in NG108-15 cells. At higher concentrations necrotic cell death was observed. At earlier time points, METH caused generation of reactive oxygen/nitrogen species like hydrogen peroxide and nitric oxide and also release of dopamine from the cells. At later time points, caspase-3, -8 and -9 activation was observed indicating activation of both extrinsic and intrinsic caspase-dependent apoptotic pathways. Sigma receptor antagonism attenuated these neurotoxic mediators and cell death. Additionally, DTG, a sigma receptor agonist shifted the dose response curve of METH-induced cell death (especially necrosis) towards left further confirming the involvement of sigma receptors in the neurotoxic effect of METH. Among the two subtypes of sigma receptors, sigma-2 receptors are highly upregulated in tumor cells and are implicated in cell death mechanism. Both CM398 and CM775, sigma-2 receptor antagonists also attenuated METH-induced neurotoxicity, providing further evidence for involvement of this subtype in METH-induced cell death mechanisms. In addition to activation of cell death mediators, METH also causes increase in body temperature. METH treatment in NG108-15 cells maintained at 40 °C versus 37 °C caused a significant and synergistic increase in cell death, indicating that increase in temperature exacerbates METH-induced toxicity. SN79 was able to attenuate the enhanced cell death observed in the METH treated cells at 40 °C. This can have important implications in *in vivo* systems where sigma receptor antagonism can protect against toxicity induced by METH at elevated temperature. This study provided evidence for the potential application of sigma receptor antagonists as drug development against METH-induced neurotoxicity, in addition to providing a mechanistic basis of neuroprotection.

Dedicated to

The loving memory of my late father

Lt. Col. Satyender Nath Kaushal

My beloved mother

Vijay Kaushal

And my dear sister

Vidushi Kaushal Bahl

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ABBREVIATIONS

AC927	N-phenethylpiperidine oxalate
ANOVA	Analysis of variance
BiP	Binding immunoglobulin protein
BT	Body temperature
CM156	3-(4-(4-cyclohexylpiperazin-1-yl)butyl)benzo[d] thiazole-2(3 <i>H</i>)-thione
CM-H ₂ DCFDA	5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester
DAT	Dopamine transporter
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
HAT	Hypoxanthine-aminopterin-thymidine
H ₂ O ₂	Hydrogen peroxide
5-HT	Serotonin
IP ₃	Inositol trisphosphate
MAM	Mitochondria-associated ER membrane
METH	Methamphetamine
Na ₂ Cr ₂ O ₇	Sodium bichromate
NAC	N-acetylcysteine
Na ₂ S ₂ O ₅	Sodium metabisulfite
NG108-15	Neuroblastomaxglioma hybridoma
L-NMMA	L-N ^G -monomethyl arginine citrate
KCl	Potassium chloride
MFI	Mean fluorescence intensity
·ONOO ⁻	Peroxynitrite;
OH ⁻	Hydroxyl
NMDA	N-methyl-D-aspartate
·NO	Nitric oxide
NOS	Nitric oxide synthase
·O ₂ ⁻	Superoxide
PBS	Phosphate buffered saline
RFU	Relative fluorescence units
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SERT	Serotonin transporter
SN79	6-acetyl-3-(4-(4-(4-florophenyl)piperazin-1-yl)butyl)benzo[d]oxazol-2(3 <i>H</i>)-one
TH	Tyrosine hydroxylase
VMAT2	Vesicular monoamine transporter 2

CHAPTER 1

BACKGROUND AND SPECIFIC AIMS

1.1 Methamphetamine

Methamphetamine (METH) is one of the amphetamine analogs and a psychomotor stimulant. It is a widely abused substance worldwide mainly due to its ease of synthesis from over the counter drugs and its long half life (10-12 h). Due to these factors, it ranks second worldwide after Cannabis as the most extensively abused drug, with an estimated 15-16 million users worldwide (Krasnova and Cadet, 2009).

Apart from its immediate stimulant effects such as euphoria and enhanced energy, METH causes harmful effects upon exposure to repeated or large doses. Some of these harmful effects include hyperthermia, addiction, altered neurological and cognitive functions, psychosis, depression, and neurodegeneration (Krasnova and Cadet, 2009).

The neurodegenerative and neurotoxic effects have been studied using neuroimaging and postmortem studies in humans, depicting damage to dopaminergic and serotonergic neurons in several regions of the brain. This damage can be measured as reductions in dopamine transporters (DAT), serotonin transporters (SERT), and dopamine and serotonin (5-HT) levels (Krasnova and Cadet, 2009). In addition, in certain postmortem studies, apoptotic markers like caspase 3 have also been reported (Kitamura et al., 2007). These neurodegenerative effects are a cause of concern because they are implicated in some of the neuropsychiatric symptoms observed due to METH (Volkow et al., 2001; Scott et al., 2007). In addition, retrospective studies have shown that many Parkinson's disease patients were former abusers of METH (Garwood et al., 2006; Kuehn, 2011). Figure 1.1 shows the comparison of DAT levels in a METH user and control brain 80 days after detoxification.

Figure 1.1 Striatal distribution volume of DAT ligand in METH abuser

FIGURE 1. Striatal Distribution Volume of the Dopamine Transporter Ligand [^{11}C]*d-threo*-Methylphenidate in a 33-Year-Old Male Comparison Subject and a 33-Year-Old Male Methamphetamine Abuser

^a PET scan was performed 80 days after detoxification.
(Volkow et al., 2001)

1.2 Models and markers of METH-induced neurotoxicity

METH-induced neurotoxic damage has been studied by researchers in *in vivo* and *in vitro* models. *In vivo* models include rodents and non-human primates. A 4xMETH (4 injections of METH at 2 hour intervals) is a commonly used dosing regimen for studying METH's neurotoxic effects as it mimics the binge pattern of METH intake by addicts (Villemagne et al., 1998). In addition, a bolus dose of METH administration has also been used, but it leads to a higher mortality rate (Fukumura et al., 1998). The neurotoxic regimen of METH causes damage to neurons in several brain regions like the striatum, cortex, and hippocampus (Deng et al., 2001). Some of the earlier methods used to detect damage were ATP loss and decrease in regional cerebral glucose utilization (Chan et al., 1994; Huang et al., 1999). METH mainly causes degeneration of nerve terminals which have previously been detected using Fluoro jade staining (Schmued and Bowyer, 1997). Damage to dopamine nerve terminals is observed as a decrease in the levels of dopamine, tyrosine hydroxylase,

DAT, and vesicular monoamine transporters (VMAT2) (Krasnova and Cadet, 2009). Similarly, damage to serotonin nerve terminals is observed as a decrease in the levels of 5-HT, tryptophan hydroxylase, and SERT (Krasnova and Cadet, 2009). In addition, it has been recently observed that METH can also cause apoptosis in these brain regions (Deng et al., 2001; Krasnova and Cadet, 2009).

In vitro studies have been done using primary neuronal cells (fetal mesencephalic dopamine neurons, midbrain neuronal cells, mesencephalic dopamine cells) (Bennett et al., 1993; Cubells et al., 1994; Kanthasamy et al., 2006) and immortalized neuronal cells (neuroblastoma NBP2 cells, CATH.a cells, PC12 cells, human neuroblastoma SH-SY5Y cells, immortalized rat mesencephalon cells) (Adams et al., 1996; Cadet et al., 1997; Hom et al., 1997; Choi et al., 2002; Wang et al., 2008) as models to further understand the mechanisms involved in METH-induced neurotoxicity and cell death (Krasnova and Cadet, 2009). Apoptotic and necrotic types of cell death and some of their mechanisms have been studied using makers like TUNEL staining (Samantaray et al., 2006), Hoechst staining (Larsen et al., 2002), annexin V, propidium iodide (Deng et al., 2002), reactive oxygen species (ROS) (Cubells et al., 1994), proapoptotic factors like Bax, Bid, AIF, and caspase levels (Jayanthi et al., 2004).

1.3 Mechanisms of action of METH

Classically, METH is known to cause an enormous increase in dopamine release by the blockade and reversal of DAT (Barr et al., 2006). METH also enters the neuron passively or via DAT and impairs VMAT2 which are present on synaptic vesicles containing the neurotransmitter dopamine. This excessive dopamine in the synapse and cytoplasm causes production of ROS through autooxidation as well as enzyme-mediated reactions (Wrona et al., 1995; Wrona and Dryhurst, 1998; LaVoie and Hastings, 1999a; LaVoie and Hastings, 1999b). This excessive ROS can cause nerve terminal degeneration (by lipid peroxidation and

oxidation of proteins and phospholipids) and cell death via apoptosis or necrosis (Krasnova and Cadet, 2009). Figure 1.2 shows a simplistic mechanism of METH-induced neurotoxicity. Figure 1.3 shows some mechanisms by which dopamine can lead to ROS generation.

Figure 1.2: Mechanisms of METH-induced neurotoxicity

Figure 1.3: Mechanisms of formation of ROS from dopamine

(DA: Dopamine, MAO: Monoamine oxidase, SQ[•]: Dopamine quinone)

Apart from the dopamine system, similar damage is seen in the 5-HT system with ROS generation being the major cause (Kita et al., 2003).

1.4 METH and sigma receptors

Until today, there is no effective treatment for psychostimulant abuse. Exploration of a novel target for medication development, and to better understand the mechanisms of METH-induced neurotoxicity led to the discovery that METH interacts with sigma (σ) receptors at physiologically relevant concentrations (Table 1.1) (Itzhak, 1993; Nguyen et al., 2005). σ Receptors seem to play an important role in many of the effects of METH. They are found to be present in the organs that mediate the actions of METH (e.g. brain, heart, lungs) (Waterhouse et al., 1997). Additionally and more specifically, they are present in the areas of the brain that are damaged by METH (Gundlach et al., 1986).

Table 1.1: Binding affinity of METH for σ receptors

	σ -1 Receptors (μ M)	σ -2 Receptors (μ M)
METH	2.16 ± 0.25	46.67 ± 10.34

(Nguyen et al., 2005)

Previous studies underscored the importance of targeting σ receptors to attenuate some of the behavioral effects of METH in rodent models:

- 1.4.1 Locomotor stimulant effect: σ -1 Receptor antisense and antagonists like BD1047 (N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(dimethylamino)ethylamine) and BD1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine) attenuate METH-induced locomotor stimulation (Nguyen et al., 2005).
- 1.4.2 Sensitization: σ Receptor antagonists like MS377 [(R)-(1)-1-(4-chlorophenyl)-3-[4-(2-methoxyethyl)piperazin-1-yl]methyl-2-pyrrolidinone-L-tartrate] and BMY14802 (alpha-(4-fluorophenyl)-4-(5-fluoro-2-pyrimidinyl)-1-piperazine-butanol) prevent the

development of behavioral sensitization to METH (Ujike et al., 1992; Takahashi et al., 2000).

- 1.4.3 Self administration: Chronic self administration of METH in rats causes an upregulation of σ -1 receptors and mRNA in various brain regions including the substantia nigra, frontal cortex, cerebellum, midbrain, and hippocampus (Stefanski et al., 2004; Hayashi et al., 2010).

1.5 σ Receptors

σ Receptors are a unique group of drug binding sites (Matsumoto et al., 2003). They were first proposed by the group of Martin in 1976 (Martin et al., 1976). They were called σ after the first letter 'S' of SKF-10047, which was then considered a prototypic σ ligand. σ Receptors represent a unique class of proteins that are non-opioidergic and non-N-methyl-D-aspartate (NMDA) receptors (Vaupel, 1983). They possess a unique drug binding profile and anatomical distribution which is very different from the other mammalian proteins (Matsumoto et al., 2003; Guitart et al., 2004). They are found extensively in the brain and periphery (Guitart et al., 2004). Through various biochemical and pharmacological experiments, the two subtypes of σ receptors, σ -1 and σ -2, were characterized (Bowen et al., 1989; Hellewell and Bowen, 1990; Walker et al., 1992).

- 1.5.1 σ -1 Receptors: They have been cloned and are not homologous to any known protein except for sharing 30% homology with fungal sterol isomerase (Moebius et al., 1996), but do not possess isomerase activity. They have a 223 amino acid sequence with a double transmembrane structure (Kekuda et al., 1996; Prasad et al., 1998; Seth et al., 1998) and a molecular weight of 25 kDa (Hellewell and Bowen, 1990). Following the binding of ligands, σ -1 receptors have been shown to operate via protein-protein interactions and translocation between subcellular compartments through chaperone-like functions (Hayashi and Su, 2003a;

Hayashi and Su, 2007; Su et al., 2010), modulation of G-protein coupled receptors, ion channels and signaling molecules like IP₃, protein kinases and calcium (Morin-Surun et al., 1999; Aydar et al., 2002; Hayashi and Su, 2003a; Hayashi and Su, 2007).

1.5.2 σ -2 Receptors: In contrast to σ -1 receptors, much less is known about σ -2 receptors, due to a lack of selective ligands and also because they have not yet been cloned. Using photoaffinity labeling studies, σ -2 receptors have been found to be 18-22 kDa in molecular weight (Hellewell et al., 1994). At the cellular level, they are localized in lipid rafts, the plasma membrane, endoplasmic reticulum (ER), mitochondria, and lysosomes of cells (Gebreselassie and Bowen, 2004; Zeng et al., 2007). σ -2 Receptors are highly upregulated in rapidly dividing tumor cell lines and have a role in cell death signaling via sphingolipid products (Mach et al., 1997; Bowen, 2000), calcium signaling (Vilner and Bowen, 2000; Cassano et al., 2009), and oxidative stress (Ostenfeld et al., 2005; Hornick et al., 2010). Recent studies also show that they can be valuable in chemotherapy and for tumor imaging studies (John et al., 1999; Bowen, 2000; Tu et al., 2010).

A recent study has proposed σ -2 receptors to be a binding site in the PGRMC1 (progesterone receptor membrane component 1) complex (Xu et al., 2011). PGRMC1 like σ -2 receptors are highly expressed in tumor cells in comparison to normal cells (Bowen, 2000; Cahill, 2007). They both are studied as important cancer biomarkers and potential therapeutic targets for anticancer drugs (Bowen, 2000; Cahill, 2007; Mir et al., 2011). This study is very promising given the lack of molecular biology tools to study σ -2 receptors. The novel compounds used in this study are selective for σ -2 over σ -1 receptors. However, additional selectivity profile of those tested compounds over other non-sigma sites may have to be determined in order to further establish the claim.

1.6 Sigma receptor ligands: agonist/antagonist

Several functional studies have been conducted in different systems to characterize agonists and antagonists at σ receptors. In classical pharmacological terms, an agonist at a receptor produces a response, whereas an antagonist is able to inhibit the action of agonist without producing any effect on its own. An antagonist causes a concentration-dependent parallel rightward shift in the dose response curve of an agonist. Among some of the early studies, classical σ ligands like di-o-tolylguanidine (DTG), (+)-pentazocine, and (+)-SKF 10047 caused a dystonic reaction when injected into the red nucleus of rats, and also increased the burst duration of red nucleus neurons following electrical stimulation of cerebellorubrospinal circuits (Matsumoto et al., 1990; Matsumoto et al., 1999). Data such as these suggested that classical compounds, such as DTG and (+)-pentazocine, act as agonists at σ receptors. In contrast, other σ compounds like BD1047 and BD1063 decreased the burst duration of red nucleus neurons following stimulation of cerebellorubrospinal circuits (Matsumoto et al., 1999). In addition, when tested *in vivo*, BD1047 and BD1063 did not show any behavioral effects when injected into the red nucleus of rats, but they prevented the dystonic reactions produced by putative σ receptor agonists such as DTG (Matsumoto et al., 1995). Therefore, these latter compounds were termed as putative antagonists.

Using these types of classical designations, the putative agonist actions of many compounds, including DTG and (+)-pentazocine, were demonstrated in other model systems. Putative antagonist actions of compounds such as BD1047 and BD1063 were also confirmed. As one example, σ receptor agonists have also been shown to modulate the activity of G-proteins by indirectly enhancing GTP γ S binding (Joseph and Bowen, 1998). σ Receptor antagonists such as BD1047 and BD1063 do not produce any effects on their own, but are able to attenuate these agonist-stimulated effects on GTP γ S binding (Joseph and Bowen, 1998). Similar patterns have also been shown with regard to σ receptor agonist-induced

cognitive enhancing effects which can be prevented by σ receptor antagonists (Hashimoto et al., 2007). σ Receptor agonists can also promote NGF-induced neurite outgrowth, and this effect is attenuated in the presence of antagonists (Takebayashi et al., 2002).

Recent studies suggest that, at a subcellular level sigma-1 receptor acts as a ligand-operated chaperone at the mitochondria associated ER membrane (MAM) (Hayashi and Su, 2007). At MAM, sigma-1 receptor is found to be associated with another chaperone protein, BiP. Sigma-1 receptor agonists like (+)Pentazocine, (+)SKF-10047 and PRE-084 cause sigma-1 and BiP dissociation, whereas sigma-1 receptor antagonists like NE-100, Progesterone and haloperidol inhibit agonist induced dissociation of sigma-1 and BiP (Hayashi and Su, 2007).

In some later studies, these antagonists were also able to attenuate various behavioral effects of cocaine (Matsumoto et al., 2003), presumably by interfering with the access of cocaine to one of its binding sites, the σ receptor (Sharkey et al., 1988). This antagonistic effect through σ receptors against cocaine was confirmed by the use of antisense oligonucleotides (ONs) targeting σ -1 receptors, which led to a reduction in the expression of σ -1 receptors in the brain by about 40% (Matsumoto et al., 2001; Matsumoto et al., 2002; Matsumoto et al., 2003). By reducing access of drugs to their target sites, the antisense ONs would be expected to act functionally like pharmacological antagonists. This was indeed confirmed, where reductions in σ -1 receptors by antisense ONs also resulted in the attenuation of the behavioral effects of cocaine (Matsumoto et al., 2001). Similar effects were also observed with behavioral effects of METH (as mentioned before), where σ receptor antisense ONs or antagonists attenuated the METH-induced locomotor stimulant (Nguyen et al., 2005) and sensitized response (Ujike et al., 1992; Takahashi et al., 2000). Similar confirmation of effects by putative antagonists and antisense ONs were obtained in other

model systems, including the cognitive enhancing and neurite outgrowth effects mentioned above. Together, the body of literature provides converging lines of evidence to support the designation of antagonist and agonist effects of σ receptor ligands, which were first assigned using classical pharmacological definitions. Therefore, with regard to their actions through σ receptors, METH is considered a σ receptor agonist, whereas σ receptor ligands that can block the effects of METH are considered putative antagonists. For all of these definitions, validation of the robustness of the effects across multiple model systems is important.

1.7 Potential involvement of σ receptors in the mechanisms of METH-induced neurotoxicity

Mechanistically, the neurotoxic effects of METH seem to involve the activation of certain events such as dopamine and 5-HT release, oxidative stress, ER stress and mitochondrial death cascades, excitotoxicity, microglial activation, and hyperthermia (Krasnova and Cadet, 2009). In addition, compelling evidence in the literature indicates that apart from being a direct target of METH, σ receptors play an important role in the modulation of other neurotransmitter systems and downstream events involved in METH-induced neurotoxicity. Systematic mechanistic studies, however, have not been conducted in the context of METH. Below are mentioned some of the mechanisms involved in the neurotoxic effects of METH and the potential role that σ receptors can play based on evidence from independent studies in the literature. In those instances where the role of an individual subtype of σ receptor is known, the subtype is specified. Otherwise, the term σ receptor, with no subtype designation, is used. Figure 1.4 shows various pathways of METH's neurotoxicity and the steps at which σ receptors could potentially act.

1.7.1 Dopamine and 5-HT release: METH acts through DAT, SERT and VMAT2 to cause excessive release of dopamine and 5-HT into the cytoplasm and synapse (Stephans and

Yamamoto, 1994; Riddle et al., 2006), which is thought to be one of the initiating factors in METH's neurotoxic cascade.

σ Receptors are found in the dopaminergic nerve terminals and cell bodies in the brain (Gundlach et al., 1986) and stimulate dopamine synthesis and release (Booth and Baldessarini, 1991; Bastianetto et al., 1995; Weiser et al., 1995). σ Receptor agonists have been shown to facilitate dopamine release, through both σ -1/2 receptors (Booth and Baldessarini, 1991; Bastianetto et al., 1995; Weiser et al., 1995; Derbez et al., 2002). σ -2 Receptors modulate DAT and the release of dopamine via protein kinase C (PKC) and Ca^{2+} -calmodulin systems (Derbez et al., 2002).

σ Receptors are also present on 5-HT neurons and have been shown to modulate them (Campbell et al., 1989; Shirayama et al., 1993; Hashimoto et al., 1997).

1.7.2 Glutamate, NMDA and excitotoxicity: Glutamate release and excitotoxic mechanisms have also been implicated in METH-induced neurotoxicity (Krasnova and Cadet, 2009). σ Receptors have an important role in modulating the glutamatergic NMDA system and display neuroprotective effects against excitotoxic mechanisms in ischemic stroke models, midbrain and cortical dopaminergic neurons, and retinal ganglion cells (DeCoster et al., 1995; Shimazu et al., 2000; Dun et al., 2007; Shen et al., 2008).

1.7.3 Reactive oxygen and nitrogen species: After excessive dopamine release, auto-oxidation reactions lead to the formation of harmful ROS, including dopamine quinones, superoxide radicals, hydroxyl radicals, and hydrogen peroxide (Krasnova and Cadet, 2009). Additionally, excessive NMDA receptor stimulation by glutamate causes production of superoxide and nitric oxide (NO) production by activation of neuronal nitric oxide synthase (nNOS) (Lafon-Cazal et al., 1993a; Lafon-Cazal et al., 1993b; Gunasekar et al., 1995).

These ROS can injure neurons and surrounding cells via oxidative damage to cellular components such as lipids, proteins, and DNA (Krasnova and Cadet, 2009). Excess 5-HT also

leads to the generation of ROS (Wrona et al., 1997; Jiang et al., 1999) and cause similar damage.

σ -2 Receptor activation has been shown to cause the release of ROS in tumor cells (Ostenfeld et al., 2005; Hornick et al., 2010). In contrast, σ receptor antagonism affords protection in brain ischemia, a condition in which ROS are generated (Schetz et al., 2007). In addition, recent studies suggest that σ receptors can modulate nNOS activity in animal models of stroke (Yang et al., 2010). These studies indicate a potential connection between σ receptors and oxidative/nitrosative stress mechanisms of METH-induced neurotoxicity.

1.7.4 ER stress, calcium and mitochondrial death cascades: METH causes ER stress and activation of mitochondrial death cascades partly due to increases in oxidative stress in the cell (Jayanthi et al., 2004). ER stress causes disturbances in cellular calcium homeostasis (Gorlach et al., 2006). ER stress and calcium dysregulation contributes to cell death observed due to METH (Jayanthi et al., 2004). Sustained ER stress and excessive calcium efflux in turn can contribute to METH-induced apoptosis via mitochondrial pore permeabilization (Jayanthi et al., 2004; Kroemer et al., 2007).

σ Receptors are located on the ER and mitochondria (Guitart et al., 2004; Zeng et al., 2007). σ -1 Receptors have been shown to function as chaperones at the mitochondrial-associated ER membrane (MAM). At MAM, σ -1 receptors play an important role in regulating calcium levels via IP₃ receptors and maintaining homeostasis in the cell against oxidative stress and perturbations in calcium (Hayashi and Su, 2007). On the other hand, σ -2 receptor activation has been shown to cause a biphasic flux of calcium from the ER and mitochondrial stores which leads to apoptotic cell death (Vilner and Bowen, 2000; Cassano et al., 2009).

1.7.5 Apoptotic factors: METH causes apoptotic cell death in certain brain areas including the striatum, cortex, and hippocampus (Deng et al., 2001). The apoptotic process can be either caspase-dependent or independent (Jayanthi et al., 2004).

σ -2 Receptor activation has been shown to cause apoptosis in tumor cells via caspase-dependent and independent processes, depending on the cell type (Bowen, 2000; Crawford and Bowen, 2002). σ -2 Receptor activation has also been shown to cause apoptotic cell death in tumor cells via alterations in cellular sphingolipid levels (Crawford et al., 2002). In contrast, σ -1 receptor agonists have been shown to have neuroprotective effects by regulating intracellular calcium levels and preventing activation of pro-apoptotic genes in retinal ganglion cells (Tchedre and Yorio, 2008). σ -1 Receptor agonists have also been reported to preserve protective genes like bcl-2 in a cerebral focal ischemia model (Yang et al., 2007). σ Receptor ligands have been shown to cause cytotoxicity and apoptotic cell death in various cell lines, including rat C6 glioma cells, NG108-15 neuroblastoma-glioma, SK-N-SH neuroblastoma, SH-SY5Y neuroblastoma, PC12 pheochromocytoma and COS-7 (Crawford and Bowen, 2002; Crawford et al., 2002).

1.7.6 Microglial activation: Recent studies have underscored the role of accessory cells like microglia in METH-induced neurotoxicity (Thomas et al., 2004; Sekine et al., 2008). Microglial activation has been observed in areas of the brain that have been damaged by METH (Thomas et al., 2004; Sekine et al., 2008). Drugs that attenuate neurotoxic effects also suppress microglial activation (Krasnova and Cadet, 2009). However, the mechanisms by which microglia influence METH's neurotoxic effects are still not very clear. Release of cytokines and ROS/RNS have been suggested as possible contributing mechanisms (Krasnova and Cadet, 2009).

σ Receptors are found on microglia and can modulate microglial activation, membrane ruffling and migration (Hall et al., 2009). The subtype of sigma receptors involved in this effect is not clear and additional experiments will need to be performed to address this issue.

1.7.7 Hyperthermia: METH increases body temperature, which is known to exacerbate neurotoxic effects (Bowyer et al., 1994). The production of ROS and release of cytokines like

IL1 β have been implicated in the generation of METH's hyperthermia (Bowyer et al., 1994; Imam et al., 1999; Riedel and Maulik, 1999; Yamashita et al., 2000; Fukami et al., 2004). Moreover, dopaminergic and serotonergic systems are likewise implicated in METH-induced body temperature alterations (Numachi et al., 2007). σ Receptor ligands, with their ability to modulate these neurotransmitter systems, cytokines, as well as ROS, can attenuate elevations in body temperature (Campbell et al., 1989; Bastianetto et al., 1995; Derbez et al., 2002; Schetz et al., 2007; Matsumoto et al., 2008). Additionally, σ receptors are located in the hypothalamic brain region and have previously been shown to modulate body temperature (Gundlach et al., 1986; Rawls et al., 2002). A combination of all these factors may explain how σ receptors can regulate METH-induced hyperthermia.

1.7.8 Other mechanisms:

1.7.8.1 Cytoskeleton: METH-induced nerve terminal damage involves the cleavage of cytoskeletal proteins (Wallace et al., 2003). σ -1 Receptors are found in association with ankyrin, which is a cytoskeletal adaptor protein (Hayashi and Su, 2001).

1.7.8.2 Neurotrophic factors: σ Receptors can play a role in signal amplification in several systems especially that of neurotrophins, which are involved in the survival and differentiation of neurons (Cass et al., 2006). σ -1 Receptor activation enhances BDNF signaling which has a protective role against METH-induced neurotoxicity (Matsuzaki et al., 2004; Yagasaki et al., 2006).

Figure 1.4: Potential involvement of σ receptors in the mechanisms of METH-induced neurotoxicity

1.8 AC927, CM156 and SN79

AC927 (N-phenethylpiperidine oxalate)

CM156 (3-(4-(4-cyclohexylpiperazin-1-yl)butyl)benzo[d] thiazole-2(3*H*)-thione)

SN79 (6-acetyl-3-(4-(4-(4-florophenyl)piperazin-1-yl)butyl)benzo[d]oxazol-2(3*H*)-one)

AC927 (N-phenethylpiperidine oxalate), CM156 (3-(4-(4-cyclohexylpiperazin-1-yl)butyl)benzo[d] thiazole-2(3*H*)-thione) and SN79 (6-acetyl-3-(4-(4-(4-florophenyl)piperazin-1-yl)butyl)benzo[d]oxazol-2(3*H*)-one) were synthesized to have high affinity and selectivity for σ receptors over other non- σ sites (Maeda et al., 2002; Mesangeau et al., 2008; Kaushal et al., 2011a). The affinities of AC927, CM156 and SN79 for σ -1/2 receptors, as well as other receptors, transporters, ion channels and binding sites are listed in Table 1.2. All these compounds have been previously tested against the behavioral effects of cocaine and were found to function as antagonists at σ receptors (Xu et al., 2010; Kaushal et al., 2011a; Matsumoto et al., 2011).

1.8.1 Receptor binding assays: The affinity of the novel compounds for σ receptors and other non- σ binding sites was determined by conducting competition binding assays in

tissues or cells that were heavily concentrated with the respective proteins of interest. This assay is based on the principle of competition between a radiolabelled ligand (specific for that site) and an unlabelled ligand for binding to a receptor or transporter.

Table 1.2 shows the results obtained from radioligand binding studies for AC927, CM156 and SN79. AC927 has nanomolar affinity for σ -1/2 receptors and micromolar to negligible affinity for the other sites tested. CM156 has high affinity for both σ -1/2 receptors in the nanomolar and subnanomolar range. Compared to its high affinity for σ receptors, CM156 had ≥ 1000 -fold weaker affinity for other non- σ binding sites tested. SN79 has high nanomolar affinity for σ -1/2 receptors. However, SN79 also exhibits moderate affinity for 5-HT₂ receptors, SERT and norepinephrine transporters (NET), as well as some affinity for DAT. For all other sites listed in Table 1.2, SN79 displayed negligible affinity ($>10,000$ nM affinity or $>1,000$ -fold selectivity compared to σ binding).

Table 1.2: Binding affinity of AC927, CM156 and SN79 for σ and non- σ sites

Binding Site	Radioligand	Nonspecific binding	Tissue	K _i		
Sigma receptors:				AC927	CM156	SN79
σ_1	5 nM [³ H](+)-pentazocine	10 μ M haloperidol	Rat brain	30 $\pm$ 2	1.3 $\pm$ 0.4	27 $\pm$ 2
σ_2	3 nM [³ H]di-o-tolylguanidine	10 μ M haloperidol	Rat brain	138 $\pm$ 18	0.6 $\pm$ 0.1	7 $\pm$ 0.09
Monoamine transporters:						
Dopamine	0.5 nM [³ H]WIN 35,428	50 μ M cocaine	Rat striatum	2939 $\pm$ 452	1175 $\pm$ 100	2610 $\pm$ 57
Serotonin	0.2 nM [³ H]paroxetine	1.5 μ M imipramine	Rat brainstem	>10,000	1402 $\pm$ 152	159 $\pm$ 15
Norepinephrine	0.5 nM [³ H]nisoxetine	4 μ M desipramine	Rat cerebral cortex	8828 $\pm$ 1197	>10,000	177 $\pm$ 14
Other neurotransmitter receptors:						
Adenosine	4.0 nM [³ H]NECA	1 μ M NECA	Bovine striatum	> 10,000	> 10,000	>10,000
Adrenergic, β_1	0.04 nM [¹²⁵ I](-)iodocyanopindolol	3 μ M alprenolol	Human Neuroepithelioma	>10,000	> 10,000	>10,000
Cannabinoid, CB1	0.5 nM [³ H]CP 55940	1 μ M HU-210	Human recombinant HEK293 cells	ND	>10,000	>10,000
Cannabinoid, CB2	0.5 nM [³ H]CP 55940	1 μ M HU-210	Human recombinant CHO cells	ND	>10,000	>10,000
Dopamine D ₂	5 nM [³ H](-)-sulpiride	1 μ M haloperidol	Rat brain	>10,000	1041 $\pm$ 9	>10,000
GABA A, agonist site	5 nM [³ H]GABA	1 μ M GABA	Bovine cerebellum	>10,000	>10,000	>10,000
GABA A, BDZ α 1	1 nM [³ H]flunitrazepam	0.5 μ M flumazenil	Bovine cortex	ND	>10,000	>10,000
GABA-B	1 nM [³ H]CGP 54626A	100 μ M baclofen	Rat cerebral cortex	ND	>10,000	>10,000
Glutamate, AMPA	5 nM [³ H]AMPA	100 μ M AMPA	Rat forebrain	ND	>10,000	>10,000
Glutamate, kainite	10 nM [³ H]kainic acid	10 μ M kainic acid	Rat forebrain	ND	>10,000	>10,000
Glutamate, NMDA agonist	2 nM [³ H]CGP 39653	300 μ M NMDA	Rat forebrain	>10,000	>10,000	>10,000
Glutamate, NMDA-glycine	4 nM [³ H]MDL-105,519	3 μ M MDL-105,519	Rat cortex/hippoc	ND	>10,000	>10,000
Glutamate, NMDA/PCP	10 nM [³ H]TCP	100 μ M (+)-MK801	Rat forebrain	ND	>10,000	>10,000
	5 nM [³ H]TCP	10 μ M cyclazocine	Rat brain	ND	>10,000	>10,000
Glutamate, mGluR1	20 nM [³ H]quisqualic acid	1 mM L-glutamate	Rat cerebellum	ND	>10,000	>10,000
Glutamate, mGluR5	10 nM [³ H]MPEP	10 μ M MPEP	Rat brain	ND	>10,000	>10,000
Glycine,strychnine	16 nM [³ H]strychnine	100 μ M strychnine nitrate	Rat spinal cord	ND	>10,000	>10,000
Histamine H ₂	0.1 nM [¹²⁵ I]aminopotentidine	3 μ M tiotidine	Guinea pig striatum	ND	2570	>10,000
Muscarinic, central	0.15 nM [³ H]QNB	0.1 μ M atropine	Rat cerebral cortex	>10,000	3080	>10,000
Muscarinic, peripheral	0.3 nM [³ H]QNB	0.1 μ M atropine	Guinea pig bladder	>10,000	2155	>10,000

Muscarinic M ₁	0.5 nM [³ H]N-methylscopolamine	1 µM (-)scopolamine	Human recombinant CHO cells	ND	>10,000	>10,000
Muscarinic M ₂	0.5 nM [³ H]N-methylscopolamine	1 µM methylscopolamine	Human recombinant CHO cells	ND	1210	>10,000
Nicotinic, muscle	1 nM [¹²⁵ I]α-bungarotoxin	10 µM nicotine	Human TE671 cells	ND	>10,000	>10,000
Nicotinic, neuronal	0.05 nM [³ H]epibatidine	20 nM epibatidine	Human SK-N-F1 cells	ND	>10,000	>10,000
Opioid, non-selective	0.5 nM [³ H]bremazocine 1 nM [³ H]naloxone	10 µM levallorphan 1 µM naloxone	Rat brain Rat forebrain	>10,000 >10,000	>10,000 >10,000	>10,000 >10,000
5-HT ₂	2 nM [³ H]ketanserin	1 µM mianserin	Rat brain	>10,000	1376 ± 159	321 ± 16
Hormone, peptide, steroid receptors:						
Angiotensin II, AT ₁	0.06 nM [¹²⁵ I](Sar ¹ -Ile ⁸)angiotensin	1 µM angiotensin II	Human KAN-TS cells	ND	>10,000	>10,000
Angiotensin II, AT ₂	0.1 nM [¹²⁵ I]Tyr ⁴ -angiotensin II	0.05 µM angiotensin II	Bovine cerebellum	ND	>10,000	>10,000
Bradykinin, BK ₂	0.2 nM [³ H]bradykinin	100 nM bradykinin TFA	Guinea pig ileum	ND	>10,000	>10,000
Cholecystokinin, CCK _A	0.02 nM [¹²⁵ I]CCK-8	1 µM CCK-8	Mouse pancreas	ND	>10,000	>10,000
Cholecystokinin, CCK _B	0.02 nM [¹²⁵ I]CCK-8	1 µM CCK-8	Mouse forebrain	ND	>10,000	>10,000
CRF, non-selective	0.1 nM [¹²⁵ I]Tyr ⁰ -Ocrf	1 µM Tyr ⁰ -oCRF	Rat cerebral cortex	ND	>10,000	>10,000
Endothelin, ET _A	0.033 nM [¹²⁵ I]endothelin-1	0.1 µM endothelin-1	Human neuroblastoma	ND	>10,000	>10,000
Endothelin, ET _B	0.025 nM [¹²⁵ I]endothelin-1	0.1 µM endothelin-1	Human astrocytoma	ND	>10,000	>10,000
Estrogen	0.1 nM [¹²⁵ I]3,7β-estradiol	10 nM 17β-estradiol	Human breast cancer	ND	>10,000	>10,000
Galanin, non-selective	0.07 nM [¹²⁵ I]galanin	100 nM galanin (porcine)	Rat brain	ND	>10,000	>10,000
Glucocorticoid	1 nM [³ H]dexamethasone	10 µM triamcinolone	Human recombinant	ND	>10,000	>10,000
Neurokinin, NK ₁	1.4 nM [³ H]substance P	1 µM substance P	Rat submaxillary gland	ND	>10,000	>10,000
Neurokinin, NK ₂	0.1 nM [¹²⁵ I]neurokinin A	1 µM neurokinin A	Human recombinant CHO cells	ND	1510	>10,000
Neurokinin, NK ₃ (NKB)	0.1 nM [¹²⁵ I]eledoisin	1 µM eledoisin	Rat cerebral cortex	ND	>10,000	>10,000
Oxytocin	1 nM [³ H]oxytocin	1 µM oxytocin	Rat uterus	ND	>10,000	>10,000
Testosterone, cytosolic	0.5 nM [³ H]methyltrienolone	0.7 µM methyltrienolone	Human LnCAP cells	ND	>10,000	>10,000
TRH	2 nM [³ H](3MeHis ²)TRH	10 µM TRH	Rat forebrain	ND	>10,000	>10,000
VIP, non-selective	0.05 nM [¹²⁵ I]VIP	1 µM VIP	Rat forebrain	ND	>10,000	>10,000
Vasopressin 1	0.5 nM [³ H]phenylalanyl-3,4,5-v	1 µM Arg ⁸ -vasopressin	Rat liver	ND	>10,000	>10,000
Ion channels:						
Calcium, type L (DHP)	0.2 nM [³ H]nitrendipine	1 µM nifedipine	Rat cerebral cortex	>10,000	1710	>10,000

Calcium, type N	0.01 nM [¹²⁵ I]ω-conotoxin GVIA	0.1 μM ω-conotoxin GVIA	Rat cerebral cortex	>10,000	>10,000	>10,000
Potassium, ATP-sensitive	0.2 nM [³ H]glibenclamide	0.1 μM glibenclamide	Rat cerebral cortex	>10,000	>10,000	>10,000
Potassium, Ca ²⁺ act VI	0.05 nM [¹²⁵ I]apamin	100 nM apamin	Rat forebrain	ND	>10,000	>10,000
Enzymes and other miscellaneous:						
Acetylcholine esterase	0.3 mM [³ H]acetylthiocholine	100 μM physostigmine	Human recombinant	ND	>10,000	>10,000
Choline acetyltransferase	0.2 nM [¹⁴ C]acetyl coenzyme	0.1 μM Ro 41-1049	Rat cerebral cortex	ND	>10,000	>10,000
Glutamic acid decarboxylase	4 μM [¹⁴ C]L-glutamic acid	100 μM aminooxy acetic acid	Rat striatum	ND	>10,000	>10,000
Leukotriene, LTB ₄ (BLT)	0.48 nM [³ H]leukotriene B ₄	500 nM leukotriene B ₄	Guinea pig spleen	ND	>10,000	>10,000
Leukotriene, LTD ₄ (CysLT1)	0.2 nM [³ H]leukotriene D ₄	1 μM leukotriene D ₄	Guinea pig lung	ND	>10,000	>10,000
MAO-A oxidase, peripheral	50 μM [¹⁴ C]5-HT	1 μM Ro 41-1049	Rat liver mitochondria	ND	>10,000	>10,000
MAO-B oxidase, peripheral	10 μM [¹⁴ C]phenylethylamine	10 μM Ro 16-6491	Rat liver mitochondria	ND	>10,000	>10,000
Nitric oxide, NOS	5 nM [³ H]NOARG	100 μM NOARG	Rat brain	ND	1220	>10,000
Platelet activating factor	1.7 nM [³ H]hexadecyl-acetyl-PAF	1 μM C ₁₆ -PAF	Rabbit platelets	ND	>10,000	>10,000
Thromboxane, TXA ₂	2 nM [³ H]SQ 29,548	10 μM pinane-thromboxane	Human platelets	ND	>10,000	>10,000

Affinities (K_i in nM) were determined in tissue or cell homogenates. The values in this table represent the mean ± S.E.M. from replicate assays. Values of >10,000 signify that there was less than 30% displacement of the radioligand at that concentration. BTZ=benzothiazepine; CRF=corticotrophin releasing factor; DHA=dihydroalprenolol; DHP=dihydropyridine; GABA=γ-aminobutyric acid; LSD=lysergic acid diethylamide; MAO=monoamine oxidase; MPEP=2-methyl-6-(phenylethynyl)pyridine; NECA=5'-N-ethylcarboxamidoadenosine; NOARG=L-N^G-nitro-arginine; PAF=platelet activating factor; QNB=quinuclidinyl benzilate; TBOB=t-butylbicycloorthobenzoate; TBPS=t-butylbicyclophosphorothionate; TCP=1-[1-(2-thienyl)cyclohexyl]piperidine; TRH=thyrotropin releasing hormone; VIP=vasoactive intestinal peptide.

1.8.2 Pharmacokinetic studies: Pharmacokinetic studies were conducted for SN79 and CM156, but not for AC927 as it displayed toxicity at supratherapeutic doses deeming it not suitable as a drug candidate.

1.8.2.1 In vitro liver microsomal assays: The metabolic stability of CM156 was evaluated through the NIDA Medications Development Program in liver microsomes prepared from rat, monkey and human (Table 1.3). Using a Tecan Liquid Handling System (Tecan, Research Triangle Park, NC), CM156 (5 μ M) was incubated at 37° C with the reaction mixture. At designated time points (0, 15, 30 and 60 min) the supernatants were collected and analyzed using LC/MS.

Table 1.3 Metabolic stability of CM156 in rat, monkey, and human liver microsomes

Species	Incubation Time (min)	Cofactor (+/-)	Area Ratio	Percent Loss Of Substrate	Percent Remaining	Half-Life (min)	Clearance (ml/min/kg)
Rat	0	+	143	0.0%	100%	4.62	270
	15	+	12.7	91.2%	8.8%		
	30	+	12.2	91.5%	8.5%		
	60	+	11.5	92.0%	8.0%		
	0	-	148	0.0%	100%		
	60	-	148	0.0%	100%		
Monkey	0	+	140	0.0%	100%	2.8	184
	15	+	3.17	97.7%	2.3%		
	30	+	2.11	98.5%	1.5%		
	60	+	1.81	98.7%	1.3%		
	0	-	146	0.0%	100%		
	60	-	144	1.4%	98.6%		
Human	0	+	133	0.0%	100%	10.6	58.8
	15	+	51.4	61.3%	38.7%		
	30	+	15.4	88.4%	11.6%		
	60	+	7.35	94.5%	5.5%		
	0	-	138	0.0%	100%		
	60	-	142	No loss	103%		

Values are the mean of triplicate determinations, except for the zero cofactor values that are the mean of duplicate determinations. CM156 was tested at a concentration of 5 μ M. Protein concentration was set at 1.0 mg protein/ml.

1.8.2.2 *In vivo pharmacokinetic studies:* Plasma pharmacokinetic parameters associated with oral dosing of SN79 are summarized in Table 1.4. In this study, blood concentration of orally administered SN79 (20 mg/kg) was determined in Sprague Dawley rats at different time intervals using ultra performance liquid chromatography. CM156 showed short half life in *in vitro* microsomal assays due to which it was not tested in *in vivo* pharmacokinetic studies.

Table 1.4: Mean plasma pharmacokinetic parameters of SN79 after oral administration in Sprague-Dawley rats

AUC	117.34 µg-hr/ml
T _{1/2}	7.51 hr
C _{max}	0.205 µg/ml
MRT	9.87 hr
V _d	115.10 l/kg
CL _{ss} /F	0.18 l/hr/kg
T _{max}	1.5 hr

C_{max}, maximum concentration; T_{max}, time to reach maximum concentration; AUC, area under the curve concentration; V_d, volume of distribution; MRT, mean residence time; CL_{ss}/F, apparent oral clearance at steady state.

1.8.3 Cytochrome P450 Interactions: Since, SN79 was the most druggable compound out of AC927 and CM156 (it did not display any toxicity like AC927 and had long half life unlike CM156), it was the only compound to be moved forward in the cytochrome P450 studies. The effect of SN79 on a number of human cytochrome P450 enzymes was assessed by NovaScreen CYP Screen (Caliper Life Sciences, Hopkinton, MA). The specific cytochrome P450 enzymes selected were those most likely to cause problematic drug-drug interactions. Table 1.5 provides a synopsis of the assay conditions, with NADPH (nicotinamide adenine dinucleotide phosphate) added to the substrate to catalyze the reaction. SN79 was evaluated at a single screening concentration of 10 μ M, with 50% used as the criterion for significant inhibition at this concentration.

Table 1.5 Human cytochrome P450 inhibition studies

Cytochrome P450	Substrate	% Inhibition by SN79
1A2	5 μ M 7-ethoxy-3-cyanocoumarin	17.8%
2A6	3 μ M coumarin	-10.4%
2C19	2.5 μ M 7-ethoxy-3-cyanocoumarin	34.8%
2C9*1	37.5 μ M 7-methoxy-4-trifluoromethyl-coumarin	15.3%
2D6	0.5 μ M AMMC	-1.7%
3A4	0.5 μ M dibenzyl fluorescein	29.0%

Duplicate assays were conducted for the inhibition of human recombinant cytochrome P450 enzymes expressed in SF9 cells by SN79. NADPH was added to the substrate to catalyze the reaction. SN79 was evaluated at a single screening concentration of 10 μ M, with 50% used as the criterion for significant inhibition at this concentration. Negative values represent slight induction. SN79 did not significantly inhibit or induce the major cytochrome P450 enzymes tested.

1.9 *In vivo* model: Male, Swiss Webster mice

This strain of mice was used for the studies in this dissertation because they are capable of exhibiting the behavioral and neurotoxic effects of METH, and have been widely used to study σ and non- σ compounds (Itzhak and Ali, 1996; Ali and Itzhak, 1998; Callahan and Ricaurte, 1998; Itzhak et al., 1998; Itzhak et al., 2003; Nguyen et al., 2005; Matsumoto et al., 2008). Their continued use will facilitate comparisons to data already in the literature. Mice were preferred over rats for these studies because the novel compounds are produced in small amounts, and the smaller size of the mice will help in optimizing their use. Male mice were used in this study because of varying levels of progesterone in the system of female mice during estrous cycle (Kosaka et al., 1988). Progesterone is considered an endogenous σ ligand and can therefore interfere with the binding of the drugs and interpretation of the results (Su, 1991).

1.10 *In vitro* model: NG108-15 cells

In order to elucidate the cellular mechanisms of METH-induced toxicity at which σ receptors may intervene, a neuronal cell culture model (neuroblastoma $\times$ glioma hybridoma, NG108-15 cells) was used. A cell culture model also provides a system where the temperature can be maintained constant and the mechanistic basis of the neuroprotective effects of σ antagonists can be studied under more controlled conditions compared to *in vivo*. NG108-15 cells when differentiated display neurite outgrowth, electrical excitability and express proteins with neuronal and glial properties (Ma et al., 1998). These cells have been used extensively as a sensitive cell line to study toxic effects of various drugs and chemicals (Canete and Diogene, 2008). Additionally, NG108-15 cells have been commonly used in the literature to study the functioning of σ receptors, thus making it a suitable model for these

studies (McLawhon et al., 1981; Georg and Friedl, 1991; Vilner et al., 1995b; Hayashi and Su, 2003a; Peeters et al., 2004).

1.11 Specific Aims

The future chapters will describe the studies conducted using sigma receptor antagonists: AC927, CM156 and SN79 in *in vivo* and *in vitro* models. The aims of this dissertation are as follows:

Specific Aim 1: To determine if antagonism of σ receptors (using AC927, CM156 and SN79) can attenuate METH-induced neurotoxicity *in vivo*: The following studies were performed:

1. To determine if σ receptor antagonists (AC927, CM156 and SN79) can attenuate METH-induced neurotoxicity *in vivo*. Male, Swiss Webster mice were pretreated with AC927, CM156 or SN79 to determine if METH-induced dopaminergic and serotonergic neurotoxicity can be attenuated. The markers studied were: striatal dopamine and 5-HT levels, striatal DAT and SERT expression levels. This study had been included in chapter 2.
2. To determine if the σ receptor antagonists can attenuate the hyperthermic effects of METH. Male, Swiss Webster mice were pretreated with AC927, CM156 or SN79 to determine if METH-induced hyperthermia can be attenuated. This study has been included in Chapter 3.
3. To determine if hyperthermia is associated with neurotoxicity of METH. Correlation between the body temperature of the mice and striatal dopamine and serotonin levels was determined. This study has been included in Chapter 3.

Specific Aim 2: To determine the mechanisms of neuroprotective effects of sigma receptor antagonists against METH-induced neurotoxicity *in vitro*: The following studies were performed:

1. Identification of the mechanisms of METH-induced neurotoxicity in differentiated NG108-15 cells that can be prevented by sigma receptor antagonists (AC927 and SN79). Differentiated NG108-15 cells were used as a model system to identify the mechanisms of METH-induced neurotoxicity that can be attenuated by sigma receptor antagonists. The neurotoxic end points and mediators detected were: apoptosis, necrosis, caspase-3, -9, -8 activation, ROS/RNS generation and dopamine release. The results from this study have been included in chapter 4 and 5.
2. Effect of elevated temperature on METH-induced neurotoxicity in differentiated NG108-15 cells: Differentiated NG108-15 cells were used to study the effect of METH treatment at 37 °C and elevated temperature (40 °C). The ability of SN79 to attenuate METH and elevated temperature-induced toxicity was also determined. The results from this study have been included in Chapter 5.

Specific Aim 3: To determine if antagonism of σ -2 receptors can attenuate METH-induced neurotoxicity *in vitro*: A series of potential σ 2 receptor selective compounds were synthesized and screened using radioligand binding assay for their high affinity and selectivity for σ 2 receptors over σ 1 receptors and other non- σ sites. Sigma-2 receptor antagonist, CM398 was tested to determine its ability to attenuate METH-induced apoptotic and necrotic end points. The results from this study have been included in Chapter 6.

It is expected that this study will help us understand the role of σ receptors especially σ 2 receptor subtype in the neurotoxic effects of METH. The work is significant because of the

impact it will have not only on the understanding of the functioning of σ receptors but also on the potential development of medications to treat METH-induced neurotoxicity.

1.12 Contributions: The compounds CM156 and SN79 were synthesized by Dr. Christophe Mésangeau and Dr. Sanju Narayanan in Dr. Christopher R. McCurdy's lab at University of Mississippi. AC927 was obtained from Dr. Andrew Coop's laboratory at University of Maryland, Baltimore.

The radioligand binding studies for SN79 were performed by Dr. Jamaluddin Shaikh, Michael Seminerio and Dr. Yan-tong Xu at the University of Mississippi and West Virginia University. The radioligand binding studies for CM156 and AC927 were performed by Dr. Jamaluddin Shaikh at the University of Mississippi. The NOVAScreen for all the three compounds were performed by Calipers Life Sciences (Hanover, MD).

The *in vivo* pharmacokinetic studies for SN79 were performed by Dr. Harsha Vinnakota in Dr. Bonnie A. Avery's lab at University of Mississippi.

CHAPTER 2

EVALUATION OF SIGMA RECEPTOR ANTAGONISTS AGAINST METH-INDUCED NEUROTOXICITY: *IN VIVO* STUDIES

2.1 Introduction

High or repeated METH administration results in hyperthermia and neurotoxicity (Cruickshank and Dyer, 2009). At high doses, neurotoxic cascades are activated due to excessive dopamine and 5-HT release into the cytoplasm and synapse (Baldwin et al., 1993; Kuczenski et al., 1995; 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 DAT, 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 (Walsh and Wagner, 1992; Cruickshank and Dyer, 2009), 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 neurotoxicity was evaluated. We focused 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 on dopamine and 5-HT neurons, which are damaged by neurotoxic doses of METH, and can modulate their function (Campbell et al., 1989; Bastianetto et al., 1995; Derbez et al., 2002). Third, σ receptors participate in cell death pathways in tumor cells (Bowen, 2000), suggesting their possible contribution in METH-induced neurotoxic cascades. Fourth, 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).

Therefore, in this study three sigma receptor antagonists (AC927, CM156 and SN79) were tested to determine if they can attenuate METH-induced neurotoxicity *in vivo*. All these compounds have previously been tested against the behavioral and toxic effects of cocaine, and were found to function as antagonists at σ receptors (Xu et al., 2010; Kaushal et al.,

2011a; Matsumoto et al., 2011). The effect of pretreatment of AC927, CM156 and SN79 was determined on the striatal dopaminergic and serotonergic neurotoxicity caused by METH. The markers used were: striatal dopamine and 5-HT levels, striatal DAT and SERT expression.

2.2 Materials and Methods

2.2.1. Animals: Male, Swiss Webster mice (18-28 g; Harlan, Indianapolis, IN) were used for the *in vivo* studies. They were housed in groups of five with a 12:12h light/dark cycle with food and water available *ad libitum*. Naïve mice were randomly assigned to different treatment groups. All procedures involving animals were performed as approved by the Institutional Animal Care and Use Committee at the West Virginia University.

2.2.2. Drugs: CM156 and SN79 were obtained from Dr. Christopher McCurdy's laboratory (University of Mississippi, University, MS). AC927 was provided by Dr. Andrew Coop (University of Maryland, Baltimore, MD). METH hydrochloride was obtained from Research Biochemicals International (Natick, MA).

2.2.3. METH neurotoxic schedule: As shown in Figure 2.1 male, Swiss Webster mice were randomly divided into four experimental groups: 1) Saline/Saline, 2) Saline/METH (1.25, 2.5, 5, 10 mg/kg) 3) Antagonist (AC927: 5, 10, 20 mg/kg; CM156: 5, 10, 20 mg/kg or SN79: 1, 3, 10 mg/kg)/Saline, 4) Antagonist (AC927: 5, 10, 20 mg/kg, i.p.; CM156: 5, 10, 20 mg/kg, i.p. or SN79: 1, 3, 10 mg/kg, i.p.)/METH (5, 10 mg/kg, i.p.). These doses of AC927, CM156 and SN79 were selected as these were effective in attenuating the behavioral and toxic effects of cocaine, another sigma receptor agonist (Xu et al., 2010; Kaushal et al., 2011a; Matsumoto et al., 2011). The first drug was administered 15 min prior to the second drug. 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 their

brains removed one week following treatment (Cappon et al., 2000). The detailed procedure for each of the end points is provided below.

Figure 2.1 Neurotoxic schedule of METH

2.2.3.1. Dopamine assay: The mice ($n = 5-9/\text{group}$) 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) AC927 (5, 10, 20 mg/kg, i.p.)/CM156 (5, 10, or 20 mg/kg, i.p.)/SN79 (1, 3, 10 mg/kg, i.p.) + Saline; (4) AC927 (5, 10, 20 mg/kg, i.p.)/CM156 (5, 10, or 20 mg/kg, i.p.)/SN79 (1, 3, 10 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. One week after the treatment schedule, the mice were

sacrificed and 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 and 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 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 (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 is inversely proportional to the dopamine concentration of the tissue sample and is quantified relative to a standard curve of known concentrations.

2.2.3.2. 5-HT assays: The mice ($n = 5-10/\text{group}$) 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) AC927 (5, 10, 20 mg/kg, i.p.)/CM156 (5, 10, or 20 mg/kg, i.p.)/SN79 (1, 3, 10 mg/kg, i.p.) + Saline; (4) AC927 (5, 10, 20 mg/kg, i.p.)/CM156 (5, 10, or 20 mg/kg, i.p.)/SN79 (1, 3, 10 mg/kg, i.p.) + METH (5, 10 mg/kg, i.p.). One week following the treatments, 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).

2.2.3.3. DAT Immunohistochemistry: Striatal sections were assessed for DAT expression. The mice ($n = 4/\text{group}$) were randomly assigned to one of the following treatment groups: (1) Saline + Saline; (2) Saline + METH (5 mg/kg, i.p.); (3) AC927 (10 mg/kg, i.p.)/CM156 (10 mg/kg, i.p.)/SN79 (3 mg/kg, i.p.) + METH (5 mg/kg, i.p.); (4) AC927 (10 mg/kg, i.p.)/CM156 (10 mg/kg, i.p.)/SN79 (3 mg/kg, i.p.) + Saline. Striatal sections were assessed for DAT expression. 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 anti-rat DAT antibody (Chemicon International, Temecula, CA; MAB369, dilution 1:10,000) were then performed for 36 h at 4 $^{\circ}\text{C}$. The labeled sections were washed twice in TBS and processed using Vectastain Elite ABC (Vector Laboratories, Burlingame, CA). Sections were incubated with biotinylated secondary anti-rat antisera (diluted 1:200) in TBS-NBS for 60 min. This was followed by incubation of the sections with avidin-biotinylated peroxidase substrate in TBS for 60 min. The staining was 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 dehydrated, cleared, and coverslipped. The images were captured digitally using a Leica DMIL microscope (Leica Microsystems, Bannockburn, IL) and optical density

readings 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 averaged.

2.2.3.4. SERT Immunohistochemistry: To confirm that depletion in 5-HT levels is due to METH-induced damage to serotonin nerve terminals, the 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 in the first part of the study. Moreover, higher mortality was observed in the 10 mg/kg METH group, therefore in the second part of the study CM156 was evaluated against the 5 mg/kg dose of METH. The mice ($n = 4/\text{group}$) were randomly assigned to one of the following treatment groups: (1) Saline + Saline; (2) Saline + METH (5, 10 mg/kg, i.p.); (3) AC927 (10 mg/kg, i.p.)/CM156 (20 mg/kg, i.p.)/SN79 (3 mg/kg, i.p.) + METH (5, 10 mg/kg, i.p.); (4) AC927 (10 mg/kg, i.p.)/CM156 (20 mg/kg, i.p.)/SN79 (3 mg/kg, i.p.) + Saline. The protocol was the same as for DAT immunohistochemistry with the exception of incubating with rabbit anti-mouse SERT antibody (Millipore, Temecula, CA; AB9726, dilution 1:5,000) for 36 h at 4 °C. The labeled sections were washed thrice in TBS and processed with Histostain-Plus kit (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. The stained sections were mounted on the slides and analysed in the same way as for DAT immunohistochemistry.

2.2.4. Statistical analysis: The data from the dopamine and 5-HT assays, and DAT and SERT immunohistochemical studies were evaluated using one-way analysis of variance (ANOVA). Post-hoc analyses were performed with Dunnett's test for comparisons to controls or Tukey's tests for pairwise comparisons. For all statistical analyses, $p < 0.05$ was considered statistically significant.

2.3 Results

2.3.1. Dopamine assays: One-way ANOVA showed that METH produced a dose dependent reduction of dopamine levels in the striatum (Figure 2.2A., $F(3,27) = 7.26$, $p < 0.0005$). 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 ($F(3,27) = 0.20$, *n.s.*).

Administration of AC927 in the absence of METH had no significant effects on dopamine levels in the striatum and cerebellum of the mouse brain (Figure 2.2B). ANOVA confirmed that there were no significant differences between the treatment groups in either the striatum ($F[3,16]=1.10$, *n.s.*) or the cerebellum ($F[3,16]=0.14$, *n.s.*). One-way ANOVA showed that AC927 pretreatment significantly protected against the striatal dopamine depletions produced by METH ($F(4,23) = 6.26$, $p < 0.005$). Post-hoc Tukey's multiple comparison tests showed that METH caused a significant decrease in dopamine levels ($q = 5.96$, $p < 0.001$) and AC927 (10 mg/kg) significantly attenuated the striatal ($q = 5.58$, $p < 0.01$) dopamine depletions caused by METH.

When the selective σ receptor antagonist CM156 was administered in the absence of METH, one-way ANOVA showed that there were no significant changes in striatal and cerebellar dopamine levels ($F(3,16) = 0.36$, *n.s.*; $F(3,16) = 0.37$, *n.s.* respectively).

Nevertheless, in the presence of METH (5 mg/kg), pretreatment with CM156 significantly protected against the neurotoxic dopamine depletions produced by METH in the striatum (Figure 2.2C, $F(4,24) = 12.32$, $p < 0.0001$). Post-hoc Tukey's tests show that METH treatment caused a significant decrease in dopamine levels ($q = 7.50$, $p < 0.001$) and that pretreatment with the following doses of CM156 differed significantly protected against METH-induced striatal dopamine depletions: 10 mg/kg ($q = 7.60$, $p < 0.001$) and 20 mg/kg ($q = 7.36$, $p < 0.001$).

When SN79 was administered in the absence of METH, one-way ANOVA showed that there were no significant changes in striatal and cerebellar dopamine levels (Figure 2.2D, $F(3,19) = 1.53$, ns ; $F(3,19) = 1.30$, ns). One-way ANOVA showed that SN79 pretreatment significantly attenuated METH-induced dopamine depletion ($F(4,24) = 31.61$, $p < 0.0001$). Post-hoc Tukey's test show that METH treatment caused a significant decrease in dopamine levels when compared with saline treatment ($q = 10.68$, $p < 0.001$) and that SN79 pretreatment is able to significantly prevent METH induced decrease in dopamine levels (SN79 dose: 1 mg/kg, $q = 11.70$, $p < 0.001$; 3 mg/kg, $q = 12.24$, $p < 0.001$; 10 mg/kg $q = 14.28$, $p < 0.001$).

Figure 2.2 Dopamine assays: A. Dose response of METH. B, C and D. Effect of AC927, CM156 and SN79 pretreatment on METH-induced striatal dopamine depletion. (A) Dose response effects of METH on dopamine (DA) levels in the striatum. Male, Swiss Webster mice ($n = 5-9$ 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, C, D) 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 AC927 (5, 10, 20 mg/kg, i.p.) or CM156 (5, 10, 20 mg/kg, i.p.) or SN79 (1, 3, 10 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, ## $p < 0.01$, ### $p < 0.001$ vs. METH.

2.3.2. 5-HT assays: Figure 2.3A 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 ($F(4,35) = 2.93, p < 0.05$). 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 ($q = 3.38, p < 0.01$). However, in the cerebellum, METH did not produce significant 5-HT depletions ($F(4,35) = 2.33, n.s.$).

Figure 2.3B shows the effects of the σ receptor antagonist AC927 (0-20 mg/kg, i.p.) on METH-induced 5-HT depletions in the striatum of mice. To determine the effects of AC927 against METH-induced 5-HT depletions, a one-way ANOVA followed by a post-hoc Tukey's multiple comparison was run. Pretreatment with AC927 (5, 10 or 20 mg/kg, i.p.) significantly attenuated the ability of METH to produce its neurotoxic effects in the striatum ($F(7,32) = 4.49, p < 0.005$). Post-hoc Tukey's multiple comparisons tests confirmed that pretreatment with the 10 mg/kg dose of AC927 significantly attenuated the striatal ($q = 6.17, p < 0.01$) 5-HT depletions caused by METH.

As the one-way ANOVA showed, administration of CM156 alone produced no significant changes in striatal and cerebellar 5-HT levels ($F(3,21) = 2.37, n.s.$; and $F(3,21) = 0.49, n.s.$, respectively). However, similar to that seen with dopamine, a one-way ANOVA showed that pretreatment with CM156 protected against METH-induced 5-HT depletions in the striatum (Figure 2.3C; $F(4,34) = 11.14, p < 0.0001$). Post-hoc Tukey's tests confirmed that METH treatment caused a significant depletion in striatal 5-HT levels ($q = 8.93, p < 0.01$) and that pretreatment with CM156 (20 mg/kg) significantly attenuated METH (10 mg/kg, i.p.)-induced striatal 5-HT depletions ($q = 4.92, p < 0.05$) when compared to pretreatment with saline.

One-way ANOVA showed that administration of SN79 alone produced no significant changes in 5-HT levels in cerebellum and striatum (2.3D, $F(3,19) = 0.24, ns$ and $F(3,38) = 0.72, ns$ respectively). When SN79 is given as a pretreatment prior to METH treatment, two-

way ANOVA showed a significant effect of METH treatment ($F(1,71) = 9.60, p < 0.005$), SN79 pretreatment ($F(3,71) = 5.26, p < 0.005$) and SN79 pretreatment $\times$ METH treatment interaction ($F(3,71) = 3.29, p < 0.05$). Post-hoc Tukey's test showed that METH treatment caused a significant decrease in striatal 5-HT levels ($q = 5.86, p < 0.01$) and that SN79 pretreatment is able to significantly prevent the METH-induced decrease in 5-HT levels (SN79 dose: 1 mg/kg, $q = 4.95, p < 0.05$; 3 mg/kg, $q = 5.29, p < 0.01$ and 10 mg/kg, $q = 6.88, p < 0.001$).

Figure 2.3 5-HT assays: A. Dose response of METH. B,C and D. Effect of AC927, CM156 and SN79 pretreatment on METH-induced striatal 5-HT depletion. (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.) or saline (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, C, D) Effect of AC927, CM156 and SN79 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 AC927 (5, 10, 20 mg/kg, i.p.) or CM156 (5, 10, 20 mg/kg, i.p.) or SN79 (1, 3, 10 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. ** $p < 0.01$ vs. saline, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. METH.

2.2.5. DAT immunohistochemistry: Figure 2.4A illustrates the effects of METH and AC927 on DAT immunoreactivity in the mouse striatum. ANOVA revealed a significant difference between the treatment groups ($F(3,12) = 43.76, p < 0.0001$). Post-hoc Tukey's multiple comparison tests confirmed that METH caused a significant reduction in DAT immunoreactivity ($q = 14.26, p < 0.001$). Pretreatment with AC927 significantly attenuated the METH-induced neurotoxicity ($q = 13.63, p < 0.001$), whereas treatment with AC927 alone had no significant effects on DAT expression ($q = 3.56, \text{n.s.}$).

Immunohistochemical analysis of striatal DAT expression confirmed that the METH-induced reductions in striatal dopamine levels were linked to degeneration of dopaminergic nerve terminals. Figure 2.4B 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 ($q = 7.90, p < 0.001$), whereas treatment with CM156 alone had no significant effects on DAT expression ($q = 0.75, \text{n.s.}$).

Figure 2.4C illustrates the effects of METH and SN79 on DAT immunoreactivity in the mouse striatum. ANOVA revealed a significant difference between the treatment groups ($F(3,71) = 162.90, p < 0.0001$). Post-hoc Tukey's multiple comparison tests confirmed that METH caused a significant reduction in DAT immunoreactivity ($q = 28.66, p < 0.001$). Pretreatment with SN79 significantly attenuated the METH-induced neurotoxicity ($q = 24.66, p < 0.001$), whereas treatment with SN79 alone had no significant effects on DAT expression ($q = 1.81, \text{n.s.}$).

Figure 2.4 DAT Immunohistochemistry: Effect of (A) AC927, (B) CM156 and (C) SN79 pretreatment on METH-induced decrease in striatal DAT levels. Male, Swiss Webster mice ($n = 4$ per group) were pretreated with saline (SAL) or AC927 (AC, 10 mg/kg, i.p.) or CM156 (CM, 10 mg/kg, i.p.) or SN79 (SN, 3 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.

2.2.6. 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 (Figure 2.5A; $F(2,20) = 56.67$, $p < 0.0001$). Post-hoc Dunnett's tests showed that both 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).

The effects of METH and AC927 on SERT immunoreactivity in the mouse striatum were explored in order to confirm that 5-HT depletions were a result of nerve terminal damage (Figure 2.5B). Significant difference between the treatment groups ($F(5,47) = 50.85$, $p < 0.0001$) were revealed using a one-way ANOVA. Post-hoc Tukey's multiple comparisons tests confirmed that METH (5 and 10 mg/kg) caused a significant reduction in SERT immunoreactivity relative to treatment with saline alone ($q = 15.90$, $p < 0.001$ and $q = 14.43$, $p < 0.001$ respectively). Pretreatment with AC927 (10 mg/kg) significantly attenuated METH (5 and 10 mg/kg)-induced neurotoxicity ($q = 12.98$, $p < 0.001$ and $q = 11.85$, $p < 0.001$ respectively), whereas treatment with AC927 alone had no significant effects on SERT expression ($q = 0.87$, n.s.).

Immunohistochemical analysis of striatal SERT expression confirmed that the METH-induced reductions in striatal 5-HT levels were linked to degeneration of serotonergic nerve terminals. Figure 2.5C depicts the effects of METH and CM156 on SERT immunoreactivity in the mouse striatum, with a significant difference between the treatment groups ($F(5,44) = 45.42$, $p < 0.0001$). Post-hoc Tukey's multiple comparisons tests confirmed that METH (5 and 10 mg/kg) caused a significant reduction in SERT immunoreactivity relative to treatment with saline alone ($q = 15.07$, $p < 0.001$ and $q = 13.67$, $p < 0.001$ respectively). Pretreatment with CM156 (20 mg/kg) significantly attenuated METH (5 and 10 mg/kg)-induced neurotoxicity ($q =$

13.17, $p < 0.001$ and $q = 8.10$, $p < 0.001$), whereas treatment with CM156 alone had no significant effects on SERT expression ($q = 0.4$, n.s.).

Figure 2.5D illustrates the effects of METH and SN79 on SERT immunoreactivity in the mouse striatum. ANOVA revealed a significant difference between the treatment groups ($F(3,54) = 53.73$, $p < 0.0001$). Post-hoc Tukey's multiple comparison tests confirmed that METH caused a significant reduction in SERT immunoreactivity ($q = 15.07$, $p < 0.001$). Pretreatment with SN79 significantly attenuated the METH-induced neurotoxicity ($q = 14.81$, $p < 0.001$), whereas treatment with SN79 alone had no significant effects on SERT expression ($q = 2.91$, n.s.).

Figure 2.5 SERT Immunohistochemistry: Effect of (A) AC927, (B) CM156 and (C) SN79 pretreatment on METH-induced decrease in striatal SERT levels. 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, C, D) Male, Swiss Webster mice ($n = 4$ per group) were pretreated with saline (SAL) or AC927 (AC, 10 mg/kg, i.p.) or CM156 (CM, 20 mg/kg, i.p.) or SN79 (SN, 3 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 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.

2.3 Discussion

The current study demonstrates that pretreatment with AC927, CM156 and SN79 can mitigate METH-induced decreases in striatal dopamine and 5-HT levels and DAT and SERT expression levels. These protective effects likely involve modulation through both σ_1 and σ_2 receptors. The implications of the results are discussed below.

Consistent with the literature, high subchronic dose administration of METH leads to dopaminergic and serotonergic neurotoxicity (Krasnova and Cadet, 2009). The striatal and cerebellar brain regions were evaluated for the neurotoxic effects of METH. Since METH is retained for a longer time in the striatum as compared to the control cerebellum brain region, this explains why no significant neurotoxicity was seen in the cerebellum (Fowler et al., 2007). 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. For the striatal 5-HT neurotoxicity studies, SERT immunohistochemistry and 5-HT depletion experiments were performed in different buildings with slightly different ambient temperatures (5-HT: 21-22 °C, SERT: 23-23.5 °C). Since 5-HT neurotoxicity is sensitive to changes in ambient room temperature (Malberg and Seiden, 1997; Malberg and Seiden, 1998), this may explain the difference in dose response between the two experiments.

In this study, AC927, CM156 and SN79 pretreatment protected against two markers of METH-induced striatal dopamine and serotonin nerve terminal degeneration (dopamine and 5-HT depletion and reductions in DAT and SERT expression levels), thus providing evidence of neuroprotection.

Although the mechanisms of METH-induced neurotoxicity are not very well understood, there are some evidences in the literature suggesting the involvement of the following

cascades of events: excessive release of monoamines like dopamine and 5-HT, autooxidation of monoamines leading to reactive oxygen species generation, ER stress and activation of mitochondrial death cascades as well as glutamatergic/NMDA-mediated excitotoxic mechanisms (Kaushal and Matsumoto, 2011). AC927, CM156 and SN79 can provide neuroprotection by acting through σ receptors and competitively or non-competitively intervening at the previously described METH's downstream mechanisms (Kaushal and Matsumoto, 2011). First, σ receptors are located on dopamine and 5-HT neurons, and can modulate the release of these neurotransmitters (Campbell et al., 1989; Bastianetto et al., 1995; Derbez et al., 2002). Second, σ receptors have been implicated in protective effects against ROS-related dysfunctions such as brain ischemia (Schetz et al., 2007). Third, presence of σ receptors at the ER and mitochondria and its function as a chaperone between these organelles can help regulate the ER stress and mitochondrial death cascades (Tsai et al., 2009; Su et al., 2010). Fourth, σ receptors can modulate NMDA receptor function, and have been shown to protect against excitotoxic mechanisms in ischemic stroke models, midbrain and cortical dopaminergic neurons, and retinal ganglion cells (DeCoster et al., 1995; Shimazu et al., 2000; Shen et al., 2008). Taking all these observations from the literature and the results obtained from this study into consideration, the neuroprotective effects of σ receptor antagonists may be due to their ability to modulate various steps in METH's neurotoxic cascade.

σ Receptors, due to their properties of intracellular translocation, protein-protein interaction and chaperone activity can help normalize a disordered system (Tsai et al., 2009). Therefore, in addition to mitigating METH-induced neurotoxicity, targeting σ receptors has also been reported to effectively attenuate neurotoxicity in several other models including β -amyloid, carbon monoxide, and excitotoxicity (Marrazzo et al., 2005; Meunier et al., 2006; Shen et al., 2008). Additionally, σ receptors may play a role in animal models of neuropsychiatric

complications like depression, psychosis, cognitive and motor deficits (Guitart et al., 2004; Mishina et al., 2005), which are also observed in METH users and have been associated with the neurotoxic effects of METH (Cruickshank and Dyer, 2009).

In conclusion, pretreatment with AC927, CM156 and SN79 significantly protects against METH's dopamine and 5-HT neurotoxicity. 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 will be conducted to provide a more complete understanding of the underlying mechanisms through which σ ligands convey neuroprotective actions.

2.4 Contributions: Nidhi Kaushal conducted the Dopamine ELISA studies for SN79; 5-HT ELISA studies for CM156 and SN79; DAT immunohistochemistry studies for SN79 and SERT immunohistochemistry studies for AC927, CM156 and SN79.

Dr. Jamaluddin Shaikh performed the DAT immunohistochemistry studies for AC927 and CM156; Dopamine ELISA studies for AC927 and CM156; and 5-HT ELISA studies for AC927.

CHAPTER 3

EVALUATION OF AC927, CM156 AND SN79 AGAINST METH- INDUCED HYPERTHERMIA

3.1. Introduction

Methamphetamine is a highly abused drug which produces neurotoxic effects and psychiatric complications (Davidson et al., 2001; Cadet et al., 2003). High or repeated METH administration also causes an elevation in body temperature (Fukumura et al., 1998; Numachi et al., 2007). Previous clinical reports and animal studies suggest the lethality produced by METH is closely related with hyperthermia, which may be the primary cause of death (Bowyer et al., 1994; Davidson et al., 2001). Earlier studies have shown that hyperthermia potentiates METH-induced dopamine and 5-HT depletions and exacerbates oxidative stress in the brain (Bowyer et al., 1994; Hirata et al., 1995; Fukumura et al., 1998), whereas hypothermia protects against these effects (Bowyer et al., 1994). Therefore, the hyperthermic effect of METH is an essential factor for the neurotoxic effects of METH. It is critical to verify if the neuroprotective effects of these σ receptor antagonists is dependent on their ability to decrease the hyperthermic effects of METH.

Recent evidence has shown that METH produces some of its physiological and behavioral effects through σ receptors (Nguyen et al., 2005). σ Receptors can be found on monoaminergic neurons (Booth and Baldessarini, 1991; Bastianetto et al., 1995) and modulate the release of neurotransmitters such as dopamine and serotonin, which have been linked to changes in body temperature (Lee et al., 1985; Gonzalez et al., 1986; Lipton and Clark, 1986; Schwartz et al., 1995; Salmi and Ahlenius, 1998). In addition, σ receptor ligands can modulate thermoregulation (Rawls et al., 2002), likely mediated through an association between thermosensitive neurons and σ receptors in the hypothalamus (Bouchard and Quirion, 1997).

The present study investigated whether METH-induced hyperthermia could be prevented using the σ receptor antagonists AC927, CM156 and SN79. Two well-known markers of neurotoxicity were also evaluated; striatal dopamine and serotonin levels, results of which have been shown in Chapter 2. Striatal tissue was used in the present investigation because it contains the terminals of monoaminergic neurons and is the region primarily affected by METH-induced neurotoxicity (Brunswick et al., 1992; Kovachich et al., 1989; Ricaurte et al., 1980; Seiden et al., 1988). Using correlation analysis, it was also determined if the body temperatures of mice in each of the treatment groups can be correlated with striatal dopamine and serotonin levels.

3.2 Methods

3.2.1. Animals: Male, Swiss Webster mice (18-28 g; Harlan, Indianapolis, IN) were used for the *in vivo* studies. They were housed in groups of five with a 12:12h light/dark cycle with food and water available *ad libitum*. Naïve mice were randomly assigned to different treatment groups. All procedures involving animals were performed as approved by the Institutional Animal Care and Use Committee at the West Virginia University.

3.2.2. Drugs: CM156 and SN79 were obtained from Dr. Christopher McCurdy's laboratory (University of Mississippi, University, MS). AC927 was provided by Dr. Andrew Coop (University of Maryland, Baltimore, MD). METH hydrochloride was obtained from Research Biochemicals International (Natick, MA).

3.2.3 Experimental schedule: As shown in Figure 3.1 male, Swiss Webster mice ($n = 5$ -10/group) were randomly divided into four experimental groups: 1) Saline/Saline, 2) Saline/METH (1.25, 2.5, 5, 10 mg/kg) 3) Antagonist (AC927: 5, 10, 20 mg/kg; CM156: 5, 10,

20 mg/kg or SN79: 1, 3, 10 mg/kg)/Saline, 4) Antagonist (AC927: 5, 10, 20 mg/kg, i.p.; CM156: 5, 10, 20 mg/kg, i.p. or SN79: 1, 3, 10 mg/kg, i.p.)/METH (5, 10 mg/kg, i.p.). The first drug was administered 15 min prior to the second drug. Each group of mice received their treatment a total of four times at two hour intervals. Core body temperature was measured 1 h following each of the four treatment injections 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 seconds. All procedures involving animals were performed as approved by the Institutional Animal Care and Use Committee at the University of Mississippi and West Virginia University.

3.2.4 Statistical analysis: The body temperature readings were evaluated using one-way analysis of variance (ANOVA). Post-hoc analyses were performed with Dunnett's test for comparisons to controls and Tukey's tests for pairwise comparisons. The body temperature of the mice from each group was correlated with the striatal dopamine and 5-HT levels obtained a week later. Results from studies in Chapter 2 measuring striatal dopamine and 5-HT levels have been used for the correlation studies. For all statistical analyses, $p < 0.05$ was considered statistically significant.

Figure 3.1 Neurotoxic schedule of METH

3.3 Results

3.3.1 Body temperature: The hyperthermic effects of METH are represented in Figure 3.2. One-way ANOVA demonstrated that METH produces a dose dependent increase in core body temperature after the first injection ($F(4,81) = 7.29, p < 0.0001$), second injection ($F(4,81) = 12.70, p < 0.0001$), third injection ($F(4,81) = 29.21, p < 0.0001$), and fourth injection ($F(4,81) = 52.72, p < 0.0001$). 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 ($q = 2.53, p < 0.05$), 5 mg/kg ($q = 3.62, p < 0.01$), and 10 mg/kg ($q = 4.99, p < 0.01$); after the second injection with METH 1.25 mg/kg ($q = 4.46, p < 0.01$), 2.5 mg/kg ($q = 4.05, p < 0.01$), 5 mg/kg ($q = 6.01, p < 0.01$), and 10 mg/kg ($q = 5.52, p < 0.01$); after the third injection with METH 1.25 mg/kg ($q = 4.53, p < 0.01$), 2.5 mg/kg ($q = 4.91, p < 0.01$), 5 mg/kg ($q = 9.01, p < 0.01$), and 10 mg/kg ($q = 8.95, p < 0.01$); and after the fourth injection with METH 1.25 mg/kg ($q = 3.91, p < 0.01$), 2.5 mg/kg ($q = 5.30, p < 0.01$), 5 mg/kg ($q = 9.46, p < 0.01$) and 10 mg/kg ($q = 13.57, p < 0.01$).

Figure 3.2 Dose response of METH-induced hyperthermia Dose response effects of METH on core body temperature. Male, Swiss Webster mice ($n = 5-10$ per group) were injected with 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. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. saline.

AC927, on the other hand, did not significantly change core body temperature after the first injection (Figure 3.3A, $F(3,19) = 1.29$, *ns*), second injection ($F(3,19) = 1.02$, *ns*), third injection ($F(3,19) = 1.25$, *ns*) and fourth injection ($F(3,19) = 1.44$, *ns*). However, pretreatment with AC927 significantly attenuated the ability of METH to produce its hyperthermic effects (Figure 3.3B; after 2nd injection, $F(4,20) = 5.86$, $p < 0.005$ and 3rd injection, $F(4,20) = 9.12$, $p < 0.0005$).

Figure 3.3 Effect of AC927 on basal body temperature, and METH-induced hyperthermia in mice. Effect of (A) AC927 (5, 10, 20 mg/kg, i.p.) alone on core body temperature, (B) Saline or AC927 (5, 10, 20 mg/kg, i.p.) pretreatment on METH (5 mg/kg, i.p.)-induced hyperthermia. Male, Swiss Webster mice ($n = 5-10$ per group) were pretreated with saline or AC927 (5, 10, 20 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 was reported as mean $\pm$ SEM. ### $p < 0.001$ vs. METH.

The effects of CM156 alone on body temperature are depicted in Figure 3.4A. One-way ANOVA showed that CM156 alone had no significant effects on body temperature after the first ($F(3,36) = 0.05$, n.s.), second ($F(3,36) = 1.81$, n.s.), third ($F(3,36) = 1.37$, n.s.), or fourth ($F(3,36) = 1.68$, n.s.) injection. Pretreatment with CM156 attenuated METH (5 mg/kg, i.p.)-induced hyperthermia after all but the first injection (Figure 3.4B): first ($F(3,51) = 0.99$, n.s.); second ($F(3,51) = 7.56$, $p < 0.0005$); third ($F(3,51) = 10.44$, $p < 0.0001$); fourth ($F(3,51) = 15.79$, $p < 0.0001$). 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 ($q = 4.47$, $p < 0.01$); after third injection, CM156 (5, 10 and 20 mg/kg, i.p.) attenuated the hyperthermic effects of METH ($q = 2.51$, $p < 0.05$; $q = 3.71$, $p < 0.01$ and $q = 5.09$, $p < 0.01$ respectively); after the fourth injection, CM156 (10 and 20 mg/kg, i.p.) attenuated the hyperthermic effects of METH ($q = 3.82$, $p < 0.01$ and $q = 6.59$, $p < 0.01$ respectively).

Figure 3.4C shows the effects of pretreatment with the μ receptor antagonist 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: $F(3,21) = 1.20$, n.s.; second: $F(3,21) = 1.29$, n.s.; third: $F(3,21) = 2.57$, n.s.; fourth: $F(3,21) = 21.18$, $p < 0.0001$). Post-hoc Tukey's tests revealed that pretreatment with CM156 (10 and 20 mg/kg, i.p.) attenuated the hyperthermic effects of METH after the fourth injection ($q = 3.68$, $p < 0.01$; $q = 7.86$, $p < 0.01$ respectively).

Figure 3.4 Effect of CM156 on basal body temperature and METH (5 and 10 mg/kg)-induced hyperthermia in mice Effects of CM156 on (A) basal body temperature, (B) METH (5 mg/kg, i.p.) or (C) 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. * $p < 0.05$, ** $p < 0.05$ vs. METH.

One-way ANOVA showed that SN79 treatment significantly reduced body temperature (Figure 3.5 A) when compared with saline treated mice after the first injection ($F(3,59) = 21.77, p < 0.0001$), second injection ($F(3,59) = 20.12, p < 0.0001$), third injection ($F(3,59) = 11.34, p < 0.0001$) and fourth injection ($F(3,59) = 18.01, p < 0.0001$). Post-hoc Dunnetts test revealed that SN79 (10 mg/kg) significantly decreased the basal body temperature after first injection ($q = 6.54, p < 0.001$), second injection ($q = 5.98, p < 0.001$), third injection ($q = 4.36, p < 0.001$) and fourth injection ($q = 5.58, p < 0.001$).

One-way ANOVA showed that SN79 pretreatment is able to significantly attenuate the hyperthermic effects of METH (Figure 3.5B and C) after the first injection ($F(11,123) = 24.04, p < 0.0001$), second injection ($F(11,123) = 15.40, p < 0.0001$), third injection ($F(11,123) = 18.25$) and fourth injection ($F(11,123) = 18.21$). Post-hoc Tukey's test revealed that the following doses of SN79 attenuated the hyperthermic effects of METH (5 mg/kg) after the first injection (3 mg/kg: $q = 4.84, p < 0.05$; 10 mg/kg: $q = 9.26, p < 0.001$), second injection (3 mg/kg: $q = 5.51, p < 0.01$; 10 mg/kg: $q = 6.86, p < 0.001$), third injection (1 mg/kg: $q = 4.80, p < 0.05$; 3 mg/kg: $q = 8.41, p < 0.001$; 10 mg/kg: $q = 7.76, p < 0.001$) and fourth injection (1 mg/kg: $q = 5.85, p < 0.01$; 3 mg/kg: $q = 7.85, p < 0.001$; 10 mg/kg: $q = 9.63, p < 0.001$). Post-hoc Tukey's test also revealed that the following doses of SN79 attenuated the hyperthermic effects of METH (10 mg/kg) after the first injection (3 mg/kg: $q = 5.71, p < 0.01$; 10 mg/kg: $q = 13.34, p < 0.001$), second injection (1 mg/kg: $q = 5.91, p < 0.01$; 3 mg/kg: $q = 9.59, p < 0.001$; 10 mg/kg: $q = 10.39, p < 0.001$), third injection (1 mg/kg: $q = 5.18, p < 0.05$; 3 mg/kg: $q = 7.39, p < 0.001$; 10 mg/kg: $q = 10.20, p < 0.001$) and fourth injection (3 mg/kg: $q = 6.94, p < 0.001$; 10 mg/kg: $q = 9.84, p < 0.001$).

Figure 3.5 Effect of SN79 treatment on basal body temperature and METH (5 and 10 mg/kg)-induced hyperthermia in mice. Effects of SN79 on (A) basal body temperature, (B) METH (5 mg/kg, i.p.) or (C) METH (10 mg/kg, i.p.) induced hyperthermia. Male, Swiss Webster mice ($n = 5-10$ per group) were pretreated with saline or SN79 (1, 3, 10 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. ### $p < 0.001$ vs. Saline; * $p < 0.05$, ** $p < 0.05$, *** $p < 0.001$ vs. METH.

3.3.2 **Correlation:** Table 3.1-3.3 summarizes the mean $\pm$ S.E.M. for each of the groups, with the statistical results shown at the bottom. The numbers in the parentheses of the treatment group column represent doses (in mg/kg, i.p.) of AC927, CM156, SN79 or METH. The correlation plot for each experiment is shown above the table.

Figure 3.6 and Table 3.1 Correlation of body temperature and striatal (A) dopamine (DA) and (B) 5-HT levels of mice pretreated with saline or AC927(5-20 mg/kg) prior to saline or METH (5 mg/kg)

Treatment Group	DA (% Saline Control)	BT1	BT2	BT3	BT4
Sal/Sal	100 ± 4.83	37.4 ± 0.16	37.3 ± 0.18	37.38 ± 0.21	37.24 ± 0.21
Sal/Meth(5)	48.21 ± 4.45	37.76 ± 0.23	38.8 ± 0.30	39.52 ± 0.40	39 ± 0.26
AC927(5)/Meth(5)	67.19 ± 11.28	37.64 ± 0.31	37.52 ± 0.23	37.74 ± 0.26	38.16 ± 0.47
AC927(10)/Meth(5)	96.64 ± 11.69	37.26 ± 0.36	37.56 ± 0.24	37.42 ± 0.26	37.36 ± 0.33
AC927(20)/Meth(5)	69.08 ± 9.24	37.85 ± 0.42	37.53 ± 0.33	37.28 ± 0.26	37.43 ± 0.42
AC927(5)/Sal	97.16 ± 0.82	37.52 ± 0.22	37.14 ± 0.17	37.68 ± 0.18	37.54 ± 0.26
AC927(10)/Sal	91.27 ± 4.46	37.64 ± 0.25	37.66 ± 0.11	37.72 ± 0.09	37.36 ± 0.19
AC927(20)/Sal	81.47 ± 7.78	37.02 ± 0.30	37.02 ± 0.44	37.34 ± 0.20	36.94 ± 0.16
<i>P</i>		<0.05	<0.01	<0.05	< 0.05
Pearson <i>r</i>		-0.3219	-0.4313	-0.3899	-0.3672
<i>r</i> ²		0.1036	0.186	0.152	0.1348

The values from individual mice were used to calculate the correlation between core body temperature at each of the four time points and striatal dopamine levels measured one week later. The table summarizes the mean ± S.E.M. for each of the groups, with the statistical results shown at the bottom. The numbers in the parentheses of the treatment group column represent doses (in mg/kg, i.p.) of AC927 or methamphetamine (METH).

Treatment Group	5-HT (% Saline Control)	BT1	BT2	BT3	BT4
Sal/Sal	100 ± 6.71	37.1 ± 0.07	37.18 ± 0.16	37.3 ± 0.11	37.38 ± 0.12
Sal/METH(5)	55.35 ± 4.99	38.24 ± 0.18	39.2 ± 0.28	39.42 ± 0.24	39.22 ± 0.23
AC927(5)/METH(5)	67.85 ± 4.16	37.52 ± 0.22	37.5 ± 0.16	37.5 ± 0.13	37.8 ± 0.27
AC927(10)/METH(5)	109.55 ± 12.86	37.32 ± 0.20	37.3 ± 0.16	37.4 ± 0.14	37.28 ± 0.23
AC927(20)/METH(5)	82.71 ± 16.50	37.5 ± 0.23	37.5 ± 0.14	37.82 ± 0.28	37.9 ± 0.12
AC927(5)/Sal	90.77 ± 6.37	37.34 ± 0.18	37.2 ± 0.19	37.62 ± 0.13	37.34 ± 0.26
AC927(10)/Sal	102.24 ± 6.23	37.34 ± 0.10	37.4 ± 0.08	37.46 ± 0.06	37.54 ± 0.13
AC927(20)/Sal	98.30 ± 3.67	37.16 ± 0.29	37.3 ± 0.22	37.48 ± 0.12	37.28 ± 0.14
<i>P</i>		< 0.01	< 0.005	< 0.0005	< 0.005
Pearson <i>r</i>		-0.4042	-0.481	-0.5693	-0.4707
<i>r</i> ²		0.1634	0.2314	0.3241	0.2216

The values from individual mice were used to calculate the correlation between core body temperature at each of the four time points and striatal serotonin levels measured one week later. The table summarizes the mean ± S.E.M. for each of the groups, with the statistical results shown at the bottom. The numbers in the parentheses of the treatment group column represent doses (in mg/kg, i.p.) of AC927 or methamphetamine (METH).

Figure 3.7 and Table 3.2 Correlation of body temperature and striatal (A) dopamine (DA) and (B) 5-HT levels of mice pretreated with saline or CM156 (5-20 mg/kg) prior to saline or METH (5 and 10 mg/kg)

Treatment Group	DA (% Saline Control)	BT1	BT2	BT3	BT4
Sal/Sal	100 ± 7.15	37.06 ± 0.10	36.62 ± 0.20	36.72 ± 0.09	36.7 ± 0.25
Sal/Meth(5)	51.06 ± 4.96	37.98 ± 0.30	38.22 ± 0.20	38.36 ± 0.25	38.5 ± 0.21
CM156(5)/Meth(5)	69.20 ± 8.17	37.58 ± 0.28	37.64 ± 0.23	37.28 ± 0.31	37.86 ± 0.49
CM156(10)/Meth(5)	101.71 ± 5.52	37.08 ± 0.48	37.12 ± 0.12	37.08 ± 0.25	37.04 ± 0.25
CM156(20)/Meth(5)	99.07 ± 6.33	36.76 ± 0.07	36.44 ± 0.07	36.76 ± 0.19	36.62 ± 0.15
CM156(5)/Sal	96.34 ± 7.94	37.04 ± 0.05	36.88 ± 0.21	36.8 ± 0.12	36.98 ± 0.07
CM156(10)/Sal	106.87 ± 7.80	37.1 ± 0.21	37.08 ± 0.12	36.76 ± 0.18	36.66 ± 0.22
CM156(20)/Sal	100.74 ± 6.07	37.06 ± 0.14	37.06 ± 0.13	37.08 ± 0.23	36.98 ± 0.12
<i>P</i>		<0.0005	<0.0001	<0.0001	<0.0001
Pearson <i>r</i>		-0.5581	-0.6725	-0.6793	-0.6923
<i>r</i> ²		0.3114	0.4523	0.4614	0.4793

The values from individual mice were used to calculate the correlation between core body temperature at each of the four time points and striatal dopamine levels measured one week later. The table summarizes the mean ± S.E.M. for each of the groups, with the statistical results shown at the bottom. The numbers in the parentheses of the treatment group column represent doses (in mg/kg, i.p.) of CM156 or methamphetamine (METH).

Treatment Group	5-HT (% Saline Control)	BT1	BT2	BT3	BT4
Sal/Sal	100 ± 8.16	36.84 ± 0.10	36.32 ± 0.07	36.5 ± 0.09	36.66 ± 0.12
Sal/Meth(10)	58.35 ± 5.91	38.48 ± 0.20	38.36 ± 0.17	38.99 ± 0.19	40.15 ± 0.33
CM156(5)/Meth(10)	76.44 ± 4.76	38 ± 0.23	37.96 ± 0.18	38.46 ± 0.23	39.14 ± 0.29
CM156(10)/Meth(10)	66.80 ± 5.42	38.6 ± 0.10	38.76 ± 0.56	38.36 ± 0.61	37.86 ± 0.79
CM156(20)/Meth(10)	86.45 ± 3.88	38.34 ± 0.25	37.72 ± 0.63	37.66 ± 0.49	36.54 ± 0.32
CM156(5)/Sal	102.04 ± 4.10	37.38 ± 0.14	37.28 ± 0.06	36.94 ± 0.07	37.4 ± 0.10
CM156(10)/Sal	96.09 ± 6.11	37.38 ± 0.19	37.42 ± 0.19	36.88 ± 0.17	37 ± 0.14
CM156(20)/Sal	98.74 ± 6.07	37.34 ± 0.13	37.48 ± 0.17	37.06 ± 0.14	36.58 ± 0.17
<i>P</i>		< 0.0001	< 0.0005	< 0.0001	< 0.0001
Pearson <i>r</i>		-0.5978	-0.5170	-0.7484	-0.6552
<i>r</i> ²		0.3573	0.2673	0.5601	0.4293

The values from individual mice were used to calculate the correlation between core body temperature at each of the four time points and striatal serotonin levels measured one week later. The table summarizes the mean ± S.E.M. for each of the groups, with the statistical results shown at the bottom. The numbers in the parentheses of the treatment group column represent doses (in mg/kg, i.p.) of CM156 or methamphetamine (METH).

Figure 3.8 and Table 3.3 Correlation of body temperature and striatal (A) dopamine (DA) and (B) 5-HT levels of mice pretreated with saline or SN79 (1-10 mg/kg) prior to saline or METH (5 and 10 mg/kg)

Treatment Group	DA (% Saline Control)	BT1	BT2	BT3	BT4
Sal/Sal	100 ± 6.9	37.86 ± 0.24	37.24 ± 0.29	37.06 ± 0.29	36.8 ± 0.30
SN79(1)/Saline	112.39 ± 4.55	37.76 ± 0.17	37.16 ± 0.14	36.92 ± 0.19	36.9 ± 0.26
SN79(3)/Saline	112.04 ± 4.75	37.06 ± 0.33	36.78 ± 0.31	36.78 ± 0.41	36.8 ± 0.26
SN79(10)/Saline	111.87 ± 4.7	35.2 ± 0.48	35.42 ± 0.41	35.54 ± 0.35	35.6 ± 0.33
Saline/Meth(5)	41.95 ± 6.59	37.96 ± 0.23	38.14 ± 0.19	38.86 ± 0.34	39.62 ± 0.32
SN79(1)/Meth(5)	105.58 ± 3.70	37.32 ± 0.26	37.14 ± 0.23	37.24 ± 0.39	37.52 ± 0.24
SN79(3)/Meth(5)	108.48 ± 4.53	36.38 ± 0.36	36.26 ± 0.36	36.02 ± 0.32	36.8 ± 0.35
SN79(10)/Meth(5)	119.58 ± 4.76	34.94 ± 0.32	35.8 ± 0.38	36.24 ± 0.32	36.16 ± 0.35
<i>P</i>		<0.01	<0.0005	<0.0001	<0.0001
Pearson <i>r</i>		-0.4164	-0.5291	-0.5819	-0.7555
<i>r</i> ²		0.1734	0.28	0.3386	0.5708

The values from individual mice were used to calculate the correlation between core body temperature at each of the four time points and striatal dopamine levels measured one week later. The table summarizes the mean ± S.E.M. for each of the groups, with the statistical results shown at the bottom. The numbers in the parentheses of the treatment group column represent doses (in mg/kg, i.p.) of SN79 or methamphetamine (METH).

Treatment Group	5-HT (% Saline Control)	BT1	BT2	BT3	BT4
Sal/Sal	100 ± 6.86	37.14 ± 0.19	36.82 ± 0.15	36.65 ± 0.20	37 ± 0.19
SN79(1)/Saline	115.59 ± 7.95	37.32 ± 0.20	37.12 ± 0.20	36.99 ± 0.16	37.24 ± 0.12
SN79(3)/Saline	107.58 ± 6.70	37.4 ± 0.15	37.11 ± 0.13	36.94 ± 0.14	37.14 ± 0.13
SN79(10)/Saline	99.51 ± 11.80	35.85 ± 0.31	35.46 ± 0.36	35.72 ± 0.36	35.6 ± 0.34
Saline/Meth(10)	55.76 ± 6.14	38.78 ± 0.22	38.45 ± 0.20	39.17 ± 0.18	38.83 ± 0.34
SN79(1)/Meth(10)	93.18 ± 5.63	38.56 ± 0.14	37.37 ± 0.22	37.9 ± 0.27	38.16 ± 0.31
SN79(3)/Meth(10)	95.81 ± 6.91	37.31 ± 0.27	36.43 ± 0.34	37.27 ± 0.34	37.53 ± 0.33
SN79(10)/Meth(10)	108.08 ± 8.83	35.88 ± 0.26	36.33 ± 0.24	36.71 ± 0.18	36.63 ± 0.17
P		<0.01	<0.005	<0.001	<0.05
Pearson r		-0.3025	-0.3208	-0.3735	-0.2875
r²		0.09152	0.1029	0.1395	0.08267

The values from individual mice were used to calculate the correlation between core body temperature at each of the four time points and striatal serotonin levels measured one week later. The table summarizes the mean ± S.E.M. for each of the groups, with the statistical results shown at the bottom. The numbers in the parentheses of the treatment group column represent doses (in mg/kg, i.p.) of SN79 or methamphetamine (METH).

3.4 Discussion

σ Receptor antagonists, AC927, CM156 and SN79 can attenuate the hyperthermic effects of METH. Mechanistically, dopaminergic and serotonergic systems are implicated in METH-induced body temperature alterations (Numachi et al., 2007). In addition, the production of ROS and release of cytokines like IL1 β have been implicated in the generation of METH's hyperthermia (Bowyer et al., 1994; Imam et al., 1999; Riedel and Maulik, 1999; Yamashita et al., 2000; Fukami et al., 2004).. Moreover, σ Receptor ligands, with their ability to modulate these neurotransmitter systems, cytokines, as well as ROS, can attenuate elevations in body temperature (Campbell et al., 1989; Bastianetto et al., 1995; Derbez et al., 2002; Schetz et al., 2007; Matsumoto et al., 2008). Additionally, σ receptors are located in the hypothalamic brain region and have previously been shown to modulate body temperature (Gundlach et al., 1986; Rawls et al., 2002). A combination of all these factors may explain how AC927, CM156 and SN79 can prevent METH-induced hyperthermia.

When body temperature of the mice was correlated with the striatal dopamine and 5-HT levels a week later, a significant correlation was observed. This indicates that the METH-induced neurotoxicity is related to the body temperature of the mice during treatment. In addition, the ability of AC927, CM156 and SN79 to attenuate the depletions in dopamine and 5-HT levels may be related to their ability to decrease the hyperthermic effects of METH. However, correlation does not always mean causation. As discussed earlier, sigma receptors may be able to attenuate the hyperthermic effects of METH through various mechanisms; they may also be able to prevent METH-induced neurotoxicity at a cellular level independent of temperature influence. However, the separation of these effects in an *in vivo* system may not be possible or very difficult to interpret.

Certain animal studies can be performed to determine if the σ antagonists can attenuate the neurotoxicity independent of decreases in body temperature. It involves maintaining the

mice at elevated ambient temperatures (under a 37 °C heating lamp) and then conducting the experiment. This would prevent the decrease in body temperature observed by pretreatment with σ receptor antagonists. However, the limitation of this study is that the positive control METH treated mice all die (Miller and O'Callaghan, 1994; Miller and O'Callaghan, 2003; Seminerio et al., 2011). Nevertheless, this study was conducted with AC927 which significantly decreased the METH-induced mortality. However, the neurotoxicity data was difficult to interpret because of the lack of a positive control (Seminerio et al., 2011).

In order to clearly differentiate between the ability of σ receptor antagonists to attenuate the neurotoxic effects of METH independent of temperature modulation, an experimental model is needed where temperature component can be controlled and separated the effects of METH alone. Therefore, in the next chapter, a neuronal cell culture model was used to begin identifying the mechanisms of METH's neurotoxicity and the pathways where σ receptors may mediate neuroprotection.

3.5 Contributions: Nidhi Kaushal conducted the body temperature experiments for CM156 (serotonin depletion experiment) and SN79 (dopamine and 5-HT depletion experiment).

Dr. Jamaluddin Shaikh performed the body temperature experiments for AC927 (dopamine and 5-HT depletion experiments) and CM156 (dopamine depletion experiment).

CHAPTER 4

EVALUATION OF AC927 AGAINST METH-INDUCED NEUROTOXICITY: *IN VITRO* STUDIES

4.1. Introduction

Methamphetamine (METH) is a highly abused psychostimulant drug that results in neurotoxicity at high or repeated drug administration (Krasnova and Cadet, 2009). This neurotoxicity is seen as dopamine nerve terminal degeneration and apoptosis in several brain regions (Krasnova and Cadet, 2009), the mechanism of which is not completely understood. However, some of the neurotoxic cascades involved are excessive release of dopamine, oxidative/nitrosative stress, endoplasmic reticulum (ER) stress, and activation of mitochondrial death cascades (Cadet and Brannock, 1998; Krasnova and Cadet, 2009).

Among its various targets, METH interacts with sigma (σ) receptors at physiologically relevant micromolar concentrations (Nguyen et al., 2005; Han and Gu, 2006). Two subtypes of σ receptors, σ_1 and σ_2 , are expressed in almost all types of mammalian cells (Guitart et al., 2004). σ_1 Receptors are highly conserved 223 amino acid membrane bound proteins (Kekuda et al., 1996). They function as chaperones at the mitochondrial-ER membrane and regulate Ca^{2+} signaling via inositol trisphosphate (IP_3) receptors (Hayashi and Su, 2007). Additionally, σ_1 receptors can undergo protein-protein interactions with several other cellular proteins such as binding immunoglobulin protein (BiP), ankyrin-B, IP_3 receptors, and dopamine D_1 receptors (Hayashi and Su, 2001; Hayashi and Su, 2007; Navarro et al., 2010; Su et al., 2010). σ_1 Receptors are also capable of translocation to different subcellular compartments upon stimulation through ligand binding and changes in intracellular conditions (Hayashi and Su, 2003a). In contrast, σ_2 receptors are less well characterized. However, they are elevated in immortalized cell lines and tumor cells (Vilner et al., 1995b), where they have been implicated in the control of cell cycle functions (Wheeler et al., 2000), alterations in cellular Ca^{2+} levels (Vilner and Bowen, 2000), and cell death signaling involving sphingolipid products (Crawford et al., 2002).

Previous *in vivo* studies have shown that pretreatment with AC927 (N-phenethylpiperidine oxalate), a σ receptor ligand, attenuates the neurotoxic effects of METH in male, Swiss Webster mice (Matsumoto et al., 2008; Seminerio et al., 2011). The ability of AC927 to mitigate neurotoxic cascades is consistent with the location of σ receptors in various subcellular organelles like ER, mitochondria, lysosomes, cell membrane, and nucleus (McCann et al., 1994; Zeng et al., 2007) as well as the functional roles of σ receptors in cells as described above. Nevertheless, the role of σ receptors in neurotoxicity resulting from drugs of abuse like METH remains unclear due to various limitations, including the lack of a suitable *in vitro* model system. Previous studies to understand the mechanisms of METH-induced toxicity employed either primary neuronal cultures or immortalized cell lines of neuronal origin (Cadet et al., 1997; Choi et al., 2002; Deng et al., 2002; Kanthasamy et al., 2006; Wu et al., 2007). In order to induce cell death/damage, millimolar concentrations of METH were required, which are rarely achieved *in vivo* (Segal and Kuczenski, 2006; Melega et al., 2008). Therefore, a model system suitable for identifying potential mechanisms leading to neurotoxicity is needed.

In order to elucidate the cellular mechanisms of METH-induced neurotoxicity at which AC927 may intervene via σ receptors, a neuronal/glial cell culture model (neuroblastoma $\times$ glioma hybridoma, NG108-15 cells) was used in the present study. NG108-15 cells when differentiated display neurite outgrowth, electrical excitability, and express proteins with neuronal and glial properties (Ma et al., 1998). These cells have been used extensively as a sensitive cell line to study the toxic effects of various drugs and chemicals (Canete and Diogene, 2008). Additionally, NG108-15 cells have been commonly used in the literature to study the functions of σ receptors (Hayashi and Su, 2003a). In the current study, the end points measured were reactive oxygen and nitrogen species (ROS/RNS) generation,

dopamine release, apoptosis, and necrosis. Finally, AC927 was tested against these cytotoxic endpoints and mediators to gain insight into the mechanisms of σ -mediated neuroprotection.

4.2 Materials and Methods

4.2.1 Cell line and culture

4.2.1.1. *Cell growth*: The neuroblastoma x glioma, hybridoma cell line NG108-15 (Hamprecht et al., 1985) were obtained from ATCC (Rockville, MD). Cells were maintained in 25 cm² (T25) or 75 cm² (T75) culture flasks (Corning-Costar, Lowell, MA) in hi-glucose (4.5 g/L) DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum), penicillin, streptomycin, and HAT (hypoxanthine-aminopterin-thymidine) at 37°C in a humidified incubator in 5% CO₂.

4.2.1.2. *Cell differentiation*: Culture medium was replaced with differentiation medium (DMEM with 0.5% FBS, penicillin, streptomycin, HAT and 1% DMSO) and the cells allowed to grow for a further 3-4 days.

4.2.2 **Reactive oxygen/nitrogen species**: Cells grown in complete or differentiation medium were used for assay when 60-70% confluent. The culture medium was replaced phenol red free medium containing 0.5% FBS and 5 μ M fluorogenic, oxidizable, dye CM-H₂DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate) and returned to the incubator at 37 °C for 45-60 min. Cells were treated with appropriate concentrations of METH or AC927. Exogenously added 100 μ M H₂O₂ and Na₂Cr₂O₇ served as a positive control in this assay. All compounds were added in replicates of 4 or 8 and the plates are returned to the incubator. In the assay, AC927 (0-300 μ M), N-acetylcysteine (NAC, 10 mM), catalase (10000 units per ml), L-NMMA (200 μ M) were added 15 min prior to the addition of METH (0.1-3000 μ M), H₂O₂ (100 μ M) or Na₂Cr₂O₇ (100 μ M). At specified time points, plates were read in a Fluostar Optima

fluorescence plate reader fitted with a 485/520 nm excitation/emission filter set (BMG Labtech GmbH, Offenberg, Germany).

4.2.3 Dopamine release: NG108-15 cells grown and differentiated in T75 flasks were harvested by replacing the medium with Ca^{++} , Mg^{++} free DPBS (Dulbecco's Phosphate-Buffered Saline), centrifuged, re-suspended in differentiation medium with reduced glucose (1.5 g/l) at 3×10^5 cells per ml and seeded in 24-well CellBind tissue culture plates (Corning-Costar, Lowell, MA) at 0.5 ml per well. After 4 hr, various concentrations of METH (0.3-300 μM) were added to sets of wells. KCl (50 mM) was used as a positive control for maximum dopamine release. In one set of wells, AC927 was added 15 min prior to the addition of METH or KCl. After 5 min of exposure to KCl or METH, supernatants were harvested (450 μl), centrifuged to remove cells, acidified with the addition of 1/10 volume of buffer (0.1 N HCl, 10 mM EDTA and 40 mM $\text{Na}_2\text{S}_2\text{O}_5$) and samples were stored at -20°C . Dopamine was quantitated in an enzyme-linked immunosorbent assay (ELISA) employing a kit from Rocky Mountain Diagnostics (Denver, CO) and manufacturer's protocols. Briefly, dopamine was extracted from the culture supernatants by binding to an affinity gel matrix, acetylated, eluted and then measured in a competitive inhibition ELISA, using a standard curve of known concentrations of dopamine.

4.2.4 Hoechst and propidium iodide staining: NG108-15 cells were grown and differentiated in 96-well culture plates and exposed to various concentrations of METH (0-10 mM), AC927 (0.3-30 μM), AC927 (0.3-30 μM)+METH (0-1 mM) for 24 hrs at 37°C . Hoechst stain (20 $\mu\text{g/ml}$ final concentration) and propidium iodide stain (20 $\mu\text{g/ml}$ final concentration) were added to each well and incubated at room temperature for 10 min. Three images are taken/well using an UV light microscope. Cells with brightly stained and fragmented nuclei were counted as apoptotic and cells that were red were counted as necrotic. The percentage of apoptotic and necrotic cells were counted with the help of Image J cell counter (NIH).

4.2.5 Statistical analysis: Statistical analysis of data was performed using ANOVA with appropriate post-hoc tests employing GraphPad Prism 4 (GraphPad Software, San Diego, CA).

4.3. Results

4.3.1. Reactive oxygen/nitrogen species: As shown in Figure 4.1, CM-H₂DCFDA detected exogenously added H₂O₂ within 10 min (168.91 ± 6.95 % of untreated control) and up to 60 min (176.69 ± 7.1 % of control). Low levels of ROS were also induced in NG108-15 cells by Na₂Cr₂O₇ starting at 10 min; ROS in this sample increased dramatically 4 hr after treatment (140.1 ± 8.6 % of control) and continued to increase up to 24 hr reaching 223.70 ± 21.92 % of that in untreated control cells. These controls established that the assay contained sufficient and stable reagents to continuously detect oxidizing radicals from within 10 min of initiation and up to 24 hr. All tested concentrations of METH except the lowest (0.1 μ M) induced the production of ROS capable of oxidizing CM-H₂DCFDA significantly above background levels within 10 min of exposure, with 3 μ M inducing the highest levels (201.97 ± 9.97 % of control). The difference between METH-induced ROS and basal ROS in NG108-15 cells diminished beyond 60 min, while ROS continued to accumulate in cells treated with Na₂Cr₂O₇ up to 24 hr. Statistical analysis of the data obtained from 10 to 60 min employing two-way repeated measures ANOVA showed a significant effect of METH concentration ($F(10,1364) = 54.41$, $p < 0.0001$), time ($F(3,1364) = 21.14$, $p < 0.0001$) and METH treatment $\times$ time interaction ($F(30,1364) = 1.84$, $p < 0.005$). Bonferroni's post-hoc tests further established that the following concentrations of METH and positive control are capable of reproducibly inducing the strongest and sustained ROS responses: 0.3 μ M (10 min: $t = 4.7$, $p < 0.001$; 20 min: $t = 3.46$, $p < 0.01$; 30 min: $t = 2.95$, $p < 0.05$ and 60 min: $t = 2.63$, $p < 0.05$), 1 μ M (10 min: $t =$

6.88, $p < 0.001$; 20 min: $t = 5.46$, $p < 0.001$; 30 min: $t = 4.58$, $p < 0.001$ and 60 min: $t = 3.82$, $p < 0.001$), 3 μM (10 min: $t = 11.46$, $p < 0.001$; 20 min: $t = 8.69$, $p < 0.001$; 30 min: $t = 7.60$, $p < 0.001$ and 60 min: $t = 5.33$, $p < 0.001$), 10 μM (10 min: $t = 3.50$, $p < 0.01$), 30 μM ($t = 3.81$, $p < 0.001$), 100 μM (10 min: $t = 6.30$, $p < 0.001$; 20 min: $t = 3.41$, $p < 0.01$ and 30 min: $t = 3.06$, $p < 0.01$), 300 μM (10 min: $t = 8.58$, $p < 0.001$; 20 min: $t = 5.23$, $p < 0.001$; 30 min: $t = 5.78$, $p < 0.001$ and 60 min: $t = 3.71$, $p < 0.001$), H_2O_2 (10 min: $t = 7.74$, $p < 0.001$; 20 min: $t = 7.46$, $p < 0.001$; 30 min: $t = 7.27$, $p < 0.001$ and 60 min: $t = 8.63$, $p < 0.001$) and Cr (10 min: $t = 3.42$, $p < 0.01$; 20 min: $t = 2.62$, $p < 0.05$; 30 min: $t = 3.20$, $p < 0.01$ and 60 min: $t = 3.63$, $p < 0.01$).

Figure 4.1 Dose response and time course of METH-induced ROS/RNS generation. The production of ROS capable of oxidizing CM-H₂DCFDA was conducted as described in the materials and methods in NG108-15 cells following exposure to 100 μM H₂O₂, 100 μM Na₂Cr₂O₇ (Cr), or 0 to 300 μM METH. Relative fluorescence units from five separate experiments obtained at each indicated time point are shown; data were normalized to the mean rfu for untreated cells to obtain percent control values (n = 8/experiment) ± SEM.

As shown in Figure 4.2A, ROS detectable by CM-H₂DCFDA induced in NG108-15 cells by METH was completely inhibited in the presence of the common antioxidant and radical scavenger NAC as seen within 20 min of addition of the drug, confirming that the assay was indeed detecting ROS. Two-way ANOVA showed significant effect of NAC treatment ($F(1,80) = 962.7, p < 0.0001$), METH and H₂O₂ treatment ($F(5,80) = 10.64, p < 0.0001$) and NAC treatment $\times$ METH and H₂O₂ treatment interaction ($F(5,80) = 8.83, p < 0.0001$). Bonferroni's post-hoc tests showed that NAC significantly decreased ROS for all tested concentrations of METH (0.1 μ M: $t = 11.66, p < 0.001$; 0.3 μ M: $t = 12.86, p < 0.001$; 1 μ M: $t = 12.91, p < 0.001$ and 3 μ M: $t = 13.29, p < 0.001$) and H₂O₂ ($t = 18.39, p < 0.001$). Significant inhibition of radical generation persisted up to 4 hr after addition of METH, at which time the experiment was terminated. Interestingly, NAC also reduced the ROS detectable in untreated control cells ($t = 7.97, p < 0.001$) at 20 min, indicating that differentiated NG108-15 cells produce significant amounts of basal ROS.

The presence of catalase in the assay medium inhibited ROS induced by METH within 20 min of addition of the drug (Figure 4.2B). Two-way ANOVA confirmed significant effect of catalase treatment ($F(1,80) = 42.85, p < 0.0001$), METH and H₂O₂ treatment ($F(5,80) = 3.79, p < 0.005$) and catalase treatment $\times$ METH and H₂O₂ treatment ($F(5,80) = 3.71, p < 0.005$). Bonferroni's post-hoc tests confirmed significant differences for 1 μ M and 3 μ M METH concentration ($t = 3.17, p < 0.05$ and $t = 3.20, p < 0.05$) and H₂O₂ treatment ($t = 6.03, p < 0.001$), suggesting that H₂O₂ was a small but significant product of reactive species induced by these concentrations of the drug.

Finally, the increase in ROS in NG108-15 cells induced by METH was also significantly inhibited in the presence of L-NMMA (Figure 4.2C). Two-way ANOVA showed a significant effect of L-NMMA treatment ($F(1,60) = 26.89, p < 0.0001$), METH and H₂O₂ treatment ($F(5,60) = 57.36, p < 0.0001$) and L-NMMA treatment $\times$ METH and H₂O₂ treatment

($F(5,60) = 5.85$, $p < 0.0005$). Bonferroni's post-hoc tests confirmed that L-NMMA inhibited the ROS/RNS generated by 1 and 3 μM METH ($t = 4.47$, $p < 0.001$ and $t = 5.09$, $p < 0.001$ respectively), suggesting that the generation of some of the reactive species detected by CM- H_2DCFDA in these test groups must proceed through intermediates requiring the synthesis of nitric oxide radicals.

Figure 4.2 Effect of antioxidants (A) NAC, (B) catalase, (C) L-NMMA on METH-induced ROS/RNS generation. Inhibition of ROS production in NG108-15 cells by N-acetylcysteine (NAC), catalase and L-N^G-monomethyl arginine (L-NMMA). Inhibition of ROS following exposure to 0 to 3 μM methamphetamine (METH) or 100 μM H₂O₂ was determined in the absence (solid bars) or presence (gray bars) of 10 mM NAC (A), 10,000 units catalase (B) or 20 mM L-NMMA (C) 20 min following exposure to METH. Data represent averages of relative fluorescence units from two separate experiments (n = 4/experiment) ± SEM. Significant differences are noted, **p* < 0.05, ***p* < 0.01 (control vs. METH treated) and #*p* < 0.05, ###*p* < 0.001 (METH alone vs. METH with ROS inhibitor).

As shown in Figure 4.3, CM-H₂DCFDA detected exogenously added H₂O₂ within 10 min (146.69 ± 4.97 % of control) and ROS generated by Na₂Cr₂O₇ in NG108-15 cells, which peaked at 24 hr (242.06 ± 11.56 % of control). In contrast, all tested concentrations of AC927 from 0 to 300 μ M failed to induce ROS significantly higher than that in untreated control cells. Statistical analyses of the data employing two-way repeated measures ANOVA showed a significant effect of AC927 and positive control treatment ($F(9,900) = 75.37$, $p < 0.0001$), time ($F(6,900) = 28.36$, $p < 0.0001$) and AC927 and positive control treatment $\times$ time interaction ($F(54,900) = 60.50$, $p < 0.0001$). Bonferroni's post-hoc tests revealed that none of the tested concentrations of AC927 were able to induce elevation of ROS above background levels, while ROS was detected in cells treated with H₂O₂ (10 min: $t = 11.26$, $p < 0.001$; 20 min: $t = 14.14$, $p < 0.001$; 30 min: $t = 14.50$, $p < 0.001$; 60 min: $t = 11.47$, $p < 0.001$; 240 min: $t = 3.03$, $p < 0.05$ and 480 min: $t = 3.00$, $p < 0.05$) and Na₂Cr₂O₇ (240 min: $t = 7.01$, $p < 0.001$; 480 min: $t = 16.52$, $p < 0.001$ and 1440 min: $t = 34.24$, $p < 0.001$). Interestingly, AC927 at 0.3 μ M led to a reduction in basal ROS in NG108-15 cells, the reduction becoming statistically significant at 240 min ($t = 3.78$, $p < 0.01$), 480 min ($t = 2.87$, $p < 0.05$) and up to 1440 min ($t = 4.38$, $p < 0.001$).

Figure 4.3 Dose response and time course of AC927-induced ROS/RNS generation. The production of ROS capable of oxidizing CM-H₂DCFDA was conducted as described in the materials and methods in NG108-15 cells following exposure to 100 μ M H₂O₂, 100 μ M Na₂Cr₂O₇ (Cr), or 0 to 300 μ M AC927. Relative fluorescence units from two separate experiments obtained at each indicated time point are shown; data were normalized to the mean rfu for untreated cells to obtain percent control values ($n = 8/\text{experiment}$) $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control treatment.

As shown in Figure 4.4, 0.3 μM AC927 attenuated the production of reactive radicals induced by METH, reducing them to levels observed in untreated control cells for all tested concentrations of METH except the highest (300 μM); data shown here were obtained 20 min following stimulation with METH. Two-way ANOVA showed a significant effect of AC927 pretreatment ($F(1,138) = 98.44, p < 0.0001$), METH or positive control treatment ($F(10,138) = 15.63, p < 0.0001$) and AC927 pretreatment $\times$ METH or positive control treatment interaction ($F(10,138) = 2.39, p < 0.05$). Bonferroni's post-hoc tests showed that the AC927 attenuated ROS/RNS generated by following METH concentrations: 0.1 μM ($t = 3.28, p < 0.05$), 0.3 μM ($t = 3.16, p < 0.05$), 1 μM ($t = 3.52, p < 0.01$), 3 μM ($t = 7.16, p < 0.001$), 10 μM ($t = 3.99, p < 0.01$), 30 μM ($t = 3.14, p < 0.05$), 100 μM ($t = 3.39, p < 0.05$) and 300 μM ($t = 3.84, p < 0.01$). As expected, AC927 did not inhibit the oxidation of the dye by exogenously added H_2O_2 ($t = 1.39, ns$). It also failed to significantly inhibit the small amount of ROS generated by $\text{Na}_2\text{Cr}_2\text{O}_7$ at this time point ($t = 0.99, ns$). However, at later time points, AC927 inhibited ROS induction by $\text{Na}_2\text{Cr}_2\text{O}_7$ as seen at 4 hr ($t = 4.05, p < 0.001$) and 24 hr ($t = 7.73, p < 0.001$).

Figure 4.4 Effect of AC927 pretreatment on METH-induced ROS/RNS generation.

Inhibition of methamphetamine (METH)-induced ROS generation in NG108-15 cells by AC927. The production of ROS capable of oxidizing CM-H₂DCFDA was assessed in differentiated NG108-15 cells in the presence of 0 to 300 μ M METH, 100 μ M H₂O₂ or 100 μ M Na₂Cr₂O₇ (Cr) in the absence (solid bars) or presence (gray bars) of 0.3 μ M AC927 as described in the methods. Data represent averages of relative fluorescence units from two different experiments ($n = 8/\text{experiment}$) obtained 20 min after addition of drug $\pm$ SEM. Significant differences are noted as ** $p < 0.01$ (control vs. METH treated) and # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ (METH alone vs. METH with AC927).

4.3.2. Dopamine release assay: As shown in Figure 4.5, in untreated cells, 0.0-41.85 pg of dopamine was detected. In contrast, when cells were treated with KCl, 268.30-389.62 pg of dopamine was detected in the culture supernatant 5 min following exposure.

Dopamine secretion was induced by all four concentrations of METH ranging from 0.3 to 300 μ M, with 3 μ M METH inducing maximum secretion with 204.75-413.12 pg per 1.5×10^5 cells, compared to that in untreated control cells. One-way ANOVA showed a significant effect of METH treatment ($F(5,23) = 8.87$, $p < 0.001$). Post-hoc Dunnett's tests show that the 0.3 and 3 μ M of METH caused a significant increase in dopamine release ($q = 2.77$, $p < 0.05$ and $q = 5.32$, $p < 0.01$). KCl (50 μ M) also caused a significant increase in dopamine release ($q = 5.78$, $p < 0.01$).

In cells exposed to 0.3 μ M AC927 for 5 min, dopamine levels were not significantly different from those in untreated controls ($F(5,18) = 0.20$, ns). Two-way ANOVA showed a significant effect of METH and KCl treatment ($F(5,30) = 19.39$, $p < 0.0001$), AC927 pretreatment ($F(1,30) = 32.21$, $p < 0.0001$) and AC927 pretreatment $\times$ METH and KCl treatment interaction ($F(5,30) = 4.86$, $p < 0.005$). Bonferroni's post-hoc tests confirm that AC927 pretreatment attenuates dopamine release caused by 0.3 and 3 μ M METH ($t = 3.19$, $p < 0.05$ and $t = 5.58$, $p < 0.001$ respectively). Pretreatment of cells with AC927 did not however, block the release of dopamine in cells treated with KCl ($t = 0.23$, $p < 0.05$).

Figure 4.5 Effect of AC927 pretreatment on METH-induced dopamine release. Release of dopamine in response to methamphetamine (METH) from NG108-15 cells. Supernatants harvested from NG108-15 cells cultured in the presence or absence of METH and/or AC927 (x-axis) and quantitated as described in the materials and methods are represented as pg dopamine per 1.5×10^5 cells in 500 μ l of medium. Data represent means from four (METH or KCl alone) or three (METH with AC927) independent experiments $\pm$ SEM. Significant differences are noted as follows, * $p < 0.05$, ** $p < 0.01$ (control vs. METH or KCl treated) and # $p < 0.05$, ### $p < 0.001$ (METH alone vs. METH with AC927).

4.3.3. Hoechst and propidium iodide staining: The apoptotic effects of various concentrations of METH (0-1 mM) on NG108-15 cells over a period of 24 hr are shown in Figure 4.6A. One-way ANOVA showed that METH caused a significant and concentration dependent increase in the percentage of apoptotic cells ($F(7,187) = 37.21, p < 0.0001$). Post-hoc analysis employing Dunnett's tests showed that the following concentrations of METH caused significant increases in apoptosis when compared with controls: 3 μM ($q = 2.87, p < 0.05$), 10 μM ($q = 3.74, p < 0.01$), 100 μM ($q = 5.99, p < 0.001$), 300 μM ($q = 7.73, p < 0.001$), and 1000 μM ($q = 13.68, p < 0.001$).

AC927 pretreatment significantly attenuated the apoptotic effects of METH. Two-way ANOVA showed a significant effect of METH treatment ($F(6,392) = 37.82, p < 0.0001$), AC927 pretreatment ($F(3,392) = 55.94, p < 0.0001$) and METH treatment $\times$ AC927 pretreatment interaction ($F(18,392) = 5.41, p < 0.0001$). Post-hoc Bonferroni's tests showed that AC927 (0.3, 3 and 30 μM) attenuated the apoptotic effects of METH. AC927 (0.3 μM) attenuated the apoptotic effects of the following concentrations of METH: 3 μM ($t = 3.34, p < 0.01$), 10 μM ($t = 3.70, p < 0.01$), 100 μM ($t = 5.05, p < 0.001$), 300 μM ($t = 5.85, p < 0.001$), and 1000 μM ($t = 9.80, p < 0.001$). AC927 (3 μM) prevented the apoptotic effects of the following concentrations of METH: 10 μM ($t = 3.52, p < 0.01$), 100 μM ($t = 3.45, p < 0.01$), 300 μM ($t = 5.67, p < 0.001$), and 1000 μM ($t = 8.84, p < 0.001$). AC927 (30 μM) attenuated the apoptotic effects of the following concentrations of METH: 100 μM ($t = 3.20, p < 0.05$), 300 μM ($t = 3.00, p < 0.05$), and 1000 μM ($t = 7.82, p < 0.001$).

The necrotic effects of various concentrations of METH (0-1 mM) on NG108-15 cells over a period of 24 hr are shown in Figure 4.6B. One-way ANOVA showed that METH caused a significant and concentration dependent increase in the percentage of necrotic cells ($F(7,187) = 7.35, p < 0.0001$). Post-hoc analysis employing Dunnett's tests showed that the

following concentrations of METH caused significant increases in necrosis when compared with no treatment controls: 300 μM ($q = 3.82$, $p < 0.01$) and 1000 μM ($q = 5.88$, $p < 0.01$).

AC927 pretreatment significantly attenuated the necrotic effects of METH. Two-way ANOVA showed a significant effect of METH treatment ($F[6,392] = 8.32$, $p < 0.0001$) and AC927 pretreatment ($F(3,392) = 17.14$, $p < 0.0001$). Post-hoc Bonferroni's tests showed that the following concentrations of AC927 attenuated the necrotic effects of METH (300 and 1000 μM): 0.3 μM , $t = 4.33$, $p < 0.001$ and $t = 4.26$, $p < 0.001$; 3 μM , $t = 3.30$, $p < 0.01$ and $t = 3.91$, $p < 0.001$; 30 μM , $t = 3.29$, $p < 0.01$ and $t = 2.87$, $p < 0.05$.

Figure 4.6 Effect of AC927 pretreatment on METH-induced (A) apoptosis and (B) necrosis. AC927 protects against methamphetamine (METH)-induced apoptosis (A) and necrosis (B). NG108-15 cells were pretreated with AC927 (0-30 μ M) prior to exposure to METH (0-1 mM) for 24 hr. After 24 hr, the wells were incubated with Hoechst 33342 and propidium iodide stains (20 μ g/ml each) to obtain percentages of apoptotic and necrotic cells. Data represent means from two separate experiments ($n = 3$ /experiment) $\pm$ SEM. Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (control vs. METH treated); # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ (METH alone vs. METH with AC927).

4.4 Discussion

This study showed that METH causes apoptosis in differentiated NG108-15 cells at physiologically relevant micromolar concentrations and can cause necrosis at higher concentrations. At earlier time points, ROS/RNS generation and release of dopamine were also observed. AC927, a σ receptor ligand, attenuated the apoptotic and necrotic cell death as well as the neurotoxicity mediators. The implications of these findings are further discussed below.

One of the key mediators of METH's neurotoxicity is ROS/RNS generation, which can occur through multiple mechanisms including excessive dopamine release, microglial activation, glutamate release, and mitochondrial dysfunction (Krasnova and Cadet, 2009). Excessive generation of ROS/RNS can injure neurons and surrounding cells via direct oxidative damage to cellular components such as lipids, proteins, and DNA, or by causing ER stress and activation of mitochondrial death cascades, ultimately leading to nerve terminal degeneration or cell death (Krasnova and Cadet, 2009). This study demonstrated that METH generates ROS/RNS in the NG108-15 model system. All tested concentrations of METH ranging from 0.1 to 300 μ M induced the production of ROS/RNS within 10 min of exposure. High and stable ROS/RNS levels were detected at 20 min and up to 60 min after exposure of NG108-15 cells to METH. After the first hour, the levels of ROS/RNS decreased, indicating that the cells may shift to the production of radicals not detected by CM-H₂DCFDA. Alternately, in METH-treated cells, endogenous peroxidases as well as antioxidants such as glutathione may be rapidly recruited after the first hour to scavenge H₂O₂ and $\cdot$ ONOO⁻ radicals.

At the early time point (20 min) that gave consistent and robust ROS/RNS signals following METH administration, the antioxidant NAC reduced the signal indicating that the fluorescent probe CM-H₂DCFDA was detecting ROS/RNS. In addition, catalase and L-NMMA

also decreased the METH-induced signal indicating that H_2O_2 and NO species were being generated. This result is consistent with the ability of METH to generate H_2O_2 and NO species from various sources including dopamine oxidation and glutamate-induced NOS activation (Krasnova and Cadet, 2009). It is also interesting to note that the dose response of METH-induced reactive species generation did not follow a classical sigmoidal pattern, displaying highest radical generation at the 3 μM concentration. This pattern of results is most likely due to multiple mechanisms and sources of METH's ROS/RNS generation (Krasnova and Cadet, 2009).

AC927 pretreatment attenuated the ROS/RNS generated by METH. This effect of AC927 does not seem to be a general antioxidant effect because AC927 did not prevent the H_2O_2 -mediated ROS signal or reduce the basal cellular ROS/RNS signal. However, at the 4 hr time point onwards, AC927 (0.3 μM) attenuated the basal cellular ROS/RNS levels as well as the ROS signal produced by $\text{Na}_2\text{Cr}_2\text{O}_7$. $\text{Na}_2\text{Cr}_2\text{O}_7$ generates $\cdot\text{O}_2^-$, H_2O_2 and $\cdot\text{OH}$ in the cell (Azad et al., 2008). In combination with NO, $\cdot\text{O}_2^-$ can produce $\cdot\text{ONOO}^-$ in the cell. The ability of AC927 to attenuate the ROS signal produced by $\text{Na}_2\text{Cr}_2\text{O}_7$ and not H_2O_2 is most likely due to increased levels of cellular antioxidant/antioxidant enzymes like superoxide dismutase which can reduce superoxide levels in the cell, or decreased levels of enzymes like NOS which can generate NO leading to the production of $\cdot\text{ONOO}^-$. Since this effect of AC927 is observed after 4 hr and not before, it suggests the induction of cellular antioxidants or a decrease in pro-oxidant/nitrosative stress enzymes, rather than the effect of generalized intrinsic antioxidant activity.

Corroborating with this interpretation, several studies in the literature demonstrate the ability of σ receptors to modulate cellular ROS/RNS generation and antioxidant systems. Previous studies in tumor cells have shown that σ_2 receptor activation causes ROS generation (Ostenfeld et al., 2005). σ_1 Receptors are also involved in oxidative stress-mediated toxicities

like ischemic stroke and have been shown to provide protective effects (Schetz et al., 2007). σ Receptors also play a regulatory role in the redox state of the cell, and can modulate cellular NOS enzyme activity (Vagnerova et al., 2006; Tsai et al., 2009; Yang et al., 2010). In addition to modulating the cellular redox system, σ receptors can also regulate other systems that are involved in ROS/RNS generation by METH. First, σ receptors can regulate glutamate release through NMDA receptors which in turn causes nNOS activation and nitrosative stress (DeCoster et al., 1995). Second, σ receptors can modulate microglial activation through Ca^{2+} dependent mechanisms, which can contribute to the release of inflammatory cytokines and ROS/RNS generation (Hall et al., 2009). Third, σ receptors can regulate the release of dopamine which in turn undergoes autooxidation and is a major cause of METH's ROS generation (Gonzalez-Alvear and Werling, 1994; Derbez et al., 2002).

Although additional studies are needed to identify the specific mechanism(s) through which AC927 reduces the generation of METH-induced ROS/RNS, the studies conducted herein suggest that the modulation of dopamine release may be an important contributor. AC927 attenuates METH-induced dopamine release from the NG108-15 cells, with peak release observed at the 3 μM concentration of METH which is consistent with the peak observed in the ROS assay. Moreover, AC927 pretreatment prevented the dopamine release caused by METH, but not the non-specific dopamine release caused by K^{+} -mediated hyperpolarization. This is consistent with the role of σ receptors in regulating DAT function and dopamine release in several *in vivo* and *in vitro* studies, including the involvement of Ca^{2+} -dependent and protein kinase C-mediated mechanisms (Booth and Baldessarini, 1991; Derbez et al., 2002).

METH also caused apoptosis and necrosis in NG108-15 cells. Oxidative and nitrosative stress in the cell can lead to the activation of pro death pathways like mitochondrial release of cytochrome c, and/or caspase 3 activation which ultimately leads to apoptotic or

necrotic cell death (Higuchi et al., 1998). AC927 significantly attenuated both the apoptotic and necrotic cell death which is consistent with the role of σ receptors in cell death mechanisms (Bowen, 2000; Marrazzo et al., 2011). Furthermore, the protective effects of AC927 observed in NG108-15 cells is complementary and consistent with the neuroprotection observed in earlier *in vivo* studies (Matsumoto et al., 2008; Seminerio et al., 2011). Moreover, in tumor cells, AC927 has been shown to prevent apoptosis caused by σ_2 receptor agonists, further underscoring an important role of σ receptors in cell death mechanisms (Marrazzo et al., 2011).

Apart from σ receptors, D_1 receptor antagonism can also protect against the apoptotic effects of METH (Jayanthi et al., 2005). Recent studies have shown that σ_1 and D_1 receptors heteromerize in cells, which has implications for the downstream effects of psychostimulants including the activation of adenylyl cyclase and MAPK pathways (Navarro et al., 2010). Therefore, AC927 may potentially modulate the σ_1 and D_1 interaction and mitigate the toxic effects of METH.

In summary, the data in this chapter demonstrates that METH can cause apoptosis and necrosis in an *in vitro* system representative of a differentiated neuronal cell, which mechanistically involves dopamine release and induction of reactive oxygen/nitrogen radicals. Additionally, a synthetic σ ligand, AC927, can attenuate the cytotoxic effects of METH and contributing neurotoxic mechanisms. Future studies will be directed towards the elucidation of the downstream neurotoxic pathways activated after ROS/RNS generation in differentiated NG108-15 cells. Also, the individual contributions of the two subtypes of σ receptors will have to be discerned, as better tools are developed.

4.5 Contributions: Nidhi Kaushal conducted the apoptosis and necrosis assays. Dr. Meenal Elliott performed the dopamine release assay and the ROS assay.

CHAPTER 5

EVALUATION OF SN79 AGAINST METH AND ELEVATED TEMPERATURE-INDUCED NEUROTOXICITY: *IN VITRO* STUDIES

5.1 Introduction

METH is known to cause cell death in various brain regions as well as *in vitro* cell culture system (Krasnova and Cadet, 2009). Previous *in vivo* studies have shown that pretreatment of σ receptor antagonists attenuated the neurotoxic effects of METH in male, Swiss Webster mice (Matsumoto et al., 2007; Kaushal et al., 2011b; Seminerio et al., 2011). Nevertheless, the role of σ -receptors in neurotoxicity resulting from drugs of abuse like METH remained unclear due to various limitations, including the lack of a suitable *in vitro* model system. In chapter 4, differentiated NG108-15 cells were used to identify some of the most well studied mechanisms of METH-induced neurotoxicity: ROS/RNS generation and dopamine release. Notably, the concentrations of METH used to cause apoptosis and the activation of these neurotoxicity mediators were within the physiologically relevant micromolar concentrations.

AC927, a sigma receptor antagonist attenuated METH-induced neurotoxicity (apoptosis and necrosis), and its mediators like ROS/RNS generation and dopamine release. In that study, AC927 was the only compound to be tested. In order to confirm that the protective effects observed were mediated through sigma receptors, another sigma receptor antagonist needed to be tested. SN79, with its long half-life and low propensity for toxicity is a better drug candidate than AC927. In this study, SN79 was therefore tested against METH-induced cell death (apoptosis and necrosis) and its mediators like ROS/RNS generation to confirm the pattern of results obtained with AC927. If a sigma receptor antagonist attenuates the neurotoxic effects of METH, the agonist is expected to enhance the toxicity. With this hypothesis, DTG (a sigma receptor agonist) was tested against METH-induced cell death and shifts in dose response curve of METH-induced cell death were evaluated. In addition to testing SN79 against METH-induced cell death and ROS/RNS generation; in this study additional mechanisms of METH-induced apoptotic cell death in differentiated NG108-15 cells were identified. The mechanisms looked into were caspase dependence or independence

(caspase-3 activation), as well as activation of intrinsic (caspase-9 activation) or extrinsic apoptotic pathway (caspase-8 activation).

In addition to identifying the sigma receptor sensitive mechanisms of METH-induced neurotoxicity, it is also important to evaluate the contribution of elevated temperature as observed during METH administration during *in vivo* studies. Although the exact mechanism of hyperthermic effects of METH is not clear, release of inflammatory cytokines like IL-1 β from microglial cells, ROS/RNS generation are thought to be contributing factors (Bowyer et al., 1994; Imam et al., 1999; Riedel and Maulik, 1999; Yamashita et al., 2000; Fukami et al., 2004). This increase in body temperature in turn can potentially exacerbate the neurotoxic effects of METH by altering blood brain barrier permeability, increasing DAT function and ROS/RNS production (Fleckenstein et al., 1997; Xie et al., 2000; Kiyatkin et al., 2007; Numachi et al., 2007; Sharma et al., 2007).

However, it is very difficult to identify the individual contribution of METH and hyperthermia, and their interaction at the cellular level in the brain in a complex *in vivo* system. This also translates to the difficulty in identifying the mechanism of action of neuroprotective pharmacological strategies *in vivo*. Therefore, in this study a differentiated NG108-15 model system was maintained at 37 and 40 °C and then treated with METH to provide information about the contribution of hyperthermia and METH separately and also their interaction towards cell death (apoptosis and necrosis). In addition, sigma receptor antagonist, SN79 was given as a pretreatment to understand which of these components can be modulated.

5.2 Materials and Methods

5.2.1. Cell line and culture

5.2.1.1. Cell growth: The neuroblastoma x glioma, hybridoma cell line NG108-15 (Hamprecht et al., 1985) were obtained from ATCC (Rockville, MD). Cells were maintained in

25 cm² (T25) or 75 cm² (T75) culture flasks (Corning-Costar, Lowell, MA) in hi-glucose (4.5 g/L) DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum), penicillin, streptomycin, and HAT (hypoxanthine-aminopterin-thymidine) at 37°C in a humidified incubator in 5% CO₂.

5.2.1.2. Cell differentiation: Culture medium was replaced with differentiation medium (DMEM with 0.5% FBS, penicillin, streptomycin, HAT and 1% DMSO) and the cells allowed to grow for a further 3-4 days.

5.2.2 **Hoechst and propidium iodide staining**: NG108-15 cells were grown and differentiated in 96-well culture plates and four types of experiments were conducted: 1) SN79 and METH: Cells were exposed to various concentrations of METH (0-1 mM), SN79 (1-100 nM), SN79 (1-100 nM)+METH (0-1 mM) for 24 hrs at 37 °C. 2) DTG and METH: Cells were exposed to various concentrations of METH (0-1 mM), DTG (0.1 nM-10 µM), DTG (0.1 nM-10 µM) + METH (0-1 mM) for 24 hrs at 37 °C. 3) METH treatment at 37 or 40 °C: Cells were exposed to various concentrations of METH (0-1 mM) at either 37 or 40 °C. 4) SN79 and METH: Cells were exposed to various concentrations of METH (0-1 mM), SN79 (1-100 nM), SN79 (1-100 nM)+METH (0-1 mM) for 24 hrs at 40 °C. Hoechst stain (20 µg/ml final concentration) and propidium iodide stain (20 µg/ml final concentration) were added to each well and incubated at room temperature for 10 min. Three images are taken/well using an UV light microscope. Cells with brightly stained and fragmented nuclei were counted as apoptotic and cells that were red were counted as necrotic. The percentage of apoptotic and necrotic cells were counted with the help of Image J cell counter (NIH). The sum of percentage of apoptotic and necrotic cells was referred to as "total cell death".

5.2.3 **MTT assay**: NG108-15 cells grown and differentiated in 96-well culture plates were exposed to various concentrations of METH (0-10 mM), SN79 (0.1 -300 nM) or SN79 (0.1 – 300 nM) + METH (1 mM) for 24 hrs. After incubation at 37 °C for the specified times, 10 µl

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) freshly prepared in PBS was added to the wells at a final concentration of 50 µg/ml and the plates were incubated at 37 °C for a further 4 hr. 100 µl of solubilization solution (10% SDS in 0.1N HCl) was then added to each well and the plates incubated overnight. Absorbance was read at 570 nm with a reference wavelength of 690 nm. Percent cell viability was computed by dividing average absorbance values for experimental wells to average absorbance values for cells in untreated control wells.

5.2.4 Trypan blue exclusion assay: Cells were grown and differentiated in 6-well plates. On the day of the experiment, the differentiation media was changed to phenol red free differentiation media with various concentrations of METH (0-3 mM), SN79 (1 -30 nM) or SN79 (1 – 30 nM)+METH (1 mM) for 24 hrs. The media was removed and the cells detached using Trypsin-EDTA and then mixed with media. 10 µl of trypan blue dye and 10 µl of cell mixture were mixed and the number of total, live and dead cells was counted using a Countess Automated Cell Counter (Invitrogen, Carlsbad, CA).

5.2.5 Caspase-3, -9 and -8 assay: NG108-15 cells were grown and differentiated in 96-well culture plates and time course and inhibition studies were conducted: 1) Time course: Cells were exposed to various concentrations of METH (0-1 mM) for 1.5, 3, 6, 9, 12, 18 and 24 hrs. 2) Inhibition: The cells were treated with either METH (0-1 mM), SN79 (1-100 nM) or SN79 (1-100 nM)+METH (0-1 mM) for the time point at which a peak response was observed for caspase-3, -9 or -8 activation. At appropriate time points for the time course or inhibition studies, reagents from the Caspase-Glo luminescence assay kit for caspase 3/7, -9, -8 (Invitrogen, Carlsbad, CA) were added to the wells and incubated for 2 hrs. The plate was read using a luminescence plate reader. The no cell blank readings were subtracted from all the treatment readings. The results were expressed as % untreated control cells at that time point.

5.2.6 Reactive oxygen/nitrogen species: Cells grown and differentiated in 96 well plate (10^4 cells per well). The culture medium was replaced phenol red free medium containing 0.5% FBS and 5 μ M fluorogenic, oxidizable, dye CM-H₂DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate) and returned to the incubator at 37 °C for 45-60 min. Cells were treated with appropriate concentrations of METH or SN79. Exogenously added 100 μ M H₂O₂ and Na₂Cr₂O₇ served as a positive control in this assay. All compounds were added in replicates of 4 or 8 and the plates are returned to the incubator. In the assay, SN79 (1-100 nM), N-acetylcysteine (NAC, 10 mM), catalase (10000 units per ml), L-NMMA (200 μ M) were added 15 min prior to the addition of METH (0.1-3000 μ M), H₂O₂ (100 μ M) or Na₂Cr₂O₇ (100 μ M). At specified time points, plates were read in a Fluostar Optima fluorescence plate reader fitted with a 485/520 nm excitation/emission filter set (BMG Labtech GmbH, Offenberg, Germany).

5.2.7 Statistical analysis: Statistical analysis of data was performed using ANOVA with appropriate post-hoc tests employing GraphPad Prism 4 (GraphPad Software, San Diego, CA).

5.3 Results

5.3.1 Apoptosis/Necrosis:

5.3.1.1. SN79 and METH:

Apoptosis: One-way ANOVA showed that METH treatment caused a significant increase in the percentage of apoptotic cells ($F(7,192) = 22.14$, $p < 0.0001$). Post-hoc Dunnett's tests confirmed that the following concentrations of METH caused a significant increase in the percentage of apoptotic cells: 10 μ M ($q = 3.79$, $p < 0.01$), 30 μ M ($q = 5.09$, $p < 0.001$), 100 μ M ($q = 7.15$, $p < 0.01$), 300 μ M ($q = 7.72$, $p < 0.01$) and 1000 μ M ($q = 9.77$, $p < 0.01$). SN79 pretreatment significantly attenuated the apoptotic effects of METH (Figure 5.1A).

Two-way ANOVA showed a significant effect of METH treatment ($F(7,538) = 14.63$, $p < 0.0001$), SN79 pretreatment ($F(3,538) = 100.90$, $p < 0.0001$), and SN79 pretreatment $\times$ METH treatment ($F(21,538) = 6.47$, $p < 0.0001$). Bonferroni's post-hoc tests showed that SN79 (1, 10 and 100 nM) pretreatment is able to attenuate the apoptotic effects of the following concentrations of METH: 3 μ M ($t = 2.80$, $p < 0.05$ and $t = 3.07$, $p < 0.05$), 10 μ M ($t = 4.36$, $p < 0.001$; $t = 4.59$, $p < 0.001$ and $t = 4.61$, $p < 0.001$ respectively), 30 μ M ($t = 5.78$, $p < 0.001$; $t = 5.32$, $p < 0.001$ and $t = 5.59$, $p < 0.001$ respectively), 100 μ M ($t = 6.40$, $p < 0.001$; $t = 6.70$, $p < 0.001$ and $t = 8.21$, $p < 0.001$ respectively), 300 μ M ($t = 5.48$, $p < 0.001$; $t = 7.30$, $p < 0.001$ and $t = 7.64$, $p < 0.001$ respectively) and 1000 μ M ($t = 11.00$, $p < 0.001$; $t = 9.52$, $p < 0.001$ and $t = 10.52$, $p < 0.001$ respectively).

Necrosis: One-way ANOVA showed that METH treatment caused a significant increase in the percentage of necrotic cells ($F(7,192) = 8.28$, $p < 0.0001$). Post-hoc Dunnett's tests confirmed that 300 and 1000 μ M METH caused a significant increase in the percentage of necrotic cells ($q = 4.45$, $p < 0.01$ and $q = 6.31$, $p < 0.01$ respectively). SN79 pretreatment significantly attenuated the necrotic effects of METH (Figure 5.1B). Two-way ANOVA showed a significant effect of SN79 pretreatment ($F(3,538) = 14.71$, $p < 0.0001$), METH treatment ($F(7,538) = 6.98$, $p < 0.0001$) and SN79 pretreatment $\times$ METH treatment interaction ($F(21,538) = 1.82$, $p < 0.05$). Post-hoc Bonferroni's test confirmed that the following concentrations of SN79 attenuate the necrotic effects of 300 μ M (1 nM: $t = 3.12$, $p < 0.05$; 10 nM: $t = 3.57$, $p < 0.01$; 100 nM: $t = 2.98$, $p < 0.05$) and 1000 μ M METH (1 nM: $t = 4.31$, $p < 0.001$; 10 nM: $t = 2.85$, $p < 0.05$; 100 nM: $t = 5.89$, $p < 0.001$).

Total cell death: One-way ANOVA showed that METH treatment caused a significant increase in the percentage of total cell death ($F(7,192) = 29.26$, $p < 0.0001$). Post-hoc Dunnett's tests confirmed that the following concentrations of METH caused a significant increase in the percentage of total cell death: 10 μ M ($q = 3.63$, $p < 0.01$), 30 μ M ($q = 5.55$, $p <$

0.01), 100 μ M ($q = 6.68$, $p < 0.01$), 300 μ M ($q = 8.84$, $p < 0.01$) and 1000 μ M ($q = 11.71$, $p < 0.01$). SN79 pretreatment significantly attenuated METH-induced cell death (Figure 5.1C). Two-way ANOVA showed a significant effect of SN79 pretreatment ($F(3,539) = 73.49$, $p < 0.0001$), METH treatment ($F(7,539) = 15.85$, $p < 0.0001$) and SN79 pretreatment $\times$ METH treatment interaction ($F(21,539) = 5.41$, $p < 0.0001$). Post-hoc Bonferroni's test confirmed that the following concentrations of SN79 attenuate cell death caused by 10 μ M (1 nM: $t = 3.52$, $p < 0.05$; 10 nM: $t = 3.67$, $p < 0.01$; 100 nM: $t = 3.60$, $p < 0.01$), 30 μ M (1 nM: $t = 4.79$, $p < 0.0001$; 10 nM: $t = 5.08$, $p < 0.0001$; 100 nM: $t = 4.90$, $p < 0.0001$), 100 μ M (1 nM: $t = 4.52$, $p < 0.001$; 10 nM: $t = 5.06$, $p < 0.0001$; 100 nM: $t = 6.65$, $p < 0.0001$), 300 μ M (1 nM: $t = 5.47$, $p < 0.0001$; 10 nM: $t = 6.84$, $p < 0.0001$; 100 nM: $t = 6.59$, $p < 0.0001$) and 1000 μ M METH (1 nM: $t = 9.52$, $p < 0.0001$; 10 nM: $t = 7.58$, $p < 0.0001$; 100 nM: $t = 10.42$, $p < 0.0001$).

Figure 5.1 Effect of SN79 pretreatment on METH-induced (A) apoptosis, (B) necrosis and (C) total cell death. SN79 protects against methamphetamine (METH)-induced apoptosis (A), necrosis (B) and total cell death (C). Differentiated NG108-15 cells were pretreated with SN79 (0-100 nM) prior to exposure to METH (0-1 mM) for 24 hr. After 24 hr, the wells were incubated with Hoechst 33342 and propidium iodide stains (20 μ g/ml each) to obtain percentages of apoptotic and necrotic cells. Total cell death = Apoptotic cell death + Necrotic cell death. Data represent means from three separate experiments ($n = 3/\text{experiment}$) $\pm$ SEM. Significant differences are indicated by ** $p < 0.01$ (control vs. METH treated); # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ (METH alone vs. METH with SN79).

5.3.1.2. DTG and METH:

Apoptosis: METH treatment on its own caused a significant increase in apoptotic cell death in differentiated NG108-15 cells. One way ANOVA showed a significant effect of METH treatment ($F(6,244) = 22.51$, $p < 0.0001$). Dunnett's post-hoc tests confirmed that the following concentrations of METH cause a significant increase in apoptosis: 10 μM ($q = 3.29$, $p < 0.01$), 100 μM ($q = 6.94$, $p < 0.01$) and 1000 μM ($q = 9.85$, $p < 0.01$).

Figure 5.2A shows that DTG pretreatment displayed a trend towards a shift in the dose response curve of METH-induced apoptosis towards the left; however, the effect was not significant. Two way ANOVA shows a significant effect of METH treatment ($F(6,889) = 37.83$, $p < 0.0001$) and DTG pretreatment ($F(6,889) = 7.49$, $p < 0.0001$) but METH treatment $\times$ DTG pretreatment interaction was not significant ($F(36,889) = 0.68$, n.s.). Bonferroni's post-hoc tests show that DTG pretreatment at 10 nM and 1 μM with METH 1 μM concentration was significantly different than METH 1 μM treatment alone ($t = 2.92$, $p < 0.05$ and $t = 2.88$, $p < 0.05$ respectively).

Necrosis: METH treatment on its own caused a significant increase in necrotic cell death in differentiated NG108-15 cells. One way ANOVA showed a significant effect of METH treatment ($F(6,249) = 48.59$, $p < 0.0001$). Dunnett's post-hoc tests confirmed that METH 1000 μM caused a significant increase in necrosis ($q = 14.25$, $p < 0.01$).

Figure 5.2B shows that DTG pretreatment at intermediate concentrations shifts the dose response curve of METH towards the left, and at even higher concentrations shows an upward and leftward shift in the dose response curve. Two way ANOVA showed a significant effect of METH treatment ($F(6,948) = 55.75$, $p < 0.0001$), DTG pretreatment ($F(6,948) = 77.15$, $p < 0.0001$) and METH treatment $\times$ DTG pretreatment interaction ($F(36,6) = 1.80$, $p < 0.005$). Bonferroni's post-hoc tests confirm that the following concentrations of DTG in combination with METH are significantly different than METH treatment alone: DTG 10 nM (10

μM : $t = 3.07$, $p < 0.05$ and $100 \mu\text{M}$: $t = 4.59$, $p < 0.001$), DTG 100 nM ($0.01 \mu\text{M}$: $t = 2.78$, $p < 0.05$; $0.1 \mu\text{M}$: $t = 2.75$, $p < 0.05$; $1 \mu\text{M}$: $t = 5.44$, $p < 0.001$; $10 \mu\text{M}$: $t = 5.07$, $p < 0.001$ and $100 \mu\text{M}$: $t = 7.01$, $p < 0.001$), DTG $1 \mu\text{M}$ ($0 \mu\text{M}$: $t = 2.85$, $p < 0.05$; $1 \mu\text{M}$: $t = 7.39$, $p < 0.001$; $10 \mu\text{M}$: $t = 7.92$, $p < 0.001$; $100 \mu\text{M}$: $t = 9.33$, $p < 0.001$ and $1000 \mu\text{M}$: $t = 4.02$, $p < 0.001$), DTG $10 \mu\text{M}$ ($0 \mu\text{M}$: $t = 6.87$, $p < 0.001$; $0.01 \mu\text{M}$: $t = 4.49$, $p < 0.001$; $0.1 \mu\text{M}$: $t = 5.18$, $p < 0.001$; $1 \mu\text{M}$: $t = 7.07$, $p < 0.001$; $10 \mu\text{M}$: $t = 8.31$, $p < 0.001$; $100 \mu\text{M}$: $t = 10.08$, $p < 0.001$ and $1000 \mu\text{M}$: $t = 5.21$, $p < 0.001$).

Total cell death: METH treatment on its own caused a significant increase in cell death in differentiated NG108-15 cells. One way ANOVA showed a significant effect of METH treatment ($F(6,249) = 62.50$, $p < 0.0001$). Dunnett's post-hoc tests confirmed that the following concentrations of METH caused a significant increase in total cell death: $1 \mu\text{M}$ ($q = 3.15$, $p < 0.05$), $10 \mu\text{M}$ ($q = 3.54$, $p < 0.01$), $100 \mu\text{M}$ ($q = 6.48$, $p < 0.01$) and $1000 \mu\text{M}$ ($q = 16.97$, $p < 0.01$). Figure 5.2C shows that DTG pretreatment at intermediate concentrations shifted the dose response curve of METH-induced cell death towards the left and at even higher concentrations displayed an upward and leftward shift in the dose response curve. Two way ANOVA showed a significant effect of METH treatment ($F(6,948) = 88.73$, $p < 0.0001$), DTG pretreatment ($F(6,948) = 46.62$, $p < 0.0001$) and METH treatment $\times$ DTG pretreatment interaction ($F(36,946) = 1.69$, $p < 0.01$). Bonferroni's post-hoc tests confirm that the following concentrations of DTG in combination with METH are significantly different than METH treatment alone: DTG 10 nM ($1 \mu\text{M}$: $t = 3.02$, $p < 0.05$; $10 \mu\text{M}$: $t = 3.75$, $p < 0.01$ and $100 \mu\text{M}$: $t = 5.22$, $p < 0.001$), DTG 100 nM ($0.1 \mu\text{M}$: $t = 2.89$, $p < 0.05$; $1 \mu\text{M}$: $t = 4.23$, $p < 0.001$; $10 \mu\text{M}$: $t = 5.52$, $p < 0.001$ and $100 \mu\text{M}$: $t = 5.25$, $p < 0.001$), DTG $1 \mu\text{M}$ ($0 \mu\text{M}$: $t = 3.29$, $p < 0.01$; $1 \mu\text{M}$: $t = 7.07$, $p < 0.001$; $10 \mu\text{M}$: $t = 6.14$, $p < 0.001$; $100 \mu\text{M}$: $t = 7.63$, $p < 0.001$ and $1000 \mu\text{M}$: $t = 3.35$, $p < 0.001$), DTG $10 \mu\text{M}$ ($0 \mu\text{M}$: $t = 5.76$, $p < 0.001$; $0.01 \mu\text{M}$: $t = 3.30$, $p < 0.01$; $0.1 \mu\text{M}$: t

= 3.44, $p < 0.01$; 1 μM : $t = 5.75$, $p < 0.001$; 10 μM : $t = 7.23$, $p < 0.001$; 100 μM : $t = 7.62$, $p < 0.001$ and 1000 μM : $t = 5.27$, $p < 0.001$).

Figure 5.2 Effect of DTG pretreatment on METH-induced (A) apoptosis, (B) necrosis and (C) total cell death. NG108-15 cells were exposed to DTG (0.1 nM-10 μM) and/or METH (0-1000 μM) for 24 hr. After 24 hr, the wells were incubated with Hoechst 33342 and propidium iodide stains (20 $\mu\text{g}/\text{ml}$ each) to obtain percentages of apoptotic and necrotic cells. Total cell death = Apoptotic cell death + Necrotic cell death. Data represent means from two separate experiments ($n = 3/\text{experiment}$) $\pm$ SEM. Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$ (control vs. METH); ## $p < 0.01$, ### $p < 0.001$ (DTG+METH vs. METH).

5.3.1.3. METH treatment at 37 and 40 °C

Apoptosis: METH treatment caused a significant increase in apoptotic cell death in NG108-15 cells maintained at 37 °C. One way ANOVA showed a significant effect of METH treatment ($F(9,436) = 33.37, p < 0.0001$). Dunnett's post-hoc tests confirmed that the following concentrations of METH caused a significant increase in apoptosis when compared to no treatment control cells: 10 μM ($q = 4.77, p < 0.01$), 30 μM ($q = 6.38, p < 0.01$), 100 μM ($q = 9.56, p < 0.01$), 300 μM ($q = 9.93, p < 0.01$) and 1000 μM ($q = 13.30, p < 0.01$).

METH treatment at elevated temperature of 40 °C caused a significant increase in apoptotic cell death when compared to control cells maintained at the same temperature. One way ANOVA showed a significant effect of METH treatment ($F(9,217) = 5.80, p < 0.0001$). Dunnett's post-hoc tests confirmed that the following concentrations of METH caused a significant increase in apoptosis when compared to control cells maintained at 40 °C: 10 μM ($q = 3.70, p < 0.01$), 30 μM ($q = 3.42, p < 0.01$), 100 μM ($q = 4.60, p < 0.01$), 300 μM ($q = 4.31, p < 0.01$) and 1000 μM ($q = 5.16, p < 0.01$).

On comparing the METH treated NG108-15 cells at 37 and 40 °C, it was observed that cells maintained at 40 °C had a higher percentage of apoptotic cells in the no treatment control as well as METH treatment when compared with the cells maintained at 37 °C (Figure 5.3A). The dose response of METH at 40 °C shows a parallel and upward shift in apoptotic cell death. Two way ANOVA showed a significant effect of METH treatment ($F(9,635) = 21.52, p < 0.0001$) and temperature ($F(1,635) = 31.49, p < 0.0001$). Bonferroni's post-hoc tests confirmed that the following concentrations of METH caused a significant increase in apoptosis when maintained at 37 and 40 °C: 1 μM ($t = 2.92, p < 0.05$), 10 μM ($t = 4.47, p < 0.001$) and 30 μM ($t = 2.90, p < 0.05$).

Necrosis: METH treatment caused a significant increase in necrotic cell death in NG108-15 cells maintained at 37 °C. One way ANOVA showed a significant effect of METH

treatment ($F(9,428) = 32.93, p < 0.0001$). Dunnett's post-hoc tests confirmed that the following concentrations of METH caused a significant increase in apoptosis when compared to no treatment control cells: 300 μM ($q = 6.75, p < 0.01$) and 1000 μM ($q = 14.02, p < 0.01$).

METH treatment at an elevated temperature of 40 °C caused a significant increase in apoptotic cell death when compared to control cells maintained at the same temperature. The necrotic cell death was more sensitive to temperature, as lower concentrations of METH also caused significant necrosis when maintained at 40 °C. One way ANOVA showed a significant effect of METH treatment ($F(9,205) = 9.63, p < 0.0001$). Dunnett's post-hoc tests confirmed that the following concentrations of METH caused a significant increase in necrosis when compared to control cells maintained at 40 °C: 1 μM ($q = 2.86, p < 0.05$), 3 μM ($q = 2.89, p < 0.05$), 10 μM ($q = 3.24, p < 0.05$), 30 μM ($q = 2.75, p < 0.05$), 100 μM ($q = 7.10, p < 0.01$), 300 μM ($q = 4.20, p < 0.01$) and 1000 μM ($q = 7.33, p < 0.01$).

On comparing the METH treated NG108-15 cells at 37 and 40 °C, it was observed that cells maintained at 40 °C had a significantly higher percentage of necrotic cells in the no treatment control as well as METH treatment when compared with the cells maintained at 37 °C (Figure 5.3B). The dose response of METH at 40 °C shows an up and leftward shift in necrotic cell death. Two way ANOVA showed a significant effect of METH treatment ($F(9,615) = 19.76, p < 0.0001$), temperature ($F(1,615) = 600, p < 0.0001$) and METH treatment $\times$ temperature interaction ($F(9,615) = 8.81, p < 0.0001$). Bonferroni's post-hoc tests confirmed that the following concentrations of METH caused a significant increase in apoptosis when maintained at 40 versus 37 °C: 0 μM ($t = 3.24, p < 0.05$), 0.01 μM ($t = 4.48, p < 0.001$), 0.1 μM ($t = 5.17, p < 0.001$), 1 μM ($t = 8.79, p < 0.001$), 3 μM ($t = 7.02, p < 0.001$), 10 μM ($t = 9.45, p < 0.001$), 30 μM ($t = 6.37, p < 0.001$), 100 μM ($t = 17.32, p < 0.001$), 300 μM ($t = 7.80, p < 0.001$) and 1000 μM ($t = 14.43, p < 0.001$).

Total cell death: METH treatment caused a significant increase in total cell death in NG108-15 cells maintained at 37 °C. One way ANOVA showed a significant effect of METH treatment ($F(9,437) = 68.14, p < 0.0001$). Dunnett's post-hoc tests confirmed that the following concentrations of METH caused a significant increase in apoptosis when compared to no treatment control cells: 10 μM ($q = 5.05, p < 0.01$), 30 μM ($q = 7.59, p < 0.01$), 100 μM ($q = 9.40, p < 0.01$), 300 μM ($q = 12.21, p < 0.01$) and 1000 μM ($q = 20.21, p < 0.01$).

METH treatment at elevated temperature of 40 °C caused a significant increase in total cell death when compared to control cells maintained at the same temperature. Similar to necrotic cell death, the total cell death was sensitive to temperature as lower concentrations of METH also caused cell death when maintained at 40 °C. One way ANOVA showed a significant effect of METH treatment ($F(9,208) = 13.78, p < 0.0001$). Dunnett's post-hoc tests confirmed that the following concentrations of METH caused a significant increase in necrosis when compared to control cells maintained at 40 °C: 1 μM ($q = 2.91, p < 0.05$), 3 μM ($q = 3.00, p < 0.05$), 10 μM ($q = 3.99, p < 0.01$), 30 μM ($q = 3.58, p < 0.01$), 100 μM ($q = 8.17, p < 0.01$), 300 μM ($q = 5.31, p < 0.01$) and 1000 μM ($q = 8.57, p < 0.01$).

On comparing the METH treated NG108-15 cells at 37 and 40 °C, it was observed that cells maintained at 40 °C had a significantly higher percentage of cell death in no treatment control as well as METH treatment when compared with the cells maintained at 37 °C (Figure 5.3C). The dose response of METH at 40 °C shows an up and leftward shift in total cell death. Two way ANOVA showed a significant effect of METH treatment ($F(9,627) = 36.68, p < 0.0001$), temperature ($F(1,627) = 652.3, p < 0.0001$) and METH treatment $\times$ temperature interaction ($F(9,627) = 7.81, p < 0.0001$). Bonferroni's post-hoc tests confirmed that the following concentrations of METH caused a significant increase in total cell death when maintained at 40 versus 37 °C: 0 μM ($t = 4.36, p < 0.001$), 0.01 μM ($t = 4.71, p < 0.001$), 0.1 μM ($t = 5.13, p < 0.001$), 1 μM ($t = 9.31, p < 0.001$), 3 μM ($t = 7.49, p < 0.001$), 10 μM ($t =$

10.74, $p < 0.001$), 30 μM ($t = 6.56$, $p < 0.001$), 100 μM ($t = 17.67$, $p < 0.001$), 300 μM ($t = 7.81$, $p < 0.001$) and 1000 μM ($t = 14.22$, $p < 0.001$).

Figure 5.3 Effect of temperature (37 versus 40 °C) on METH-induced (A) apoptosis, (B) necrosis and (C) total cell death. NG108-15 cells were exposed to METH (0-1000 μM) for 24 hr at 37 or 40 °C. After 24 hr, the wells were incubated with Hoechst 33342 and propidium iodide stains (20 $\mu\text{g}/\text{ml}$ each) to obtain percentages of apoptotic and necrotic cells. Total cell death = Apoptotic cell death + Necrotic cell death. Data represent means from two separate experiments ($n = 3/\text{experiment}$) $\pm$ SEM. Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$ (control vs. METH treated at a particular temperature); # $p < 0.05$, ### $p < 0.001$ (treatment at 37 °C vs. 40 °C).

5.3.1.4: SN79 and METH at 40 °C:

Apoptosis: SN79 treatment was able to significantly reduce the elevated apoptotic cell death caused by increased temperature (40 °C). One way ANOVA showed a significant effect of SN79 treatment ($F(3,120) = 8.21, p < 0.0001$). Dunnett's post-hoc tests confirmed that the following concentrations of SN79 significantly attenuated the apoptotic effect of elevated temperature in control untreated cells: 1 nM ($q = 4.14, p < 0.01$), 10 nM ($q = 4.32, p < 0.01$) and 10 nM ($q = 3.16, p < 0.01$).

SN79 also attenuated the combined apoptotic effect of elevated temperature and all the concentrations of METH (Figure 5.4A). One way ANOVA showed a significant effect of SN79 pretreatment prior to the following concentrations of METH treatment: 0.01 μM ($F(3,40) = 12.06, p < 0.0001$), 0.1 μM ($F(3,39) = 3.18, p < 0.05$), 1 μM ($F(3,114) = 20.05, p < 0.0001$), 3 μM ($F(3,65) = 7.63, p < 0.0005$), 10 μM ($F(3,108) = 30.55, p < 0.0001$), 30 μM ($F(3,74) = 23.44, p < 0.0001$), 100 μM ($F(3,115) = 31.41, p < 0.0001$), 300 μM ($F(3,73) = 15.99, p < 0.0001$) and 1000 μM ($F(3,110) = 13.29, p < 0.0001$). Dunnett's post-hoc tests confirmed that SN79 pretreatment is able to attenuate the apoptotic effects of the following concentrations of METH: 0.01 μM (1 nM: $q = 3.90, p < 0.01$; 10 nM: $q = 4.97, p < 0.01$; 100 nM: $q = 5.28, p < 0.01$), 0.1 μM (100 nM: $q = 2.96, p < 0.05$), 1 μM (1 nM: $q = 5.51, p < 0.01$; 10 nM: $q = 6.00, p < 0.01$; 100 nM: $q = 7.00, p < 0.01$), 3 μM (1 nM: $q = 3.98, p < 0.01$; 10 nM: $q = 4.20, p < 0.01$; 100 nM: $q = 3.28, p < 0.01$), 10 μM (1 nM: $q = 8.05, p < 0.01$; 10 nM: $q = 8.29, p < 0.01$; 100 nM: $q = 6.64, p < 0.01$), 30 μM (1 nM: $q = 7.06, p < 0.01$; 10 nM: $q = 7.33, p < 0.01$; 100 nM: $q = 6.30, p < 0.01$), 100 μM (1 nM: $q = 8.27, p < 0.01$; 10 nM: $q = 8.04, p < 0.01$; 100 nM: $q = 7.35, p < 0.01$), 300 μM (1 nM: $q = 5.72, p < 0.01$; 10 nM: $q = 6.07, p < 0.01$; 100 nM: $q = 5.50, p < 0.01$) and 1000 μM (1 nM: $q = 5.01, p < 0.01$; 10 nM: $q = 4.58, p < 0.01$; 100 nM: $q = 5.64, p < 0.01$).

Necrosis: SN79 treatment was able to significantly reduce the elevated necrotic cell death caused by increased temperature (40 °C) in control untreated cells. One way ANOVA showed a significant effect of SN79 treatment ($F(3,120) = 12.57, p < 0.0001$). Dunnett's post-hoc tests confirmed that the following concentrations of SN79 significantly attenuated the apoptotic effect of elevated temperature: 1 nM ($q = 4.82, p < 0.01$), 10 nM ($q = 4.95, p < 0.01$) and 100 nM ($q = 5.04, p < 0.01$).

SN79 also attenuated the combined necrotic effect of elevated temperature and all the concentrations of METH (Figure 5.4B). One way ANOVA showed a significant effect of SN79 pretreatment prior to the following concentrations of METH treatment: 0.01 μ M ($F(3,40) = 11.98, p < 0.0001$), 0.1 μ M ($F(3,70) = 36.99, p < 0.0001$), 1 μ M ($F(3,114) = 18.84, p < 0.0001$), 3 μ M ($F(3,65) = 29.95, p < 0.0001$), 10 μ M ($F(3,108) = 10.09, p < 0.0001$), 30 μ M ($F(3,74) = 15.68, p < 0.0001$), 100 μ M ($F(3,115) = 24.65, p < 0.0001$), 300 μ M ($F(3,73) = 17.50, p < 0.0001$) and 1000 μ M ($F(3,110) = 24.19, p < 0.0001$). Dunnett's post-hoc tests confirmed that SN79 pretreatment is able to attenuate the necrotic effects of the following concentrations of METH: 0.01 μ M (1 nM: $q = 5.19, p < 0.01$; 10 nM: $q = 5.06, p < 0.01$; 100 nM: $q = 3.82, p < 0.01$), 0.1 μ M (1 nM: $q = 9.43, p < 0.01$; 10 nM: $q = 9.61, p < 0.01$; 100 nM: $q = 9.15, p < 0.01$), 1 μ M (1 nM: $q = 5.84, p < 0.01$; 10 nM: $q = 6.65, p < 0.01$; 100 nM: $q = 5.58, p < 0.01$), 3 μ M (1 nM: $q = 7.22, p < 0.01$; 10 nM: $q = 8.64, p < 0.01$; 100 nM: $q = 6.79, p < 0.01$), 10 μ M (1 nM: $q = 4.30, p < 0.01$; 10 nM: $q = 4.90, p < 0.01$; 100 nM: $q = 4.06, p < 0.01$), 30 μ M (1 nM: $q = 4.35, p < 0.01$; 10 nM: $q = 5.34, p < 0.01$; 100 nM: $q = 6.47, p < 0.01$), 100 μ M (1 nM: $q = 7.34, p < 0.01$; 10 nM: $q = 6.68, p < 0.01$; 100 nM: $q = 6.98, p < 0.01$), 300 μ M (1 nM: $q = 5.88, p < 0.01$; 10 nM: $q = 6.13, p < 0.01$; 100 nM: $q = 6.13, p < 0.01$) and 1000 μ M (1 nM: $q = 6.46, p < 0.01$; 10 nM: $q = 6.75, p < 0.01$; 100 nM: $q = 7.47, p < 0.01$).

Total cell death: SN79 treatment was able to significantly reduce the elevated cell death caused by increased temperature (40 °C). One way ANOVA showed a significant effect

of SN79 treatment ($F(3,120) = 20.41, p < 0.0001$). Dunnett's post-hoc tests confirmed that the following concentrations of SN79 significantly attenuated the cell death of elevated temperature: 1 nM ($q = 6.23, p < 0.01$), 10 nM ($q = 6.41, p < 0.01$) and 10 nM ($q = 6.24, p < 0.01$).

SN79 also attenuated the combined effect elevated temperature and all the concentrations of METH on cell death in differentiated NG108-15 cells (Figure 5.4C). One way ANOVA showed a significant effect of SN79 pretreatment prior to the following concentrations of METH treatment: 0.01 μM ($F(3,40) = 27.03, p < 0.0001$), 0.1 μM ($F(3,39) = 20.74, p < 0.0001$), 1 μM ($F(3,114) = 28.69, p < 0.0001$), 3 μM ($F(3,65) = 42.27, p < 0.0001$), 10 μM ($F(3,108) = 21.87, p < 0.0001$), 30 μM ($F(3,74) = 40.99, p < 0.0001$), 100 μM ($F(3,115) = 40.66, p < 0.0001$), 300 μM ($F(3,73) = 36.07, p < 0.0001$) and 1000 μM ($F(3,110) = 32.66, p < 0.0001$). Dunnett's post-hoc tests confirmed that SN79 pretreatment is able to attenuate the cell death caused by the following concentrations of METH: 0.01 μM (1 nM: $q = 7.38, p < 0.01$; 10 nM: $q = 7.71, p < 0.01$; 100 nM: $q = 6.50, p < 0.01$), 0.1 μM (1 nM: $q = 6.43, p < 0.01$; 10 nM: $q = 6.59, p < 0.01$; 100 nM: $q = 6.62, p < 0.01$), 1 μM (1 nM: $q = 7.10, p < 0.01$; 10 nM: $q = 8.00, p < 0.01$; 100 nM: $q = 7.35, p < 0.01$), 3 μM (1 nM: $q = 8.76, p < 0.01$; 10 nM: $q = 10.19, p < 0.01$; 100 nM: $q = 8.00, p < 0.01$), 10 μM (1 nM: $q = 6.53, p < 0.01$; 10 nM: $q = 7.14, p < 0.01$; 100 nM: $q = 5.85, p < 0.01$), 30 μM (1 nM: $q = 8.06, p < 0.01$; 10 nM: $q = 9.22, p < 0.01$; 100 nM: $q = 9.89, p < 0.01$), 100 μM (1 nM: $q = 9.44, p < 0.01$; 10 nM: $q = 8.71, p < 0.01$; 100 nM: $q = 8.84, p < 0.01$), 300 μM (1 nM: $q = 8.50, p < 0.01$; 10 nM: $q = 8.90, p < 0.01$; 100 nM: $q = 8.65, p < 0.01$) and 1000 μM (1 nM: $q = 7.59, p < 0.01$; 10 nM: $q = 7.72, p < 0.01$; 100 nM: $q = 8.72, p < 0.01$).

Figure 5.4 Effect of SN79 pretreatment on METH-induced (A) apoptosis, (B) necrosis and (C) total cell death at 40 °C. SN79 protects against methamphetamine (METH) and elevated temperature (40 °C)-induced apoptosis (A), necrosis (B) and total cell death (C). NG108-15 cells were pretreated with SN79 (1-100 nM) prior to exposure to METH (0-1000 μ M) for 24 hr at 40 °C. After 24 hr, the wells were incubated with Hoechst 33342 and propidium iodide stains (20 μ g/ml each) to obtain percentages of apoptotic and necrotic cells. Total cell death = Apoptotic cell death + Necrotic cell death. Data represent means from two separate experiments ($n = 3/\text{experiment}$) $\pm$ SEM. Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$ (control vs. METH treated); ## $p < 0.01$ (control/METH alone vs. control/METH with SN79).

5.3.2 **MTT Assay**: Figure 5.5A shows a dose response of METH-induced decrease in cell viability. One-way ANOVA shows a significant effect of METH treatment ($F(11,165) = 66.67$, $p < 0.0001$). Post-hoc Dunnett's tests reveal a significant effect of the following concentrations of METH: 1 mM ($q = 8.13$, $p < 0.001$), 3 mM ($q = 17.24$, $p < 0.001$) and 10 mM ($q = 18.09$, $p < 0.001$).

One-way ANOVA showed that SN79 (0.1-10000 nM) pretreatment significantly attenuated the decrease in cell viability caused by METH (1 mM) (Figure 5.5B, $F(12,269) = 13.94$, $p < 0.0001$). Post-hoc Dunnett's tests revealed that the following concentration of SN79 pretreatment were significantly different from METH treatment alone (0.1 nM: $q = 3.59$, $p < 0.01$; 0.3 nM: $q = 3.48$, $p < 0.01$; 1 nM: $q = 7.26$, $p < 0.001$; 3 nM: $q = 4.97$, $p < 0.001$; 10 nM: $q = 4.99$, $p < 0.001$; 30 nM: $q = 6.23$, $p < 0.001$; 100 nM: $q = 3.56$, $p < 0.01$; 300 nM: $q = 3.82$, $p < 0.01$; 1000 nM: $q = 4.05$, $p < 0.001$).

Figure 5.5 MTT Assay: A. Dose response of METH-induced decrease in cell viability B. Effect of SN79 pretreatment on METH (1000 μM)-induced decrease in cell viability. (A) Differentiated NG108-15 cells were treated with METH (0-1000 μM). At 24 hour time point cell viability was determined using MTT assay. METH 1-10 mM caused significant decrease in cell viability. (B) Differentiated NG108-15 cells were pretreated with SN79 (0-10 μM), 15 min prior to adding METH (1 mM). SN79 attenuated the decrease in cell viability caused by METH at 1000 μM without significantly altering the cell viability on its own (** $P < 0.001$, ## $P < 0.01$, ### $P < 0.001$; post hoc tests, * vs. no treatment control, # vs. METH 1000 μM).

5.3.3 Trypan blue exclusion assay: METH treatment caused a significant decrease in NG108 cell viability (Figure 5.6A). One-way ANOVA showed a significant effect of METH treatment ($F(10,38) = 9.64, p < 0.0001$). Dunnett's post-hoc tests confirmed that the following doses of METH cause a significant decrease in cell viability (1 mM: $q = 7.52, p < 0.01$ and 3 mM: $q = 6.66, p < 0.01$). SN79 pretreatment attenuated METH (1 mM)-induced decrease in cell viability (Figure 5.6B). Two-way ANOVA showed a significant effect of METH treatment ($F(1,54) = 5.83, p < 0.05$), SN79 pretreatment ($F(4,54) = 5.24, p < 0.005$) and METH treatment $\times$ SN79 pretreatment interaction ($F(4,54) = 6.11, p < 0.0005$). Bonferroni's post-hoc tests show that SN79 (1, 3, 10 and 30 nM) attenuate METH (1 mM)-induced decreases in cell viability ($t = 4.10, p < 0.001$; $t = 5.18, p < 0.001$; $t = 3.71, p < 0.01$ and $t = 3.73, p < 0.01$ respectively).

Figure 5.6 A. Dose response of METH-induced decrease in cell viability B. Effect of SN79 pretreatment on METH (1000 μM)-induced decrease in cell viability. (A) Differentiated NG108-15 cells were treated with METH (0-3000 μM) and at 24 hour time point cell viability was determined using Trypan blue exclusion assay. METH 1-3000 μM caused significant decrease in cell viability. (B) Differentiated NG108-15 cells were pretreated with SN79 (0-100 nM), 15 min prior to adding METH (1000 μM). SN79 attenuated the decrease in cell viability caused by METH at 1000 μM without significantly altering the cell viability on its own (**P<0.01, ##P<0.01, ###P<0.001; post hoc tests, * vs. no treatment control, # vs. METH 1000 μM).

5.3.4 Caspase-3, -8, -9 assay:

5.3.4.1. Caspase-3 activation: METH treatment caused a significant increase in caspase 3 activation in differentiated NG108-15 cells (Figure 5.7A). Two way ANOVA showed a significant effect of METH treatment ($F(3,104) = 16.78, p < 0.0001$), time ($F(4,104) = 47.59, p < 0.0001$) and METH treatment $\times$ time interaction ($F(12,104) = 16.17, p < 0.0001$). Bonferroni's multiple comparison post-hoc tests confirmed that 10, 100 and 1000 μM concentrations of METH caused a significant increase in caspase-3 activation at the 24 hour time point ($t = 3.34, p < 0.05$; $t = 7.74, p < 0.0001$ and $t = 19.27, p < 0.0001$ respectively).

SN79 pretreatment is significantly able to attenuate METH-induced caspase-3 activation at the 24 hour time point (Figure 5.7B). Two way ANOVA showed a significant effect of METH treatment ($F(4,128) = 109.5, p < 0.0001$), SN79 pretreatment ($F(3,128) = 14.82, p < 0.0001$) and METH $\times$ SN79 pretreatment interaction ($F(12,128) = 4.73, p < 0.0001$). Bonferroni's multiple comparison post-hoc tests showed that the following concentrations of SN79 can attenuate caspase-3 activation caused by METH 100 μM (1 nM: $t = 3.2, p < 0.05$; 10 nM: $t = 4.02, p < 0.01$) and 1000 μM (1 nM: $t = 5.33, p < 0.0001$; 10 nM: $t = 7.52, p < 0.0001$; 100 nM: $t = 5.32, p < 0.0001$) treatment.

Figure 5.7 A. Dose response and time course of METH treatment on Caspase-3 activation B. Effect of SN79 pretreatment on METH-induced Caspase-3 activation at 24 hours. (A) Differentiated NG108-15 cells were treated with METH (0-1000 μ M) and caspase-3 activation determined using Caspase-3/7 Glo Luminescence assay at 1.5, 3, 6, 12, 24 hour time points. At 24 hour time point, METH (10, 100 and 1000 μ M) caused significant increase in caspase-3 activation. (B) Differentiated NG108-15 cells were pretreated with SN79 (0-100 nM), 15 min prior to adding METH (1, 10, 100, 1000 μ M). At 24 hour time point, SN79 attenuated the caspase-3 activation caused by METH at 100 and 1000 μ M without significantly altering the caspase-3 activation on its own (* p <0.05, **** p < 0.0001, # p < 0.05, ## p <0.01,#### p <0.0001; post hoc tests, * vs. no treatment control, # vs. METH).

5.3.4.2. Caspase-8 activation: METH treatment caused a significant increase in caspase-8 activation in differentiated NG108-15 cells (Figure 5.8A). Two way ANOVA showed a significant effect of METH treatment ($F(4,319) = 41.49, p < 0.0001$), time ($F(5,319) = 10.95, p < 0.0001$) and METH treatment $\times$ time interaction ($F(20,319) = 3.13, p < 0.0001$). Bonferroni's multiple comparison post-hoc tests confirmed that 1, 10, 100 and 1000 μM concentrations of METH caused a significant increase in caspase-8 activation at the 9 hour time point ($t = 3.56, p < 0.05$; $t = 5.65, p < 0.0001$, $t = 9.02, p < 0.0001$ and $q = 16.39, p < 0.0001$ respectively). METH (1000 μM concentration) also caused caspase-8 activation at the 1.5, 3, 6, 12, and 24 hour time points ($t = 4.09, p < 0.01$; $t = 3.37, p < 0.05$; $t = 4.32, p < 0.001$; $t = 3.71, p < 0.01$ and $t = 4.93, p < 0.0001$ respectively).

SN79 pretreatment is significantly able to attenuate METH-induced caspase-8 activation at the 9 hour time point (Figure 5.8B). Two way ANOVA showed a significant effect of METH treatment ($F(4,278) = 88.61, p < 0.0001$), SN79 pretreatment ($F(3,278) = 19.50, p < 0.0001$) and METH $\times$ SN79 pretreatment interaction ($F(12,278) = 3.60, p < 0.0001$). Bonferroni's multiple comparison post-hoc tests showed that the following concentrations of SN79 can attenuate caspase-8 activation caused by METH 10 μM (100 nM: $t = 3.26, p < 0.05$), 100 μM (1 nM: $t = 4.22, p < 0.001$; 10 nM: $t = 4.25, p < 0.001$ and 100 nM: $t = 3.36, p < 0.05$) and 1000 μM (1 nM: $t = 3.43, p < 0.05$; 10 nM: $t = 3.92, p < 0.01$ and 100 nM: $t = 3.05, p < 0.05$) treatment.

Figure 5.8 A. Dose response and time course of METH treatment on Caspase-8 activation B. Effect of SN79 pretreatment on METH-induced Caspase-3 activation at 9 hours. (A) Differentiated NG108-15 cells were treated with METH (0-1000 μ M) and caspase-8 activation determined using Caspase-8 Glo Luminescence assay at 1.5, 3, 6, 9, 12, 24 hour time points. At 9 hour time point, METH (1, 10, 100 and 1000 μ M) caused significant increase in caspase-8 activation. (B) Differentiated NG108-15 cells were pretreated with SN79 (0-100 nM), 15 min prior to adding METH (1, 10, 100, 1000 μ M). At 9 hour time point, SN79 attenuated the caspase-8 activation caused by METH at 10, 100 and 1000 μ M without significantly altering the caspase-8 activation on its own (* p <0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, # p < 0.05, ## p <0.01,### p <0.001; post hoc tests, * vs. no treatment control, # vs. METH).

5.3.4.3. Caspase-9 activation: METH treatment caused a significant increase in caspase-9 activation in differentiated NG108-15 cells (Figure 5.9A). Two way ANOVA showed a significant effect of METH treatment ($F(4,511) = 52.31, p < 0.0001$), time ($F(6,511) = 19.18, p < 0.0001$) and METH treatment $\times$ time interaction ($F(24,511) = 3.13, p < 0.0001$). Bonferroni's multiple comparison post-hoc tests confirmed that 1000 μ M concentration of METH caused a significant increase in caspase-9 activation at the 3 hour ($t = 2.74, p < 0.05$), 6 hour ($t = 3.32, p < 0.01$), 9 hour ($t = 2.76, p < 0.05$), 12 hour ($t = 5.73, p < 0.001$), 18 hour ($t = 3.81, p < 0.01$) and 24 hour ($t = 20.93, p < 0.001$). METH 100 μ M concentration also caused caspase-9 activation at 9 hour ($t = 4.12, p < 0.001$) and 24 hour ($t = 6.24, p < 0.001$).

SN79 pretreatment is significantly able to attenuate METH-induced caspase-9 activation at the 24 hour time point (Figure 5.9B). Two way ANOVA showed a significant effect of METH treatment ($F(4,269) = 82.60, p < 0.0001$), SN79 pretreatment ($F(3,269) = 13.00, p < 0.0001$) and METH $\times$ SN79 pretreatment interaction ($F(12,269) = 3.89, p < 0.0001$). Bonferroni's multiple comparison post-hoc tests showed that 10 nM SN79 concentration can attenuate caspase-9 activation caused by METH 1000 μ M ($t = 5.24, p < 0.001$) and 100 nM SN79 concentration can attenuate caspase-9 activation caused by METH 100 μ M ($t = 2.91, p < 0.05$) and 1000 μ M ($t = 7.69, p < 0.001$) treatment.

Figure 5.9 A. Dose response and time course of METH treatment on Caspase-9 activation B. Effect of SN79 pretreatment on METH-induced Caspase-9 activation at 24 hours. (A) Differentiated NG108-15 cells were treated with METH (0-1000 μ M) and caspase-9 activation determined using Caspase-9 Glo Luminescence assay at 1.5, 3, 6, 9, 12, 18, 24 hour time points. At 9 hour time point, METH (1, 10, 100 and 1000 μ M) caused significant increase in caspase-8 activation. (B) Differentiated NG108-15 cells were pretreated with SN79 (0-100 nM), 15 min prior to adding METH (1, 10, 100, 1000 μ M). At 24 hour time point, SN79 attenuated the caspase-9 activation caused by METH at 10, 100 and 1000 μ M without significantly altering the caspase-8 activation on its own (* p <0.05, ** p < 0.01, *** p < 0.001, # p < 0.05, ### P <0.001; post hoc tests, * vs. no treatment control, # vs. METH).

5.3.5 Reactive oxygen/nitrogen species:

SN79 pretreatment was able to significantly attenuate the ROS generated by METH (Figure 5.10). Two-way ANOVA shows a significant effect of SN79 pretreatment ($F(4,824) = 35.27$, $p < 0.0001$), METH or H_2O_2 treatment ($F(9,824) = 75.77$, $p < 0.0001$) and SN79 pretreatment $\times$ METH or H_2O_2 treatment interaction ($F(36,824) = 2.24$, $p < 0.001$). Dunnett's post-hoc tests confirmed that the following concentrations of METH and H_2O_2 generated ROS: 1 μM ($q = 5.96$, $p < 0.01$), 3 μM ($q = 4.46$, $p < 0.01$), 10 μM ($q = 4.57$, $p < 0.01$), 30 μM ($q = 4.82$, $p < 0.01$), 100 μM ($q = 6.32$, $p < 0.01$), 300 μM ($q = 7.03$, $p < 0.01$), 1000 μM ($q = 7.15$, $p < 0.01$), 3000 μM ($q = 5.71$, $p < 0.01$) and 100 μM H_2O_2 ($q = 14.86$, $p < 0.01$). Bonferroni's post-hoc tests confirmed that SN79 can inhibit the ROS/RNS generated by the following concentration of METH: 1 μM (1 nM: $t = 2.99$, $p < 0.05$), 3 μM (1 nM: $t = 3.56$, $p < 0.01$; 10 nM: $t = 3.18$, $p < 0.05$), 30 μM (1 nM: $t = 2.91$, $p < 0.05$; 10 nM: $t = 3.71$, $p < 0.01$; 100 nM: $t = 3.30$, $p < 0.05$ and 1000 nM: $t = 4.08$, $p < 0.001$), 100 μM (1 nM: $t = 3.54$, $p < 0.01$), 300 μM (1 nM: $t = 5.09$, $p < 0.001$; 10 nM: $t = 4.88$, $p < 0.001$; 100 nM, $t = 4.63$, $p < 0.001$ and 1000 nM: $t = 5.29$, $p < 0.001$), 1000 μM (1 nM: $t = 3.07$, $p < 0.05$; 10 nM: $t = 3.64$, $p < 0.01$ and 1000 nM: $t = 2.88$, $p < 0.05$), 3000 μM (1 nM: $t = 3.96$, $p < 0.001$; 10 nM: $t = 4.23$, $p < 0.001$; 100 nM: $t = 3.95$, $p < 0.001$ and 1000 nM: $t = 3.77$, $p < 0.01$). SN79 (1000 nM) is able to attenuate the ROS generated by H_2O_2 ($t = 4.26$, $p < 0.001$).

Figure 5.10 Effect of SN79 pretreatment on METH-induced ROS/RNS generation. The production of ROS capable of oxidizing CM-H₂DCFDA was assessed in differentiated NG108-15 cells in the presence of 0 to 300 μM METH, 100 μM H₂O₂ or 100 μM Na₂Cr₂O₇ (Cr) in the absence (solid bars) or presence (cross hatched bars) of SN79 (1-1000 nM) as described in the methods. Data represent averages of relative fluorescence units (rfu) from 3-6 different experiments (n = 3-4/experiment) obtained 20 min after addition of drug ± SEM. Significant differences are noted as ***p* < 0.01 (control vs. METH or H₂O₂ treated) and #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 (METH alone vs. METH with SN79).

5.4 Discussion

This study showed that METH caused apoptosis in differentiated NG108-15 cells at physiologically relevant micromolar concentrations and necrosis at higher millimolar concentrations. At earlier time points, caspase-3, -8, and -9 activation, and ROS/RNS generation were also observed. SN79, a σ receptor antagonist, attenuated METH-induced cell death as well as the toxicity mediators. The implications of these findings are further discussed below.

In the first part of the study, the neurotoxic concentration range of METH in differentiated NG108-15 cells was determined. METH caused apoptosis at physiologically relevant micromolar concentrations. In contrast to apoptosis, necrotic cell death was observed at higher micromolar concentrations. SN79 attenuated the apoptotic cell death which is consistent with the role of σ receptors in this type of cell death in tumor models (Bowen, 2000; Marrazzo et al., 2011). SN79 also attenuated the necrotic cell death caused by METH. Even though there is not much evidence for the role of σ receptors in necrotic cell death, the following explanations may help in the better understanding of the results: First, studies suggest there is a considerable overlap between the two forms of cell death as evidenced by coining of terms like 'necroptosis' and 'aponecrosis' (Formigli et al., 2000; Papucci et al., 2004; Yakovlev and Faden, 2004; Galluzzi and Kroemer, 2008; Kroemer et al., 2009; Berghe et al., 2010; Christofferson and Yuan, 2010; Dunai et al., 2011; Jiang et al., 2011; Wu et al., 2011). Second, since the *in vitro* system lacks the phagocyte cells, these apoptotic cells may appear necrotic at later time points.

Another confirmation that METH-induced cell death is σ receptor sensitive comes from the ability of DTG to shift the dose response curve of METH-induced cell death towards the left. At higher concentrations DTG displayed toxicity on its own which is consistent with the ability of σ receptor (especially σ -2 receptor subtype) agonist to cause toxicity (Vilner et al.,

1995a; Bowen, 2000; Crawford and Bowen, 2002). In this study, the necrotic type of cell death was more sensitive to DTG's effect on METH. This is most likely because NG108-15 is a sensitive cell line and induction of stressors/pro-death mediators beyond a certain threshold may shift the cell death from apoptosis to necrosis (Leist et al., 1997; Nicotera et al., 1998; Leist et al., 1999; Proskuryakov et al., 2003; Soti et al., 2003; Nicotera and Melino, 2004).

The next part of the study was conducted to determine whether METH-induced apoptosis in NG108-15 cells is caspase-dependent or independent event. Studies in the literature suggest involvement of both caspase-dependent as well as independent pathways (Jayanthi et al., 2004). Caspase-3 activation was observed at the 24 hour time point, indicating that caspase dependent mechanism is involved in this apoptotic cell death. SN79 attenuated METH-induced caspase-3 activation, which is consistent with the role of σ receptors in caspase-dependent cell death mechanisms (Crawford and Bowen, 2002; Hornick et al., 2010; Jonhede et al., 2010).

Caspase-3 can be activated via extrinsic death receptors or intrinsic mitochondrial death pathways. Activation of caspase-8 at 9 hour time point confirms the involvement of an extrinsic cell death pathway. Caspase-8 activation was followed by caspase-9 activation which peaked at the 24 hour time point. This indicates that NG108-15 cells may be type II cells that involve mitochondrial death loop for the processing of apoptotic cell death. One of the most likely mechanisms of METH-induced caspase-8 activation in NG108-15 cells is ROS/RNS production which can increase the cellular death receptor expression (Bauer et al., 1998), and also ROS-induced death receptor independent caspase-8 activation via p38 MAPK pathway (El Mchichi et al., 2007). Once activated, caspase-8 can cleave Bid and cause cytochrome c release from the mitochondria, resulting in apoptosome formation with Apaf1 and hence caspase-9 activation (Li et al., 1998).

The ability of SN79 to attenuate caspase-8 activation at 9 hour time point is most likely due to the role of σ receptors in modulation of cellular ROS levels (Ostenfeld et al., 2005; Schetz et al., 2007). In addition, the localization of σ receptors in lipid rafts (Hayashi and Su, 2003b; Gebreselassie and Bowen, 2004; Gajate and Mollinedo, 2011) may allow them to modulate death receptor signaling (Cahuzac et al., 2006). σ -2 Receptors may play a predominant role in this pathway as a previous study using SK-N-SH neuroblastoma cells showed that activation of σ -2 receptors caused caspase-10 activation which in turn cleaved Bid thereby causing activation of mitochondrial death cascade (Wang and Bowen, 2006). The decrease in METH-induced caspase-9 activation at 24 hour time point may be due to the ability of SN79 to attenuate the caspase-8 signal. Alternately, σ receptors are located in the ER and mitochondria and can regulate the calcium signaling between these organelles (Vilner and Bowen, 2000; Wei et al., 2006; Cassano et al., 2009). Previous studies in tumor models have indicated a role of σ receptor in the modulation of mitochondrial death cascades (Wei et al., 2006). Caspase-9 can also be generated by other cellular stress signals like ROS/RNS generation in the cell (Kadenbach et al., 2004) which is activated by METH, which may also be regulated by σ receptors.

ROS/RNS generation is one of the main mediators of METH's neurotoxicity, which occurs through various sources including excessive dopamine release, microglial activation, excessive glutamate release, and mitochondrial dysfunction (Krasnova and Cadet, 2009). These ROS/RNS generated can injure neurons and surrounding cells via oxidative damage to cellular components such as lipids, proteins, and DNA, or cause ER stress and activation of mitochondrial death cascades, ultimately leading to nerve terminal degeneration or cell death (Krasnova and Cadet, 2009). In this study, METH was shown to cause ROS/RNS generation. SN79 pretreatment attenuated the ROS/RNS generated by METH. This effect of SN79 does not seem to be a general antioxidant effect as it is not able to prevent the H_2O_2 -mediated

ROS signal. Although, at high concentration of 1000 nM, SN79 causes decrease in H₂O₂-mediated ROS, which may be due to some intrinsic antioxidant activity at this concentration or due to potential upregulation of cellular antioxidant defenses. Mechanistically, SN79 can act via σ receptors to modulate cellular ROS/RNS generation and antioxidant systems. Previous studies in tumor cells have shown that σ -2 receptor activation causes ROS generation (Ostenfeld et al., 2005). σ -1 Receptors are involved in oxidative stress mediated toxicities and have been shown to provide protective effects (Schetz et al., 2007). σ Receptors also play a regulatory role in the redox state of the cell, and can also modulate the nNOS and iNOS enzyme activity (Vagnerova et al., 2006; Tsai et al., 2009; Yang et al., 2010).

In addition to modulating the cellular redox system, σ receptors can also regulate other systems that are involved in ROS/RNS generation by METH. First, σ receptors can regulate glutamate/NMDA release which in turn causes nNOS activation and nitrosative stress (DeCoster et al., 1995). Second, σ receptors can modulate microglial activation through calcium dependent mechanisms, which can contribute to the release of inflammatory cytokines and ROS/RNS generation (Hall et al., 2009). Third, σ receptors can regulate the release of dopamine which in turn undergoes autooxidation and is a major cause of METH's ROS generation (Gonzalez-Alvear and Werling, 1994; Derbez et al., 2002).

Apart from the activation of cellular death cascades, METH also caused hyperthermia *in vivo*. In order to separate the contribution of METH, hyperthermia and their interaction, NG108-15 cells were treated with METH at 37 or 40 °C. The results demonstrated that by maintaining the cells at elevated temperature, an increase in cell death (apoptosis and necrosis) was observed. The type of cell death shifted from apoptotic to necrotic cell death. This is consistent with the effect observed with treatment with DTG, where beyond a certain level of stress cell death in NG108-15 cells may be predominantly necrotic. The combination of METH and elevated temperature synergistically enhanced cell death (especially necrosis).

This potentiation effect is most likely due to high temperature induced increase in DAT activity and enhanced ROS/RNS levels (Fleckenstein et al., 1997; Xie et al., 2000; Kiyatkin et al., 2007; Numachi et al., 2007; Sharma et al., 2007). The ability of SN79 to attenuate both the elevated temperature induced toxicity as well as the enhanced toxicity caused by interaction of METH and elevated temperature is consistent with the potential role of σ receptors in various cell stress and death mechanisms. σ Receptors, due to their properties of intracellular translocation, protein-protein interaction and chaperone activity can help normalize a disordered system (Tsai et al., 2009).

In summary, the data from both aims of the *in vitro* studies demonstrates that physiologically significant concentrations of METH can cause apoptosis in an *in vitro* system representative of a differentiated neuronal cell, which mechanistically involves dopamine release and induction of reactive oxygen/nitrogen radicals, activation of caspase-8, -9 and -3. Additionally, σ receptor antagonist, SN79, can attenuate METH-induced cell death and the causative neurotoxic cascade. σ Receptor antagonists can also attenuate the elevated temperature-induced toxicity as well as toxicity caused by the interaction of METH and elevated temperature, which can have important implications *in vivo*. The ability of σ receptor antagonists to intervene at multiple points and mechanisms in the neurotoxic cascade is consistent with the location of σ receptors in various subcellular organelles like the ER, mitochondria, lysosomes, cell membrane, and nucleus (McCann et al., 1994; Zeng et al., 2007) and its functional role as a chaperone, with subcellular translocation and participation in protein-protein interactions (Su et al., 2010). Future studies are needed to elucidate the individual contributions of the two subtypes of σ receptors, as better tools are developed.

5.5 Contributions: Nidhi Kaushal conducted all the studies in this chapter. Abigail Rosen helped with the data analysis for some of the apoptosis and necrosis assays.

CHAPTER 6

EVALUATION OF σ -2 RECEPTOR ANTAGONIST AGAINST METH- INDUCED NEUROTOXICITY *IN VITRO*

6.1 Introduction

Recent studies suggest that σ -1 receptors function as a chaperone modulating inter-organelle signaling (Su et al., 2010). In comparison, activation of σ -2 receptors in tumor cells causes cell death by increasing cellular levels of calcium, ceramide and sphingolipids (Bowen, 2000). This effect is inhibited by the administration of σ receptor antagonists confirming the role of σ -2 receptors in these effects (Vilner and Bowen, 2000; Crawford and Bowen, 2002; Crawford et al., 2002). From independent experiments, it has also been shown that σ receptor antagonists can attenuate METH's neurotoxicity (Matsumoto et al., 2008; Kaushal et al., 2011b; Seminerio et al., 2011). Taking all this into consideration, σ -2 receptors seem to play a more dominant role in the neurotoxic effects of METH.

Table 6.1 displays a series of putative selective sigma-2 ligands. CM398 possesses highest affinity and selectivity for sigma-2 receptors over sigma-1 receptors. *In vivo* functional assay suggests that CM398 is functioning as an antagonist. In this part of the study therefore, it was determined whether METH induced cytotoxicity can be attenuated by CM398 in differentiated NG108-15 cells. This will further help in demonstrating the role of σ -2 receptors in METH toxicity and also the various signaling pathways that will be involved.

Table 6.1 Binding affinity of compounds for σ -1 and σ -2 receptors

	σ -1 (Ki in nM)	σ -2 (Ki in nM)	Ratio (σ -1/ σ -2)
CM360	139.47 $\pm$ 3.51	1.26 $\pm$ 0.06	110.98
CM361	3834.33 $\pm$ 580.30	30.53 $\pm$ 13.79	125.59
NF12	273.5 $\pm$ 1.33	1.81 $\pm$ 0.019	151
CM781	724.6 $\pm$ 15.7	4.55 $\pm$ 0.21	159.12
CM397	86.41 $\pm$ 2.02	0.48 $\pm$ 0.02	181.28
SN230	3128.00 $\pm$ 70.89	14.70 $\pm$ 1.18	212.79
CM775	2274 $\pm$ 186.8	4.27 $\pm$ 0.29	532.55
CM777	752.4 $\pm$ 51.44	0.66 $\pm$ 0.01	1140
CM699	16.56 $\pm$ 1.09	0.014 $\pm$ 0.0003	1183
CM398	560.4 $\pm$ 8.72	0.43 $\pm$ 0.015	1303

6.2. Materials and Methods

6.2.1. *Cell line and culture*

6.2.1.1. Cell growth: The neuroblastoma x glioma, hybridoma cell line NG108-15 (Hamprecht et al., 1985) were obtained from ATCC (Rockville, MD). Cells were maintained in 25 cm² (T25) or 75 cm² (T75) culture flasks (Corning-Costar, Lowell, MA) in hi-glucose (4.5 g/L) DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum), penicillin, streptomycin, and HAT (hypoxanthine-aminopterin-thymidine) at 37°C in a humidified incubator in 5% CO₂.

6.2.1.2. Cell differentiation: Culture medium was replaced with differentiation medium (DMEM with 0.5% FBS, penicillin, streptomycin, HAT and 1% DMSO) and the cells allowed to grow for a further 3-4 days.

6.2.2. ***Hoechst and propidium iodide staining***: NG108-15 cells were grown and differentiated in 96-well culture plates and exposed to various concentrations of METH (0-1000 µM), CM398 (1-100 nM), CM398 (1-100 nM)+METH (0-1000 µM) for 24 hrs at 37 °C. Hoechst stain (20 µg/ml final concentration) and propidium iodide stain (20 µg/ml final concentration) were added to each well and incubated at room temperature for 10 min. Three images are taken/well using an UV light microscope. Cells with brightly stained and fragmented nuclei were counted as apoptotic and cells that were red were counted as necrotic. The percentage of apoptotic and necrotic cells were counted with the help of Image J cell counter (NIH). The sum of percentage of apoptotic and necrotic cells was referred to as "total cell death".

6.3. Results

6.3.1. CM398 and METH

Apoptosis: One-way ANOVA showed that METH treatment caused a significant increase in the percentage of apoptotic cells ($F(7,144) = 29.38$, $p < 0.0001$). Post-hoc Dunnett's tests confirmed that the following concentrations of METH caused a significant increase in the percentage of apoptotic cells: 10 μM ($q = 2.77$, $p < 0.05$), 30 μM ($q = 4.23$, $p < 0.01$), 100 μM ($q = 5.55$, $p < 0.01$), 300 μM ($q = 7.90$, $p < 0.01$) and 1000 μM ($q = 12.34$, $p < 0.01$). CM398 pretreatment significantly attenuated the apoptotic effects of METH (Figure 6.1A). Two-way ANOVA showed a significant effect of METH treatment ($F(7,519) = 46.77$, $p < 0.0001$), CM398 pretreatment ($F(3,519) = 26.15$, $p < 0.0001$), and CM398 pretreatment $\times$ METH treatment ($F(21,519) = 3.68$, $p < 0.0001$). Bonferroni's post-hoc tests showed that CM398 (1, 10 and 100 nM) pretreatment is able to attenuate the apoptotic effects of the following concentrations of METH: 300 μM ($t = 4.10$, $p < 0.001$; $t = 4.49$, $p < 0.0001$ and $t = 3.03$, $p < 0.05$ respectively) and 1000 μM ($t = 4.28$, $p < 0.001$; $t = 7.35$, $p < 0.001$ and $t = 8.90$, $p < 0.001$ respectively).

Necrosis: One-way ANOVA showed that METH treatment caused a significant increase in the percentage of necrotic cells ($F(7,143) = 4.76$, $p < 0.0001$). Post-hoc Dunnett's tests confirmed that 1000 μM METH caused a significant increase in the percentage of necrotic cells ($q = 5.50$, $p < 0.01$). CM398 pretreatment significantly attenuated the necrotic effects of METH (Figure 6.1B). Two-way ANOVA showed a significant effect of CM398 pretreatment ($F(3,508) = 10.82$, $p < 0.0001$), METH treatment ($F(7,508) = 12.95$, $p < 0.0001$). Bonferroni posttests confirmed that 1 nM CM398 attenuated the necrotic effects of 1000 μM METH ($t = 3.74$, $p < 0.01$).

Total cell death: One-way ANOVA showed that METH treatment caused a significant increase in the percentage of total cell death ($F(7,146) = 17.65$, $p < 0.0001$). Post-hoc

Dunnett's tests confirmed that the following concentrations of METH caused a significant increase in the percentage of total cell death: 10 μM ($q = 3.54$, $p < 0.01$), 30 μM ($q = 5.10$, $p < 0.01$), 100 μM ($q = 4.10$, $p < 0.01$), 300 μM ($q = 5.55$, $p < 0.01$) and 1000 μM ($q = 10.25$, $p < 0.01$). CM398 pretreatment significantly attenuated METH-induced cell death (Figure 6.1CB). Two-way ANOVA showed a significant effect of CM398 pretreatment ($F(3,512) = 30.88$, $p < 0.0001$), METH treatment ($F(7,512) = 43.05$, $p < 0.0001$) and CM398 pretreatment $\times$ METH treatment interaction ($F(21,512) = 1.96$, $p < 0.01$). Bonferroni posttests confirmed that CM398 (1, 10, 100 nM) attenuate cell death caused by the following concentrations of METH: 10 μM (1 nM: $t = 3.08$, $p < 0.05$; 10 nM: $t = 3.61$, $p < 0.01$; 100 nM: $t = 2.94$, $p < 0.05$), 30 μM (1 nM: $t = 4.83$, $p < 0.001$; 10 nM: $t = 3.84$, $p < 0.01$), 100 μM (100 nM: $t = 3.05$, $p < 0.05$), 300 μM (1 nM: $t = 4.08$, $p < 0.001$; 10 nM: $t = 3.73$, $p < 0.01$; 100 nM: $t = 3.49$, $p < 0.01$) and 1000 μM METH (1 nM: $t = 5.24$, $p < 0.001$; 10 nM: $t = 4.24$, $p < 0.001$; 100 nM: $t = 5.79$, $p < 0.001$).

Figure 6.1 Effect of CM398 pretreatment on METH-induced (A) apoptosis, (B) necrosis and (C) total cell death. CM398 protects against methamphetamine (METH)-induced apoptosis (A), necrosis (B) and total cell death (C). Differentiated NG108-15 cells were pretreated with CM398 (0-100 nM) prior to exposure to METH (0-1000 μ M) for 24 hr. After 24 hr, the wells were incubated with Hoechst 33342 and propidium iodide stains (20 μ g/ml each) to obtain percentages of apoptotic and necrotic cells. Total cell death = Apoptotic cell death + Necrotic cell death. Data represent means from two separate experiments ($n = 3/\text{experiment}$) $\pm$ SEM. Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$ (control vs. METH); # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ (METH alone vs. METH with CM398).

6.3.2. CM775 and METH

Apoptosis: One-way ANOVA showed that METH treatment caused a significant increase in the percentage of apoptotic cells ($F(7,78) = 12.38, p < 0.0001$). Post-hoc Dunnett's tests confirmed that the following concentrations of METH caused a significant increase in the percentage of apoptotic cells: 100 μM ($q = 3.58, p < 0.01$), 300 μM ($q = 3.35, p < 0.01$) and 1000 μM ($q = 8.46, p < 0.001$). CM775 pretreatment significantly attenuated the apoptotic effects of METH (Figure 6.2A). Two-way ANOVA showed a significant effect of METH treatment ($F(7,286) = 23.35, p < 0.0001$), CM775 pretreatment ($F(3,286) = 23.35, p < 0.0001$), and CM775 pretreatment $\times$ METH treatment ($F(21,286) = 3.89, p < 0.0001$). Bonferroni's post-hoc tests showed that CM775 (1, 10 and 100 nM) pretreatment is able to attenuate the apoptotic effects of the following concentrations of METH: 3 μM (10nM CM775; $t = 3.44, p < 0.05$), 100 μM ($t = 4.29, p < 0.001$; $t = 4.39, p < 0.001$ and $t = 3.94, p < 0.01$ respectively), 300 μM ($t = 3.85, p < 0.01$ and $t = 3.78, p < 0.01$ respectively) and 1000 μM ($t = 9.95, p < 0.0001$; $t = 5.61, p < 0.0001$ and $t = 9.32, p < 0.0001$ respectively).

Necrosis: One-way ANOVA showed that METH treatment caused a significant increase in the percentage of necrotic cells ($F(7,77) = 3.63, p < 0.005$). Post-hoc Dunnett's tests confirmed that 300 and 1000 μM METH caused a significant increase in the percentage of necrotic cells ($q = 3.00, p < 0.05$ and $q = 4.29, p < 0.001$ respectively). CM775 pretreatment did not significantly attenuate the necrotic effects of METH (Figure 6.2B).

Total cell death: One-way ANOVA showed that METH treatment caused a significant increase in the percentage of total cell death ($F(7,79) = 10.65, p < 0.0001$). Post-hoc Dunnett's tests confirmed that the following concentrations of METH caused a significant increase in the percentage of total cell death: 10 μM ($q = 3.58, p < 0.01$), 300 μM ($q = 3.71, p < 0.01$) and 1000 μM ($q = 7.71, p < 0.001$). CM775 pretreatment significantly attenuated METH-induced cell death (Figure 6.2C). Two-way ANOVA showed a significant effect of

CM775 pretreatment ($F(3,287) = 13.09$, $p < 0.0001$), METH treatment ($F(7,287) = 20.67$, $p < 0.0001$) and CM775 pretreatment $\times$ METH treatment interaction ($F(21,287) = 2.39$, $p < 0.001$). Bonferroni posttests confirmed that CM775 (1, 10, 100 nM) attenuate cell death caused by the following concentrations of METH: 10 μ M (1 nM: $t = 3.24$, $p < 0.05$; 10 nM: $t = 4.06$, $p < 0.01$) and 1000 μ M METH (1 nM: $t = 6.07$, $p < 0.0001$; 10 nM: $t = 5.20$, $p < 0.0001$; 100 nM: $t = 3.52$, $p < 0.05$).

Figure 6.2 Effect of CM775 pretreatment on METH-induced (A) apoptosis, (B) necrosis and (C) total cell death. CM775 protects against methamphetamine (METH)-induced apoptosis (A), necrosis (B) and total cell death (C). Differentiated NG108-15 cells were pretreated with CM775 (0-100 nM) prior to exposure to METH (0-1000 μM) for 24 hr. After 24 hr, the wells were incubated with Hoechst 33342 and propidium iodide stains (20 μg/ml each) to obtain percentages of apoptotic and necrotic cells. Total cell death = Apoptotic cell death + Necrotic cell death. Data represent means from one experiment (n = 3/experiment) ± SEM. Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (control vs. METH); # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ (METH alone vs. METH with CM775).

6.3.3. CM699 and METH

Apoptosis: METH treatment on its own caused a significant increase in apoptotic cell death in differentiated NG108-15 cells. One way ANOVA showed a significant effect of METH treatment ($F(6,67) = 14.43$, $p < 0.0001$). Dunnett's post-hoc tests confirmed that the following concentrations of METH cause a significant increase in apoptosis: 100 μM ($q = 3.60$, $p < 0.01$) and 1000 μM ($q = 8.41$, $p < 0.001$).

Figure 6.3A shows that CM699 pretreatment displayed a trend towards a shift in the dose response curve of METH-induced apoptosis towards the left; however, the effect was not significant.

Necrosis: METH treatment on its own caused a significant increase in necrotic cell death in differentiated NG108-15 cells. One way ANOVA showed a significant effect of METH treatment ($F(6,249) = 4.84$, $p < 0.0001$). Dunnett's post-hoc tests confirmed that METH 100 and 1000 μM caused a significant increase in necrosis ($q = 3.47$, $p < 0.01$ and $q = 8.75$, $p < 0.001$ respectively).

Figure 6.3B shows that CM699 pretreatment at intermediate concentrations shifts the dose response curve of METH towards the left. Two way ANOVA showed a significant effect of METH treatment ($F(6,242) = 23.23$, $p < 0.0001$) and CM699 pretreatment ($F(3,242) = 11.42$, $p < 0.0001$). Bonferroni's post-hoc tests confirm that the following concentrations of CM699 in combination with METH are significantly different than METH treatment alone: CM699 1 nM (0.1 μM : $t = 3.60$, $p < 0.01$ and 1 μM : $t = 4.14$, $p < 0.01$) and CM699 10 nM (0.1 μM : $t = 3.70$, $p < 0.01$; 1 μM : $t = 3.30$, $p < 0.05$).

Total cell death: METH treatment on its own caused a significant increase in cell death in differentiated NG108-15 cells. One way ANOVA showed a significant effect of METH treatment ($F(6,67) = 35.67$, $p < 0.0001$). Dunnett's post-hoc tests confirmed that the following concentrations of METH caused a significant increase in total cell death: 100 μM ($q = 4.81$, p

< 0.001) and 1000 μ M ($q = 11.78$, $p < 0.001$). Figure 6.3C shows that CM699 pretreatment at intermediate concentrations shifted the dose response curve of METH-induced cell death towards the left. Two way ANOVA showed a significant effect of METH treatment ($F(6,242) = 35.45$, $p < 0.0001$) and CM699 pretreatment ($F(3,242) = 10.38$, $p < 0.0001$). Bonferroni's post-hoc tests confirm that the following concentrations of CM699 in combination with METH are significantly different than METH treatment alone: CM699 1 nM (0.1 μ M: $t = 3.09$, $p < 0.05$ and 1 μ M: $t = 3.59$, $p < 0.01$) and CM699 10 nM (0.1 μ M: $t = 3.10$, $p < 0.05$ and 1 μ M: $t = 3.30$, $p < 0.05$).

Figure 6.3 Effect of CM699 pretreatment on METH-induced (A) apoptosis, (B) necrosis and (C) total cell death. NG108-15 cells were exposed to CM699 (1 -100 nM) and/or METH (0-1000 μM) for 24 hr. After 24 hr, the wells were incubated with Hoechst 33342 and propidium iodide stains (20 μg/ml each) to obtain percentages of apoptotic and necrotic cells. Total cell death = Apoptotic cell death + Necrotic cell death. Data represent means from one experiment (n = 3/experiment) ± SEM. Significant differences are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (control vs. METH); # $p < 0.05$, ## $p < 0.01$ (CM699+METH vs. METH).

6.4 Discussion

This study demonstrated that σ -2 receptors may be an important subtype for mediating the effects of METH. CM398 and CM775 attenuated predominantly the apoptotic effect of METH in differentiated NG108-15 cells which is consistent with reports in the literature suggesting activation of σ -2 receptors can activate apoptotic type of cell death (Bowen, 2000; Crawford and Bowen, 2002). In addition CM669 seems to shift the dose response curve of METH-induced cell death towards the left, indicating that σ -2 receptors are an important subtype through which METH mediates its effects. This also provides additional evidence that the leftward dose response shift observed with DTG in the previous chapter is most likely due to the engagement of σ -2 receptor subtype. Additionally, σ -2 Receptors may play a predominant role in this cell death pathway as a previous study using SK-N-SH neuroblastoma cells showed that activation of σ -2 receptors caused caspase-10 activation which in turn cleaved Bid thereby causing activation of mitochondrial death cascade (Wang and Bowen, 2006). Similar phenomenon was observed with METH in the previous chapter, whereby METH caused caspase-8 activation followed by mitochondrial death cascade activation in NG108-15 cells. Studies have also shown that activation of σ -2 receptors has been shown to cause elevation of ceramide levels in the cell which can be linked with caspase activation (Crawford et al., 2002; Wang and Bowen, 2006). Ceramide levels are also elevated by the activation of death receptors like Fas (Cifone et al., 1994; Tepper et al., 1995; Skowronski et al., 1996). Additionally, The localization of both the death receptors and σ -2 receptors in the cellular lipid raft compartment indicates potential regulation of the death receptor signaling by σ -2 receptors. In the previous chapter, activation of caspase-8 levels was observed after METH treatment. This could also involve METH interaction with σ -2 receptors which in turn may regulate the death receptors to cause caspase-8 activation.

A recent study has proposed σ -2 receptors to be a binding site in the PGRMC1 (progesterone receptor membrane component 1) complex (Xu et al., 2011). PGRMC1 like σ -2 receptors are highly expressed in tumor cells in comparison to normal cells (Bowen, 2000; Cahill, 2007). They both are studied as important cancer biomarkers and potential therapeutic targets for anticancer drugs (Bowen, 2000; Cahill, 2007; Mir et al., 2011). This study is very promising given the lack of molecular biology tools to study σ -2 receptors. The novel compounds used in this study are selective for σ -2 over σ -1 receptors. However, additional selectivity profile of those tested compounds over other non- σ sites may have to be determined in order to further establish the claim.

Additional studies may be performed using other σ -2 receptor agonists like CB-64D to observe dose response shifts in METH-induced cytotoxicity. In addition, further future studies may be required to fully elucidate and compare the potential involvement of σ -1 receptors in the neurotoxic effects of METH.

6.5 Contribution: Nidhi Kaushal conducted the apoptosis and necrosis assays. Cory Weaver helped with the data analysis of some of the experiments.

Radioligand binding assays were conducted by Dr. Yantong Xu and Michael Seminerio

CHAPTER 7

OVERALL CONCLUSIONS AND FUTURE STUDIES

7.1 Summary:

In summary, the studies conducted herewith have demonstrated that:

- σ Receptor antagonism (using AC927, CM156 and SN79) attenuated METH-induced striatal dopaminergic and serotonergic neurotoxicity *in vivo* (measured as depletion in dopamine and 5-HT levels and DAT and SERT expression levels). The presence of σ receptors on the dopaminergic and serotonergic neurons along with their role in cell death pathways explains these neuroprotective effects (Campbell et al., 1989; Bastianetto et al., 1995; Derbez et al., 2002; Bowen, 2000). Therefore, AC927, CM156 and SN79 may provide neuroprotection by acting through σ receptors and competitively or non-competitively intervening at the previously described (in Chapter 1) METH's downstream mechanisms (Kaushal and Matsumoto, 2011).
- σ Receptor antagonists attenuated METH-induced hyperthermia in mice. Mechanistically, dopaminergic and serotonergic systems are implicated in METH-induced body temperature alterations (Numachi et al., 2007). In addition, the production of ROS and release of cytokines like IL1 β have been implicated in the generation of METH's hyperthermia (Bowyer et al., 1994; Imam et al., 1999; Riedel and Maulik, 1999; Yamashita et al., 2000; Fukami et al., 2004). Moreover, σ Receptor ligands, with their ability to modulate these neurotransmitter systems, cytokines, as well as ROS, can attenuate elevations in body temperature (Campbell et al., 1989; Bastianetto et al., 1995; Derbez et al., 2002; Schetz et al., 2007; Matsumoto et al., 2008). Additionally, σ receptors are located in the hypothalamic brain region and have previously been shown to modulate body temperature (Gundlach et al., 1986; Rawls et al., 2002). A combination of all these factors may explain how AC927, CM156 and SN79 can prevent METH-induced hyperthermia.

- Striatal dopamine and serotonin levels in all the treatment groups correlated with their body temperature during the experiment. This indicates that the METH-induced neurotoxicity is related to the body temperature of the mice during treatment. In addition, the ability of AC927, CM156 and SN79 to attenuate the depletions in dopamine and 5-HT levels may be related to their ability to decrease the hyperthermic effects of METH. However, correlation does not always mean causation. As discussed earlier, sigma receptors may be able to attenuate the hyperthermic effects of METH through various mechanisms; they may also be able to prevent METH-induced neurotoxicity at a cellular level independent of temperature influence. However, the separation of these effects in an *in vivo* system may not be possible or very difficult to interpret.
- σ Receptor antagonism provided protection against METH-induced cell death (apoptosis and necrosis) in differentiated NG108-15 cells. Both AC927 and SN79 attenuated METH-induced cell death which is consistent with the role of σ receptors in cell death mechanisms (Bowen, 2000; Marrazzo et al., 2011).
- METH caused dopamine release, ROS/RNS generation, caspase-8, -9 and -3 activation in differentiated NG108 cells. Sigma receptor antagonism attenuated all the above mentioned METH-induced neurotoxic endpoints. Figure 7.1 depicts the time frame of METH-induced neurotoxic cascade activation observed using NG108-15 cells. The green boxes indicate the events that were blocked by SN79 and/or AC927. The ability of σ receptor antagonists to intervene at multiple points and mechanisms in the neurotoxic cascade is consistent with the location of σ receptors in various subcellular organelles like the ER, mitochondria, lysosomes, cell membrane, and nucleus (McCann et al., 1994; Zeng et al., 2007) and its functional role as a

chaperone, with subcellular translocation and participation in protein-protein interactions (Su et al., 2010).

Figure 7.1 Time course of METH-induced activation of neurotoxic cascades in differentiated NG108-15 cells

- Elevated temperature (40 °C) exacerbated METH-induced neurotoxicity. Sigma receptor antagonism (using SN79) attenuated the enhanced toxicity caused by the interaction of METH and elevated temperature. Figure 7.2 below depicts the enhancement of METH-induced cell death in differentiated NG108-15 cells which may also have implications in vivo. Green boxes indicate that SN79 was able to attenuate this enhanced toxicity caused by the combination of elevated temperature and METH. The combination of METH and elevated temperature synergistically enhanced cell death (especially necrosis). This potentiation effect is most likely due to high temperature induced increase in DAT activity and enhanced ROS/RNS levels (Fleckenstein et al., 1997; Xie et al., 2000; Kiyatkin et al., 2007; Numachi et al., 2007; Sharma et al., 2007). The ability of SN79 to attenuate both the elevated temperature induced toxicity as well as the enhanced toxicity caused by interaction of METH and elevated temperature is consistent with the potential role of σ receptors in various cell stress and death mechanisms. σ Receptors, due to their properties of intracellular translocation, protein-protein interaction and chaperone activity can help normalize a disordered system (Tsai et al., 2009).

Figure 7.2 Effect of elevated temperature on METH-induced cell death in differentiated NG108-15 cells

- σ -2 Receptor antagonism (using CM398 and CM775) is able to attenuate METH-induced cell death in differentiated NG108-15 cells, indicating an important role of σ -2 receptors in METH mediated toxicity. σ -2 Receptor subtype has previously been shown to play an important role in cell death pathways in tumor cells (Bowen, 2000), further indicating a greater significance of this subtype in METH's effects. Further studies may be needed to compare the role of the two σ receptor subtypes in METH-induced neurotoxicity.

Overall, the ability of σ receptor antagonists in attenuating multiple end points in METH-induced toxicity may be due to its presence in multiple organelles like ER, mitochondria, lysosomes, nucleus, plasma membrane, and ability to undergo intracellular translocation, protein-protein interaction and chaperone activity which can help normalize a disordered system (Tsai et al., 2009). Alternately, the protective effects may be due to inhibition of early mediators like dopamine release or ROS/RNS generation. In order to determine this, additional posttreatment studies may have to be conducted whereby σ receptor antagonist will be administered at various time points after METH administration.

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EDUCATION

West Virginia University (2008- May 2012), Pharmaceutical and Pharmacological Sciences [GPA: 4/4]

University of Mississippi (2006-2008), MS, Pharmacology and Toxicology [GPA: 3.96/4]

University of Delhi (2002-2006), BS, Pharmacy [I Division]

RESEARCH AND TEACHING EXPERIENCE

West Virginia University

- Research assistant (Since June 2008): Screened and evaluated the neuroprotective effects of novel sigma receptor antagonists against methamphetamine-induced neurotoxicity in mice and NG108 cells
- Teaching assistant (Fall 2008): PHAR 792 - Introduction to Research elective course for Pharm.D. students

University of Mississippi

- Research assistant (2006-2008): Screened and evaluated novel sigma receptor compounds against behavioral effects of cocaine in rodents
- Teaching assistant and PBL facilitator (2006-2007): PHCL 341, 342 - Human Physiology & Pathophysiology required course and lab for Pharm.D. students

University of Delhi

- Undergraduate research assistant (2004-2006): Isolated and characterized chemical compounds extracted from medicinal plant (Swertia chirata)

OTHER RESEARCH TRAINING

- Dr. Wayne D. Bowen's lab at Brown University, Providence, RI (Summer 2010): Summer research trainee - Granted Stitzel award from West Virginia University for career development training in molecular biology techniques
- Tanuk Pharma and KARE Labs, India (2005): Trained in production and quality control of pharmaceutical drugs

HONORS AND AWARDS

- West Virginia University School of Pharmacy Graduate Student Research Award, 2011
- Behavioral and Biomedical Sciences Training Scholarship, 2010, 2011
- Robert E. Stitzel Graduate Student Award, 2009
- First place, E.J. Van Liere Research Day poster competition, 2009
- Travel Award from the American Society of Pharmacology and Experimental Therapeutics (ASPET) to attend Experimental Biology annual meeting, New Orleans, LA, 2009
- Travel Award from the NIH and the University of Texas San Antonio to attend the Behavior, Biology and Chemistry: Translational Research in Addiction meeting, San Antonio, TX, 2009
- Travel Award from the WVU School of Pharmacy to attend the International Drug Abuse Research Society meeting, Seoul, South Korea, 2009; Experimental Biology meeting, New Orleans, LA, 2009; Society for Neuroscience meeting, San Diego, CA, 2010

- Natural Products Neuroscience Fellowship, University of Mississippi, 2007
- Honorable Mention, graduate student poster presentation, South Central Society for Toxicology meeting, 2007

MANUSCRIPTS PUBLISHED

- **Kaushal N**, Elliott M, Robson MJ, Iyer A, Rojanasakul Y, Coop A, Matsumoto RR (2012) AC927, a σ ligand, blocks the release of dopamine and generation of reactive oxygen species by methamphetamine in NG108 cells. *Mol Pharmacol* 81(3): 299-308.
- **Kaushal N**, Seminerio MJ, Shaikh J, Medina MA, Mésangeau C, McCurdy CR, Matsumoto RR (2011) CM156, A high affinity sigma receptor ligand attenuates the stimulant and neurotoxic effects of methamphetamine in mice. *Neuropharmacology* 61(5-6): 992-1000.
- **Kaushal N**, Robson MJ, Vinnakota H, Narayanan S, Avery BA, McCurdy CR, Matsumoto RR (2011) Synthesis and pharmacological evaluation of SN79, a putative sigma receptor antagonist against cocaine-induced behaviors in mice. *AAPS Journal* 13(3): 336-346.
- Review: **Kaushal N**, Matsumoto RR (2011) Role of sigma receptors in methamphetamine-induced neurotoxicity. *Curr Neuropharmacol* 9(1): 54-57.
- Seminerio MJ, **Kaushal N**, Shaikh J, Huber JD, Coop A, Matsumoto RR (2010) Sigma receptor ligand AC927 attenuates methamphetamine-induced hyperthermia and serotonin damage in mice. *Pharmacol Biochem Behav* 98(1): 12-20.
- Xu Y, **Kaushal N**, Shaikh J, Wilson LL, Mésangeau C, McCurdy CR, Matsumoto RR (2010) A novel substituted piperazine, CM156, attenuates the stimulant and toxic effects of cocaine in mice. *JPET* 333(2): 491-500.
- Mésangeau C, Narayanan S, Green AM, Shaikh J, **Kaushal N**, Viard E, Xu Y, Fishback JA, Poupaert JH, Matsumoto RR, McCurdy CR (2008) Conversion of a highly selective sigma-1 receptor ligand to sigma-2 receptor preferring ligands with anti-cocaine activity. *J Med Chem* 51(5): 1482-1486.
- Review: Sharma N, Minocha S, **Kaushal N**, Chawla A, Minocha M, Sharma Y (2008) *Butea frondosa* [flame of the forest]: A review. *Hamdard Medicus* 51(2): 46-50.

MANUSCRIPTS UNDER REVIEW AND IN PREPARATION

- Seminerio MJ, Hansen R, **Kaushal N**, Zhang H, McCurdy CR, Matsumoto RR. The evaluation of AZ66, an optimized sigma receptor ligand, against methamphetamine-induced dopaminergic neurotoxicity and memory impairment in mice. (Submitted to *The International Journal of Neuropsychopharmacology*).
- **Kaushal N**, Seminerio MJ, McCurdy CR, Matsumoto RR. Pharmacological evaluation of SN79, a sigma receptor ligand against methamphetamine-induced neurotoxicity in mice. (In preparation for *JPET*).
- **Kaushal N**, McCurdy CR, Matsumoto RR. SN79 attenuates methamphetamine-induced apoptosis and necrosis in NG108 cells: Role of sigma receptors. (In preparation for *JPET*).

PRESENTATIONS

Platform Presentations:

- AC927, a selective sigma receptor ligand, blocks the generation of reactive oxygen species induced by methamphetamine in NG108-15 cells. Society for Neuroscience 2010, San Diego, CA.
- SN79, a novel sigma-2 receptor antagonist, attenuates methamphetamine-induced neurotoxicity. E.J. Van Liere Annual Convocation 2010, Morgantown, WV.
- CM156, a novel sigma receptor antagonist, mitigates stimulant and neurotoxic effects of methamphetamine in mice. 2nd Annual International Drug Abuse Research Society Meeting 2009, Seoul, South Korea.

Poster Presentations and Other Abstracts at National and International Meetings:

- **Kaushal N**, McCurdy CR, Matsumoto RR. SN79 attenuates the neurotoxic effects of methamphetamine: in vivo and in vitro studies. Society for Neuroscience 2011, Washington, DC.
- **Kaushal N**, McCurdy CR, Matsumoto RR. Attenuation of methamphetamine-induced neurotoxicity by SN79: Involvement of sigma-2 receptors. Society for Neuroscience 2010, San Diego, CA.
- **Kaushal N**, McCurdy CR, Matsumoto RR. Attenuation of psychostimulant effects by SN79, a selective sigma-2 receptor ligand: implications for medication development for cocaine and methamphetamine abuse. 2nd Annual International Drug Abuse Research Society Meeting 2009, Seoul, South Korea.
- **Kaushal N**, Seminerio MJ, Shaikh J, Medina MA, Mésangeau C, McCurdy CR, Matsumoto RR. CM156, a novel sigma receptor antagonist, mitigates stimulant and neurotoxic effects of methamphetamine in mice. Experimental Biology 2009, New Orleans, LA.
- **Kaushal N**, Croom C, Shaikh J, Narayanan S, Mésangeau C, Poupaert JH, McCurdy CR, Matsumoto RR. SN79, a novel sigma-2 receptor antagonist, attenuates cocaine induced behaviors in mice. Behavior, Biology and Chemistry: Translational Research in Addiction 2009, San Antonio, TX.
- **Kaushal N**, Seminerio MJ, Shaikh J, Medina MA, Mésangeau C, McCurdy CR, Matsumoto RR. CM156, a novel sigma receptor antagonist, attenuates the neurotoxic effects of methamphetamine in mice," Behavior, Biology and Chemistry: Translational Research in Addiction 2009, San Antonio, TX.
- Seminerio MJ, **Kaushal N**, Shaikh J, Medina M, McCurdy CR, Coop A, Matsumoto RR. Sigma antagonists, AC927 and CM156, protect against methamphetamine-induced serotonergic neurotoxicity while attenuating hyperthermia. 71st Annual College on Problems of Drug Dependence 2009, Reno, NV.
- **Kaushal N**, Shaikh J, Croom C, Mésangeau C, Narayanan S, Poupaert J, McCurdy CR, Matsumoto RR. Novel substituted piperazines with high affinity and selectivity for sigma receptors attenuate cocaine-induced behaviors in mice: role for sigma-1 and sigma-2 subtypes. College on Problems of Drug Dependence 2008, San Juan, Puerto Rico.
- **Kaushal N**, Croom C, Shaikh J, Narayanan S, Mésangeau C, Poupaert JH, McCurdy CR, Matsumoto RR. SN79, A novel sigma-2 receptor antagonist, attenuates cocaine induced behaviors in mice. Experimental Biology 2008, San Diego, CA.
- McCurdy CR, Mésangeau C, Narayanan S, Shaikh J, **Kaushal N**, Matsumoto RR, Poupaert JH. Discovery of a selective sigma-2 receptor ligand and the potential involvement of sigma-2 receptors in cocaine toxicity. 70th Annual Meeting of the College on Problems of Drug Dependence 2008, San Juan, Puerto Rico.

- Xu Y, Shaikh J, Viard E, Fishback J, **Kaushal N**, Mésangeau C, Narayanan S, Poupaert JH, McCurdy CR, Matsumoto RR. Synthesis and evaluation of subtype selective sigma receptor ligands from substituted 2(3*H*)-Benzoxazolone and 2(3*H*)-Benzothiazolone derivatives. South Central Society for Toxicology 2007, Oxford, MS.
- **Kaushal N**, Croom C, Shaikh J, Narayanan S, Mésangeau C, Poupaert JH, McCurdy CR, Matsumoto RR. SN79, a novel sigma-2 receptor antagonist, attenuates cocaine induced behaviors in mice. South Central Society for Toxicology 2007, Oxford, MS.
- Matsumoto RR, Wilson LL, Mésangeau C, Shaikh J, **Kaushal N**, Poupaert JH, McCurdy CR. CM156, a novel substituted piperazine, attenuates the behavioural effects of cocaine in mice. South Central Society for Toxicology 2007, Oxford, MS.

SOCIETY MEMBERSHIPS

- Society for Neuroscience (SfN)
- American Association of Pharmaceutical Sciences (AAPS)
- International Drug Abuse Research Society (IDARS)
- American Society of Pharmacology and Experimental Therapeutics (ASPET)
- American Scientists of Indian Origin Association (ASIOA)
- Indian Pharmaceutical Graduate Association (IPGA)

SERVICE

- Mentored undergraduate research students:
 - Ms. Abigail Rosen (West Virginia University, Undergraduate honor's thesis student), 2011
 - Mr. Mark A. Medina (West Virginia University, Pharmacy research student), Summer 2008
 - Ms. Caroline Croom (University of Mississippi, Natural Products Neuroscience Summer Research Program), Summer 2007
- Served as Senator (Department of Pharmacology), Graduate Student Council, University of Mississippi, 2007
- Participated in the polio campaign organized by the Government of India, 2005
- Participated in the blood donation camp organized by the Rotary Blood Bank, India, 2005
- Participated in social service on the Indian Railway Service Day as a member of the Bharat Scouts and Guides, 1996