The Role of Striatal Neuropeptides on Glutamate and Methamphetamine-Induced Neurotoxicity in the Murine Brain

By

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ABSTRACT

THE ROLE OF STRIATAL NEUROPEPTIDES ON GLUTAMATE AND METHAMPHETAMINE-INDUCED NEUROTOXICITY IN THE MURINE BRAIN

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Lauriaselle Afanador

Adviser: Dr. Jesus A. Angulo

The rising worldwide epidemic in addiction to methamphetamine (METH) and the well-documented neurological detriments it causes emphasizes the importance of elucidating the mechanisms by which METH causes widespread and prolonged damage. Also, METH's pathophysiology resembles a number of neurodegenerative diseases. Therefore a better understanding of the mechanisms involved would provide more effective therapeutic targets for the treatment of these neurological disorders.

METH toxicity is a complex interplay of various factors however a number of necessary components have been identified such as dopamine overflow (DA), glutamate signaling, and oxidative stress. Although METH-induced DA overflow is the initiating event, it is not the direct cause of damage. Oxidative stress is thought to be the mediator of METH toxicity and nitric oxide (NO) as a contributor.

We have found that substance P (SP) exacerbates METH-induced NO. Inhibition of SP signaling mitigated NO synthesis and conferred protection. Considering the role SP is playing in

METH toxicity we wanted to investigate the role that other striatal neuropeptides play in these events, notably the inhibitory peptides neuropeptide Y (NPY) and somatostatin (SST).

We hypothesized that SP is augmenting NMDA signaling and thus magnifying NO production. Whereas NPY and SST would serve as a counteracting force thus dampening oxidative stress and conferring protection. Overall, our data demonstrated that SP does augment NMDA signaling as inhibition of the neurokinin-1 receptor (NK-1R) decreased NMDA-induced striatal cell loss. We found that SP was potentiating NMDA-induced NO production. Although the predominant source of NO was the inducible form of nitric oxide synthase (NOS).

In support of our hypothesis, NPY and SST proved to attenuate NO. Also, they were protective from METH-induced cell death although SST failed to protect DA terminals. However, an agonist for the NPY-Y2 receptor was successful in maintaining DA terminal viability. Of interest is that neither NPY nor SST modulated NMDA-induced NO or cell loss suggesting that their protective mechanism does not include modulation glutamate signaling within the striatum.

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This doctoral dissertation at the surface encapsulates a scientific project and the methodology necessary to complete it however below that it serves as an unspoken testament to a valuable life journey rife with indispensable lessons. More importantly it is a physical manifestation of all the amazing people that played various although necessary roles so that this document could come to fruition. First of which is my mother Mercedes Delgado, to whom I am eternally grateful. She has always claimed not to ever understand one thing about the work I do but has lovingly always been there with me every single step of the way, thank you mom. Also, to my brother Angel Rodriguez who makes a similar claim as my mom but has always let me know how proud he is and that he is there.

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TABLE OF CONTENTS

LIST OF FIGURES

Number ***Page***

LIST OF ABBREVIATIONS

3-NT: 3-Nitrotyrosine

5-HT: Serotonin

7-NI: 7-Nitroindazole

aCSF: Artificial cerebrospinal fluid

CaM: Calmodulin

cGMP: Cyclic guanosine monophosphate

chAT: Choline acetyltransferase

CHO: Chinese hamster ovary

CNS : Central nervous system

CuZnSOD: Copper-zinc superoxide dismutase

Cy3: Cyanine 3

DA: Dopamine

D_1: Dopamine 1 receptor

D_2: Dopamine 2 receptor

DAPI: 4',6-diamidino-2-phenylindole

DAT: Dopamine transporter

eNOS: Endothelial nitric oxide synthase

ERK: Extracellular signal-regulated kinase

GABA: Gamma-aminobutyric Acid

GIRK: G-protein-activated inwardly rectifying K^+

GPCR: G-Protein coupled receptor

iNOS: Inducible nitric oxide synthase

IP: Intraperitoneal

K^+: Potassium

MDMA: 3,4-methylenedioxymethamphetamine

METH: Methamphetamine

MnSOD: Manganese superoxide dismutase

NK-1R: Neurokinin-1 receptor

NMDA: N-methyl-D-aspartate

nNOS: Neuronal nitric oxide synthase

NOS: Nitric oxide synthase

NO: Nitric oxide

NPY: Neuropeptide Y

NPY-Y1R/Y1: Neuropeptide Y-Y1 receptor

NPY-Y2R/Y2: Neuropeptide Y-Y2 receptor

NPY-Y5R/Y5: Neuropeptide Y-Y5 receptor

OCT: Octreotide

PARP: Poly (ADP-ribose) polymerase

PBS: Phosphate buffered saline

ROS: Reactive oxygen species

RNS: Reactive nitrogen species

SP: Substance P

SST: Somatostatin

TH: Tyrosine hydroxylase

VMAT-2: Vesicular monoamine transporter-2

Chapter 1 Introduction

1.1. About Methamphetamine

1.1.1. History, Pharmacology, and Addictive Properties

Methamphetamine (METH) is a member of the amphetamine class of stimulants. It is a longer lasting, more potent derivative with multiple forms (powder, tablet, paste) and routes of administration. Depending upon the route of administration its mean elimination half-life can range from 10.1 hours after oral consumption to 12.2 hours for intravenous (Logan, 2002). First developed in the early 1900's, it was sold as an over-the-counter nasal decongestant and several other licit uses until the 1970's when it was deemed to be limited in its therapeutic potential (United Nations Office on Drugs and Crime, 2010). After which it became a controlled substance although its demand continued to grow as did its illicit use and manufacture. Synthetic stimulants such as METH hold an appeal to drug organizations due to several factors such as the ease of acquiring the base ingredients or adequate substitutes, the potential of a high profit margin, and the advantages of a highly mobile operation.

According to the report from the United Nations Office on Drugs and Crime, METH is considered a rising worldwide epidemic with a growing consumer market in regions such as in South Africa, South-East Asia and East Asia (United Nations Office on Drugs and Crime, 2010). In Thailand from 2004 to 2008 there was a fourfold increase in METH related arrests as well as a 50% increase in the seizure of its tablet form; both are indicators of its increasing availability and consumption (United Nations Office on Drugs and Crime, 2010).

METH is a psychostimulant that at first creates an intense sense of euphoria, exhilaration, increased mental alertness, and improved physical capacity (Logan, 2002; Krasnova and Cadet, 2009). However, its long-term usage can lead to addiction, paranoia, growing anxiety, lack of concentration, and delusion (Logan, 2002; Cadet et al., 2003). An alarming association with METH usage is the incidence of violent behavior including homicide and also suicide (Logan, 2002; Cadet et al., 2003). METH exerts its effects on the central nervous system (CNS) through its influence on the monoaminergic system, specifically on the dopaminergic and serotonergic pathways. Its effectiveness and potency are governed by the resemblance of its chemical structure to the endogenous transmitter dopamine (Logan, 2002; Krasnova and Cadet, 2009). Allowing it to easily enter the DA terminal, cause the release of excessive amounts of DA, and prevent its subsequent uptake.

Decades of research have proven that METH is neurotoxic. In addition, METH's resulting pathology in the regions of the brain it affects resembles a number of neurodegenerative diseases such as Parkinson and Huntington's (Villemagne et al., 1998; Cadet et al., 2003). One such area under intense scrutiny is the striatum; progressive loss of dopaminergic innervation from the nigrostriatal pathway to the striatum is the hallmark of Parkinson's disease (Block et al., 2006; Liu, 2006). In long-term users of METH there are indicators that a similar although much less prevalent loss may be occurring, leading to the concern that chronic METH users may eventually become vulnerable to developing Parkinson's like symptoms (Villemagne et al., 1998; Volkow et al., 2001a; Volkow et al., 2001c). Thus a more thorough understanding of the mechanisms involved in METH neurotoxicity could prove invaluable to the development of effective treatments for these neurological disorders.

1.1.2. Striatum

The basal ganglia is a cohesive functional unit composed of a collection of nuclei situated at the base of the forebrain (Cicchetti et al., 2000). The largest of these nuclei is the striatum, which receives inputs from several areas of the brain among them the cerebral cortex and the thalamus (Kawagushi et al., 1995; Tepper and Bolam, 2004). It also projects to and is innervated by several of the other nuclei that form the basal ganglia (Cicchetti et al., 2000). Multiple functions have been attributed to the striatum such as voluntary motor control, learning, motivation, habit-forming, and addictive behaviors (Kawagushi, 1997; Koob and Le Moal, 1997; Balleine et al., 2007).

Striatal neurons are classified into two broad categories, the medium spiny projection neurons and the aspiny interneurons (Cicchetti et al., 2000). The projection neurons are the most abundant neuronal type, comprising greater than 90% of the neuronal population and all contain gamma-aminobutyric acid (GABA) as their main transmitter (Kawagushi et al, 1995).They are the main output of the striatum and receive the greater part of the synaptic inputs (Kawagushi, 1997). Projection neurons are further subdivided and characterized by their compliment of neuropeptides and their afferent pathway. The direct pathway or striatonigral projection neurons contain the neuropeptides SP and dynorphin whereas neurons of the indirect pathway (striatopallidal) contain enkephalin (Cicchetti et al., 2000).

The aspiny interneurons of which there are four identified subtypes, may comprise a small portion of the striatum but they have been attributed with modulating the firing pattern of the projection neurons and thus the output of the striatum (Tepper and Bolam, 2004). The largest in size of this group are the cholinergic neurons, so named due to the presence of choline

acetyltransferase (ChAT). The remaining three subtypes are GABAergic; they are the parvalbumin, calretinin, and the SST/NPY/neuronal NOS (nNOS) (Cicchetti et al., 2000; Tepper and Bolam, 2004). Throughout we will be paying particular attention to the SST/NPY/nNOS interneuron since it contains many of the substrates such as the peptides (SST & NPY), receptors [NK-1R & N-methyl-D-Aspartate (NMDA)], and enzyme (nNOS) that play prominent roles in our hypothesis as well as METH-toxicity.

Among the major inputs to the striatum are the dopaminergic afferent fibers of the nigrostriatal tract, which originate from the substantia nigra pars compacta (Kawagushi et al., 1995). In addition, there are the glutamatergic inputs (corticostriatal) from the cortex (Kawagushi et al., 1995). Mentioned previously are the major efferent pathways from the striatum, the direct and indirect pathway. The direct pathway is a shorter loop in which the projection neurons extend their axons to the substantia nigra/internal pallidum and through GABA remove the substantia nigra's inhibition on the thalamus. Disinhibition of the thalamus results in stimulation of the motor cortex via glutamate release. The indirect pathway has the opposite effect but through a more involved loop which goes through the external pallidus, subthalamic nucleus, and finally the substantia nigra/internal pallidum with the sum effect of maintaining the internal pallidum's inhibition on the thalamus. The dopaminergic and glutamatergic inputs supply two of the major transmitters that are necessary components of METH neurotoxicity. Furthermore, sustained activation of the direct pathway is considered the means by which the excitotoxic levels of glutamate are released into the striatum during METH.

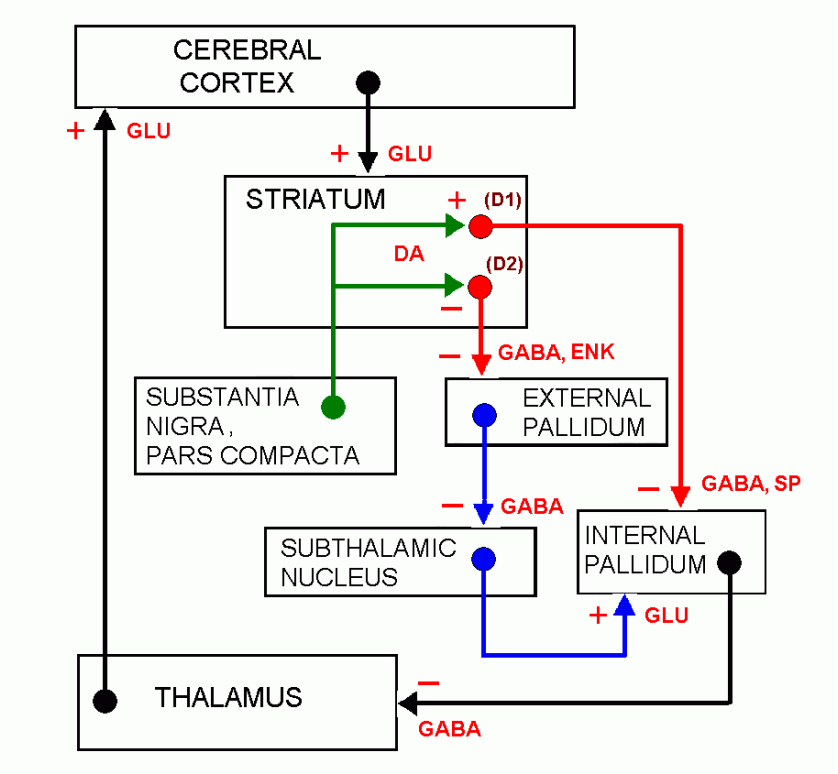


Figure 1-1: Direct and indirect pathways in the basal ganglia. The direct pathway begins in the striatum and is a simple loop represented by the red arrow that connects with the internal pallidum. The indirect pathway is polysynaptic and is represented by a combination of red and blue arrows that eventually synapse on the internal pallidum as well. (Adapted from Kiernan, 2007)

1.2. Methamphetamine-Induced Neurotoxicity

1.2.1. What Do We Know So Far: Hallmarks and Contributing Factors

Formative studies by Fibiger and McGeer (1971) unveiled the deleterious effects of METH on the dopaminergic system and in particular the nigrostriatal pathway. It was soon followed by Hotchkiss and Gibb's (1980) uncovering of METH's damaging effects on the serotonergic system in the hippocampus and the neostriatum. These influential studies emphasized the urgent need to conduct more thorough investigations into the neurotoxic capacity of METH and its mode of action. Decades of research into METH have revealed a plethora of global neurochemical dysfunctions primarily in the monoaminergic systems including but not limited to the prolonged decrease in the enzymes TH, tryptophan hydroxylase (Hotchkiss and Gibb, 1980), tissue DA and serotonin levels as well as their associated metabolites (Fumagalli et al., 1998). In addition, there is a reduction in dopamine transporters (DAT) attributed to membrane degeneration of striatal DA terminals (Villemagne et al., 1998) and evidence of cell death in multiple regions of the brain including the striatum and the cortex (Pu et al., 1996; Eisch and Marshall, 1998; Deng et al., 2001).

These markers of METH-induced toxicity are seen in various species including mice (Fumagalli et al., 1998; Zhu et al., 2005), rats (O'Dell, 1992; Eisch and Marshall, 1998), and nonhuman primates (Villegmane et al., 1998). In a study utilizing PET scanning and the DAT ligand [11C]WIN-35,428, baboons were treated with dosages of METH comparable to those used by human addicts (Villegmane et al., 1998). Imaging revealed a dose-dependent decrease in DAT binding, which was further confirmed in postmortem neurochemical analysis. There was a concomitant decline in the caudate levels of DA and 5-HT axonal markers such as DA, DOPAC,

5-HT, and 5-HIAA. Work conducted in animal models and the subsequent overwhelming evidence of the toxic nature of METH has proven relevant to human METH users as corroborated by a number of human in vivo imaging studies. For example, Ernst and colleagues (2000) found that abstinent METH users showed signs of abnormal brain chemistry even 21 months after discontinuing drug use. 1H MRS imaging revealed a decline in a mature neuronal marker suggesting cell loss in the frontal lobe and the basal ganglia in addition to elevated levels of metabolites associated with glia, which may indicate a response to neuronal injury (Ernst et al., 2000).

Persistent deficits are further substantiated in PET studies, revealing a significant reduction in striatal DAT binding in detoxified METH users (McCann et al., 1998; Volkow et al., 2001a). Furthermore, the decline in DAT density was correlated with impaired motor and cognitive function (Volkow et al. 2001a). Work by this same group demonstrated a prolonged reduction in the metabolic levels of the striatum and the thalamus as measured by glucose utilization (Volkow et al., 2001b). Although there was an increase in metabolism seen in the parietal cortex, however one possible explanation is increased activity as a result of an inflammatory response as seen in many animal studies and implicated by Ernst et al. (2000). Of note is that with protracted abstinence from METH abuse there appeared to be an increase in striatal DAT levels (Volkow et al., 2001c) as well as recovery in thalamic metabolism whereas striatal metabolism still remained depressed (Volkow et al., 2004). Recovery in DAT levels may be indicative that striatal DA terminals have remained intact after exposure to METH and thus the subsequent reductions in DAT may be a downregulation in expression. In light of the continual reduction in striatal metabolism (Volkow et al., 2004) and indication of diminished

neuronal content in the striatum up to 36 months of abstinence (Ernst et al., 2000), the increased DAT levels as measured by radioligands binding may be due to an adaptive process of the remaining dopaminergic terminals rather than DA terminal recovery.

Although the myriad toxic effects of METH as delineated above have been for the most part identified, the intricate mechanisms by which METH causes such far-reaching damage are still elusive. Thus far METH's mode of action has been pieced together sufficiently to illuminate many of the main characters in this complex interplay. At the forefront is the excessive release of DA into the extracellular space, which is a necessary component for toxicity (O'Dell et al., 1992; Yamamoto et al., 1998). It accomplishes this by disrupting vesicular monoamine transporter-2 (VMAT-2) function thus increasing the availability of DA in the cytoplasmic compartment as well as reversing the direction of the DAT therefore dumping the now readily available DA into the synapse (Sulzer et al., 1995; Jones et al., 1998; Fumagelli et al., 1998; Riddle et al., 2006). In addition to striatal DA overflow, METH is also known to cause an overflow in glutamate, which can be diminished with DA antagonists (Stephans and Yamamoto, 1994; Yamamoto and Zhu, 1998). It is postulated that the generation of reactive oxygen species (ROS) from NMDA-mediated excitotoxicity and oxidation of excessive cytoplasmic DA may serve as the mediator of damage in METH neurotoxicity through oxidative stress (Yamamoto and Zhu, 1998).

1.2.2. Glutamate and METH Toxicity

Both DA and glutamate transmission are necessary components for METH-induced toxicity to occur (Sonsalla et al., 1989; Stephans and Yamamoto, 1994). Glutamate has been implicated in potentiating METH's effect on striatal DA release (O'Dell et al., 1992).

Pretreatment with MK-801, an NMDA receptor antagonist, decreased DA overflow and damage to dopaminergic terminals. METH has been shown to raise the extracellular levels of glutamate in the striatum (Sonsalla et al., 1991; Stephans and Yamamoto, 1994). The magnitude of glutamate release is proportional to the amount of METH-induced damage exhibited. Regions of the brain in which METH induced significant DA release but not of glutamate showed marked reduction in neuronal damage such as in the prefrontal cortex and the nucleus accumbens (Eisch et al., 1996; Yamamoto et al., 1998). In response to increased striatal glutamate signaling there appears to be a compensatory downregulation in striatal NMDA receptors within 1 week of exposure to METH (Eisch et al., 1996). Reduced toxicity due to pharmacological inhibition of the NMDA receptor enforces that glutamate plays an intrinsic role in METH-induced toxicity (Sonsalla et al., 1990; O'Dell et al, 1992; Riddle et al., 2006). Also intrastriatal infusion of NMDA augmented a subtoxic dose of METH reducing DA content by up to 54% relative to control (Sonsalla et al., 1990).

Multiple lines of research have indicated that glutamate may contribute to METH toxicity through the generation of harmful oxidants (Yamamoto et al., 1998). Oxidants appear to serve as mediators of damage in METH toxicity since the introduction of various radical scavengers prior to METH attenuated the depletion of DA content (Yamamoto and Zhu, 1998; Kawasaki et al., 2006). Also NMDA receptor mediated increases in intracellular calcium influx has been linked to the activation of multiple cascades whose possible end products are ROS (Yamamoto and Zhu, 1998). One such pathway garnering intensive scrutiny is involved in NO synthesis through the enzyme nNOS (Dawson and Dawson, 1996). Cortical neuronal cultures from nNOS null mice had marked resistance to NMDA-induced cell loss, which was completely reversed by the

addition of NO donors (Dawson et al., 1996). In mice, the pharmacological inhibition or complete suppression of nNOS through gene manipulation was also protective against NMDA mediated excitotoxicity in the striatum (Schulz et al., 1995; Ayata et al., 1997). There were significant reductions in striatal lesion volume as well as cell death. Excessive NO synthesis can result in the formation of a number of toxic oxidants (Dawson and Dawson, 1996). Is METH activating the NMDA/NO cascade leading to oxidative stress?

1.2.3. Oxidative Stress

METH promotes a pro-oxidant state as seen by the in vitro and in vivo increases in indicators of ROS formation (Yamamoto and Zhu, 1998). The contribution of ROS formation to METH-induced toxicity has been implied in a study utilizing transgenic mice that overexpress the antioxidant copper-zinc superoxide dismutase (CuZnSOD) (Cadet et al., 1994; Hirata et al., 1996). Homozygous mice, with enzyme expression levels 5.7-fold relative to wildtype, only had a 2% reduction in DAT compared to 48% in the wildtype (Hirata et al., 1996). Furthermore, auto-oxidation of excessive cytosolic DA produces superoxide radicals and has been suggested as causing METH-induced DA terminal degradation (Yamamoto and Zhu, 1998). This is further substantiated in mice that have compromised vesicular packaging consequently possessing elevated levels of cytoplasmic DA. Mice heterozygous for VMAT-2 showed increased levels of METH toxicity to striatal DA terminals (Fumagelli et a., 1999). In addition, the diffusible signaling molecule NO can also react with DA's oxidative products to form a potent toxin (Cadet and Brannon, 1998).

During pathophysiological conditions NO can function in a neurotoxic capacity (Dawson and Dawson, 1996). One manner is through its formation of the oxidant peroxynitrite, which can serve as both a reactive nitrogen and oxygen species (Beckman, 1996; Boje, 2004; Bruckdorfer, 2005). During a pathological state such as excitotoxicity, an abundant level of NO can be manufactured to successfully outcompete the endogenous antioxidant CuZnSOD thus reacting with superoxide anion to form peroxynitrite (Beckman, 1996; Dawson, 1996). Peroxynitrite is known to cause lipid peroxidation, damage DNA, irreversibly inhibit the electron complex chain, and nitrate proteins (Almeida et al., 1998; Boje, 2004; Bruckdorfer, 2005). Lipid peroxidation could lead to cell membrane destabilization and eventually cytotoxicity (Boje, 2004). Whereas DNA damage can activate the DNA repair enzyme poly(ADP-Ribose) polymerase (PARP), which can undergo nitrosylation. Nitrosylation enhances PARP's activity exacerbating the already critical condition of the cells energy supply due to inhibition of respiration (Almeida et al., 1998; Cadet & Brannock, 1998; Boje, 2004). Mitochondrial dysfunction and the subsequent depletion of cellular energy stores can be especially lethal to neurons as they are highly active cells and voracious consumers of energy (Chang and Reynolds, 2006).

NO is synthesized by three different isoforms of the enzyme nitric oxide synthase (NOS), each one is a product of separate genes (Boje, 2004; Bruckdorfer, 2005). They are nNOS (Type I), immunological NOS (iNOS/Type II), and endothelial NOS (eNOS/Type III). nNOS and eNOS are activated via a calcium-dependent mechanism primarily through the NMDA receptor whereas iNOS is mobilized as part of an immune response (Dawson et al., 1998; Desaiah et al., 2000). nNOS has become the focal point of METH neurotoxicity research as the primary source of NO in the toxic cascade set-off by METH. This is supported by recent data that suggests that

nNOS expression can be dynamically regulated by toxic insults including METH (Dawson et al., 1998; Deng and Cadet, 1999; Desaiah et al., 2000). Treatment with METH increased the number of striatal neurons staining positive for nNOS in mice 1 and 24 hours after METH administration (Deng and Cadet, 1999). METH-induced elevation in NO formation has proven damaging to striatal DA terminals since pharmacological inhibition of nNOS resulted in significant attenuation of DA terminal toxicity (Itzhak and Ali, 1996; Itzhak al., 2000; Desaiah et al., 2000). Also nNOS knockout (-/-) mice were protected from the toxic effects of METH on the striatum (Itzhak et al., 2000; Desaiah et al., 2000). Agents that prevent NO synthesis confer significant protection from METH-induced death of mesencephalic neurons in vitro (Cadet and Brannock, 1998).

Previous work in our lab corroborates excessive NO formation as playing a role in METH toxicity. METH treatment increased the levels of 3-nitrotyrosine (3-NT) in the striatum seven-fold; 3-NT is an indirect measure of NO/peroxynitrite production (Schulz et al., 1995; Ayata et al., 1997; Wang et al., 2008). 3-NT is formed when NO reacts with the superoxide anion to produce peroxynitrite, which goes on to nitrate the amino acid tyrosine thus forming 3-NT (Bruckdorfer, 2005). We also observed that pretreatment with the murine neurokinin-1 receptor (NK-1R) antagonist WIN-51,708 resulted in significant reduction in 3-NT levels. In the striatum the NK-1R is located on the cholinergic and somatostatin/Neuropeptide Y/nNOS (SST/NPY/nNOS) interneuron (Kawagushi, 1997), exposure to METH resulted in robust internalization of NK-1R's on the nNOS producing interneuron; internalization of the receptor is indicative of SP binding and signaling (Wang et al., 2008; Wang and Angulo, 2011b). All of which suggest that signaling through the NK-1R is connected to NO formation in response to

METH and consequently to toxicity. As demonstrated by pretreatment with WIN-51,708, which decreased METH-induced striatal cell loss (Yu et al., 2004; Wang et al., 2008). Substance P (SP), an excitatory striatal neuropeptide is the endogenous ligand to the NK-1R (Saria, 1999). Is SP signaling through the NK-1R mediating METH-induced NO formation and thus toxicity?

1.3. Neurotoxic Role of Substance P During METH-Induced Toxicity

METH has been shown to increase striatal levels of various neuropeptides including SP (Frankel et al., 2007). In the striatum SP is synthesized solely by one subtype of the medium spiny projection neurons; projection neurons comprise approximately 90% of the neuronal population and are the primary output of the striatum (Kawagushi et al., 1995). Its receptor (NK-1) is localized on the cholinergic and SST/NPY/nNOS interneurons as confirmed by immunohistochemical studies (Kaneko et al., 1993; Li et al., 2001). SP's local axon collaterals interact with SST/NPY/nNOS interneurons (Kawagushi et al., 1997; Li et al., 2000; Li et al., 2001). Furthermore, ultrastructure imaging reveals that SST/NPY/nNOS interneurons also establish synaptic contact with SP projection neurons (Morello et al., 1997). SP's location as well as the location of its receptor places it in a position to have a pivotal role in the events following METH.

In the murine brain, SP has been implicated in glutamate-mediated excitotoxicity since SP release subsequently stimulates glutamate release in the hippocampus (Liu et al., 1999a). SP signaling through the NK-1R can augment NMDA receptor activity in the spinal cord, hippocampus, and in primary sensory neurons (Lieberman and Mody, 1998; Wu et al., 2004). Therefore SP is providing the signal and amplifying the receptors response to said signal, both

actions are creating the necessary conditions for excitotoxicity. Additionally, knockout mice that lack the gene required for SP synthesis were resistant to kainate-mediated hippocampal cell loss compared to wildtype (Liu et al., 1999b).

Our laboratory has shown that SP plays a pro-toxic role in METH-induced toxicity. Suppression of SP signaling through the NK-1R afforded protection from METH-induced striatal injury (Yu et al., 2004; Zhu et al., 2006a). Neurochemical assessment of DAT density and TH levels showed drastic attenuation of toxicity in groups pretreated with the NK-1R antagonist WIN-51,708. Inhibition of the NK-1R and ablation of striatal interneurons expressing the NK-1R demonstrated a significant reduction in striatal apoptotic cell loss (Zhu et al., 2006a). However, it did not diminish the damage of dopaminergic striatal terminals, indicating that SP's contribution to DA terminal damage is through an indirect mechanism perhaps through manipulation of corticostriatal glutamatergic transmission or a polysynaptic extrastriatal pathway. Whereas its involvement in striatal cell loss is an intrastriatal mechanism mediated through the NK-1R located therein. This seems more plausible when one considers the disparity in appearance of striatal injury markers. Striatal cell loss reaches its peak at 24 hours whereas DA terminal markers of degeneration reach their peak in an additional 24 hours (Zhu et al., 2005). Our observations as to striatal cell loss pertain to apoptotic cell loss as we have yet to explore necrotic cell death.

Histological verification of SP receptor activation in the nNOS interneuron as well as the reduction in 3-NT level with WIN51,708 pretreatment implies that SP is directly involved in METH-induced NO formation (Wang et al. 2008). SP can contribute to METH-induced NO synthesis though its modulation of glutamatergic transmission but also directly through

mobilization of intracellular calcium. The NK-1R is a G-protein coupled receptor (GPCR) that activates a number of second messenger pathways (Saria, 1999). One cascade liberates calcium from the endoplasmic reticulum thus increasing intracellular calcium concentration (Wu et al., 2004). It is through this pathway (DAG/IP3) that it is speculated SP is able to potentiate NMDA receptor activity (Wu et al, 2004;). Thus providing two means by which SP can influence NO formation within the nNOS interneuron. First, by directly increasing intracellular calcium, this is necessary for nNOS activation and NO synthesis. Second, indirectly increasing calcium influx by maintaining the NMDA receptor open longer (Lieberman and Mody, 1998; Wu et al., 2004). Overall, SP is participating in pushing the system towards a pro-oxidant state and thus toxicity.

1.4. **NPY and Somatostatin as Agents of Neuroprotection**

Sufficient evidence has implicated SP as being involved in METH-induced toxicity. Bringing to the fore the question that if SP, an excitatory neuropeptide can be pro-toxicity then can any of the inhibitory neuropeptides synthesized in the striatum be protective? In all organisms there exist mechanisms to maintain homeostasis therefore an allostatic counterbalance must exist, the inhibitory neuropeptides NPY and SST are prime candidates. Foremost, in the striatum they are both synthesized and packaged in the SST/NPY/nNOS interneuron (Kawagushi et al., 1995; Thiriet et al., 2005). Their neuroanatomical location throughout the striatum, their long axons, and their synaptic contact with the dendrites of SP projection neurons place them in a prime position to modulate striatal activity and in particular SP signaling (Westwood and Hanson, 1999; Galarraga et al., 2007).

In spite of substantial decrements in other striatal cell types due METH exposure, SST/NPY/nNOS interneurons are relatively resistant (Horner et al., 2006; Zhu et al., 2006b); they exhibit the same resistance in other pathological conditions that affect the striatum such as Huntington's (Forloni et al., 1997; Silva 2005). Therefore their function is not compromised during METH. Their survival from NO-induced oxidative stress is partially attributed to their elevated expression of the mitochondrial radical scavenger manganese superoxide dismutase (MnSOD) (Gonzalez-Zulueta et al., 1998). MnSOD would isolate the superoxide anion at its source before it is able to interact with NO within the interneuron thus preventing the formation of peroxynitrite and circumventing a destructive cascade (Gonzalez-Zulueta et al., 1998). Although this leaves NO free to passively diffuse to other neurons and exert its affect.

NPY is abundantly expressed throughout the murine CNS (Westwood and Hanson, 1999). In the brain it acts upon three GPCRs: Y1, Y2, and Y5 (Thiriet et al., 2005). It is attributed with modifying neuronal excitability through the regulation of calcium signaling pathways both pre- and postsynaptic (Silva et al., 2003; Silva et al., 2005). Consequently it can modulate the release of a number of neurotransmitters and neuropeptides, including depression of glutamate and stimulation of somatostatin release (Silva et al., 2005). There have been a number of studies that suggest that NPY may act as a neuroprotective agent in excitotoxicity and METH toxicity. During excitotoxicity NPY receptor and peptide expression has been shown to increase within the affected region (Silva et al., 2003; Silva et al., 2005). In hippocampal organotypic cultures, pharmacological manipulation of NPY receptors considerably reduced AMPA and kainite-induced degeneration (Silva et al., 2003).

METH stimulates the release of NPY in the paraventricular nucleus (Yoshihara et al., 1996). Within the striatum, METH increases the expression of NPY mRNA neurons commencing 3 hours post-METH and remaining up 7 days (Thiriet et al., 2005; Horner et al., 2006). Westwood and Hanson (1999) found that 18 hours after METH administration there was a 54% decrease in striatal NPY immunoreactivity. They hypothesized that reduced levels were either indicative of depressed synthesis or increase in release. Based on previous findings of elevated mRNA expression 24 hours post-METH the latter interpretation seems more feasible (Thiriet et al., 2005). Especially, since use of a D1 agonist was seen to cause a reduction in striatal NPY tissue content (Westwood and Hanson, 2005) where as a pretreatment with a D1 antagonist negates the METH-induced increase in NPY mRNA expression (Horner et al., 2006). Intracerebroventricular (ICV) infusion of NPY significantly reduced METH-induced apoptosis in the striatum (Thiriet et al., 2005). However, NPY knockout mice were even more susceptible to neuronal cell loss than wildtype.

SST is also expressed throughout the mammalian CNS and signals through five GPCRs: SST1-5 (Forloni et al., 1997; Kumar, 2008). In the striatum the receptor subtypes SST_1, SST_2, SST_4, and SST_5 are primarily expressed (Santis et al, 2009, Rajput et al, 2011a; Rajput et al, 2011b). Striatal projection neurons appear to express SST_2 (Allen et al., 2003; Galarraga et al., 2007) and in the nucleus accumbens SST_1 is speculated to function as an autoreceptor (Thermos et al., 2006). Functionally, SST is attributed with regulating the release of a number of transmitters, including GABA, glutamate, and DA (Hathway et al., 1999; Momiyama and Zaborszky, 2006; Thermos et al., 2006). Like NPY, SST is thought to influence the membrane potential of a neuron by manipulating voltage-gated calcium and K+ channels at multiple sites

(Rodriguez-Sanchez et al., 1997). A study of the basal forebrain demonstrated that exogenous application of SST to brain slices caused presynaptic inhibition of glutamate release in a calcium dependent way (Momiyama and Zaborszky, 2006). SST modulates the firing pattern of striatal projection neurons through SST2 and therefore the functional output of the striatum (Allen et al., 2003; Galarraga et al., 2007). Shifting the firing rate from a tonic pattern to a stuttering pattern with interspike intervals (Galarraga et al., 2007).

SST is considered a neuroprotectant in pathologies attributed to glutamate-induced excitotoxicity (Forloni et al., 1997; Cervia et al., 2008). Cortical and striatal neuronal cultures secrete SST in response to NMDA receptor activation (Forloni et al., 1997). In addition, NMDA or quinolinic acid mediated toxicity elevates SST mRNA expression (Kumar, 2008). In an in vitro model of ischemia in the mouse retina, the addition of SST and SST analogues decreased cell death and glutamate release (Cervia et al., 2008). However application of an SST_2 receptor antagonist negated the protective effect. In rats the SST_2 receptor ligand octreotide (OCT) diminishes seizure activity caused by kainite and quisqualic acid (Rauca et al., 1999). Following middle cerebral artery occlusion, ICV infusion of SST or OCT reduced infarct volume (Rauca et al., 1999). In rat cortical cells, SST curtailed NMDA mediated cytotoxicity through cyclic guanosine monophosphate (cGMP) perhaps via alteration of NMDA receptor function (Forloni et al., 1997). SST is hypothesized to serve as a key element in the survival of SST/NPY/nNOS neurons during excitotoxicity since elimination of SST through antisense or immunoabsorption enhanced cell death including the once refractory SST/NPY/nNOS interneurons (Kumar, 2008). Of interest to METH-toxicity is that DA has been shown to stimulate a transient increase in

striatal SST receptor binding and SST peptide immunoreactivity, within 24 hours all parameters return to control levels (Rodriguez-Sanchez et al., 1997).

Both NPY and SST appear to exert an inhibitory influence on glutamatergic release and transmission in addition to intracellular calcium influx, which have been attributed as the means by which these peptides protect from excitotoxic insults (Forloni et al., 1997; Thiriet et al., 2005). NPY has been shown to depress the concentration of cytosolic calcium for up to an hour after its removal (Silva et al, 2005). Whereas activation of SST receptors have been known to inhibit the NMDA receptor (Kumar, 2008). SST/NPY/nNOS interneurons receive direct glutamatergic input from the corticostriatal pathway (Kawaguchi et al., 1995). Said pathway is the source of excessive glutamate released into the striatum due to METH (Eisch and Marshall, 1998).

There are then at least two means by which these peptides could serve in a protective capacity in METH neurotoxicity. First, they could depress presynaptic glutamate release from the corticostriatal neurons thus reducing NMDA mediated NO formation. Second, in the striatum itself they could inhibit calcium influx in projection neurons ultimately reducing the excitability of the neuron. This could serve three purposes: through the direct pathway it would prevent thalamic neurons from exciting corticostriatal neurons, which release glutamate into the striatum. Also attenuating SP release and thus its downstream effects on NO formation. Finally, excessive calcium influx into striatal neurons can contribute to the overproduction of superoxide anion thus creating the environment needed for NO to form peroxynitrite therefore diminishing available calcium reduces the probability of oxidant synthesis (Almeida et al., 1998). In addition, excessive intracellular calcium has been implicated in the deleterious disruption of mitochondrial

function eventually leading to cell death (Chang and Reynolds, 2006). SST has the additional ability to depress NMDA receptor function thus affecting calcium influx into the SST/NPY/nNOS interneuron, which would lessen NO formation (Kumar, 2008).

Significance: A large body of evidence shows that METH causes sustained damage to the brain in animal models and human METH users. The exact mechanism of toxicity still remains undefined. Although, there is a general consensus that the following must be present for toxicity to occur: DA overflow and glutamate release. Compiling data implicates oxidative stress as the causative agent of METH toxicity and NO as a major contributor of ROS. We have found that METH-induced excessive signaling of SP may participate in pushing the system to a pro-oxidant pathological state through excessive NO synthesis. Pharmacological inhibition of SP significantly reduced injury to the striatum including cell loss. Leading us to consider whether other neuromodulators may serve as an allostatic counterbalance such as the inhibitory neuropeptides NPY and SST. Their influence on striatal signaling and growing evidence of neuroprotection in several excitotoxic models make them compelling candidates of further investigation in METH-toxicity. Also a deeper understanding of the role these peptides play in a pathological state and their molecular mechanisms may provide novel therapeutic targets not only in drug addiction but also in neurodegenerative diseases that affect the same systems.

Chapter 2 Central Hypothesis and Research Design

2.1. Central Hypothesis

It is our working hypothesis that METH-induced SP signaling pushes the striatum into a pro-oxidant state trough intra- and extrastriatal upregulation of glutamate signaling. Activation of the NK-1R localized on the SST/NPY/nNOS interneuron will result in potentiation of NMDA activation of neuronal nitric oxide synthase (nNOS) and excessive NO produced. However, NPY and SST will serve as opposing protective forces, through their depressive action on calcium influx and glutamate signaling.

2.2. Research Design

2.2.1 Specific Aim 1: Does the Neuropeptide Substance P Modulate Glutamate-Induced Striatal Lesions?

a) To test the hypothesis that a NK-1R antagonist attenuates the NMDA-induced striatal injury as measured by apoptosis.

b) To test the hypothesis that pharmacological blockade of the NK-1R attenuates the NMDA-induced production of striatal NO.

c) To test the hypothesis that NMDA-induced production of striatal NO involves nNOS and iNOS.

Hypothesis:

SP contributes to METH toxicity through the potentiation of NMDA mediated NO production.

2.2.2. Specific Aim 2: Do Striatal Neuropeptides Modulate in a Homeostatic Fashion the Neurotoxic Effects of METH and Glutamate in the Striatum?

a) To test the hypothesis that a NPY agonist ameliorates the METH- and NMDA-induced damage to the striatum.

b) To test the hypothesis that the neuropeptide SST attenuates the METH- and NMDA-induced injury to the striatum.

Hypothesis:

SP augmentation of METH toxicity through NO formation is counterbalanced by the inhibitory neuropeptides SST and NPY through modulation of glutamatergic transmission.

Chapter 3 Materials and Methods

3.1. Animals, Drugs, and Systemic Drug Administration

Ten-week old male ICR mice (Taconic, Germantown, NY) were singly housed on a 12 hour light/dark cycle. Food and water is available *ad libitum*. They are habituated for two weeks so as to acclimate to their environment prior to all procedures and treatments. All animal-use procedures were performed according to the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at Hunter College of the City University of New York. The Hunter College animal facility is certified by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

Systemic Drug Treatment: (+)-Methamphetamine hydrochloride (Sigma, St. Louis, MO) was dissolved in 0.9% physiological saline and administered as a bolus intraperitoneal (i.p.) injection (30 mg/kg). The NK-1R antagonist WIN-51,708 (Sigma, St. Louis, MO) was solubilized in vehicle [45% (w/v) 2-hydroxypropyl-β-cyclodextrin, Sigma, St. Louis, MO]. Control animals received an equivalent volume of the vehicle. Vehicle or WIN-51,708 (5 mg/kg) were injected i.p. 30 minutes prior to intrastriatal infusion of NMDA (20 nM, Sigma, St. Louis, MO), which was dissolved in artificial cerebrospinal fluid (aCSF). The nNOS antagonist 7-nitroindazole (7-NI, Sigma, St. Louis, MO) was dissolved in a 1:3:6 mixture of dimethyl sulfoxide, propylene glycol, and distilled water. It was administered as a single I.P. injection (25 mg/kg) 30 minutes prior to NMDA infusion.

Microinjected Drugs: The iNOS antagonist 1400W (Tocris Biosciences, Ellisville, MO) was solubilized in aCSF and injected (100 µM) simultaneously with NMDA. The NPY-Y1R agonist (Leu31, Pro34) NPY (20 µM, Bachem, Torrance, CA), NPY-Y2R agonist NPY (3-36) (20 µM, Bachem, Torrance, CA) and SST analogue octreotide (10 nM, Bachem, Torrance, CA) were all dissolved in aCSF. In addition, they were either injected together with NMDA or 15 minutes prior to METH treatment.

Animals used for western blot analysis of tyrosine hydroxylase levels were sacrificed 72 hours after treatment by decapitation. Their brains were then dissected, frozen on dry ice, and stored in -80˚C until use. Animals used for 3-NT immunofluorescence or Fluoro-Jade C staining were sacrificed either 6 hours or 24 hours post-treatment via intracardial perfusion. They were anesthetized by an i.p. injection of ketamine (100mg/kg) and acepromazine (3 mg/kg), perfused intracardially with 20 ml of phosphate buffered saline (PBS), followed by 20 ml 4% paraformaldehyde in PBS. Brains were removed and post-fixed overnight in 4% paraformaldehyde at 4˚C. Brains were then cryo-protected in 30% sucrose in 0.1M PBS until they sank in the solution and then stored at -80˚C until use.

3.2. Intrastriatal Infusion of NMDA and Peptides (Adapted from Ayata et al., 1997)

Mice were anesthetized with inhaled isoflurane (2.5% for induction, 2.0% for maintenance). Their heads were immobilized in a stereotaxic frame (Model 5000; David Kopf Instruments, Tujnga, CA) and a burr hole was drilled into the skull at the following coordinates: +0.5 mm rostral-caudal; +/- 2.0 mm medial-lateral from bregma; -2.5 mm dorsal-ventral from dura (Franklin and Paxinos, 1997). A 2 µL microinjection needle (25 ga, Hamilton, Reno, NV)

was lowered into the striatum and allowed to remain in position for 5 minutes. NMDA or peptides were injected into the striatum using the quintessential stereotaxic injector (Stoelting, Wheat Lane, IL) at a rate of 0.1µl/minute and the needle remained in place for an additional 5 minutes before its removal. The wound was closed with VetBond (n-butyl cyanoacrylate, 3M) tissue adhesive and the animal was allowed to recover.

The NPY-Y1R agonist [(Leu31, Pro34) NPY], NPY-Y2R agonist [NPY (3-36)], and SST analogue (octreotide) were all injected at the same coordinates and using the same procedure as described above for microinjection of NMDA. They were all dissolved in aCSF and a 1.0 µL volume was injected into the striatum at a rate of 0.1 µL/minute. However for the NMDA-induced lesion, the agonists and NMDA were infused concomitantly.

3.3. 3-Nitrotyrosine Fluorescent Immunohistochemistry

Sectioning and staining was carried out by the free-floating method. Striatal 30 µm coronal sections were cut on a cryostat at -20ºC. The sections were collected serially between Bregma 0.02 and 1.4 mm, with each tissue sample separated from the proceeding sample in the series by 180 µM. Thus each sample well represents an entire striatum. They were then stored in a solution of 30% glycerin, 30% ethylene glycol, 40% PBS at -20ºC until used. The sections were then rinsed in PBS and incubated 3X for 10 minutes in 10 mM citric acid at 65ºC. Washed with PBS 3X for 5 minutes each; followed by incubation in the M.O.M (mouse on mouse) kit blocking reagent (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. Rinsed with PBS 3X for 5 minutes each and then incubated for 10 minutes at room temperature in M.O.M kit diluent solution. Proceeded by incubation overnight at 4ºC in mouse monoclonal anti-

3-NT (1:200, Santa Cruz Biotech, Santa Cruz, CA) in M.O.M diluent buffer. The next day they were washed with PBS 3X for 5 minutes each. Sections were incubated in a solution of 5% goat serum in 0.2% triton PBS for 1 hour at room temperature. Then another 1 hour at room temperature in Cy3 goat anti-mouse (1:500, Millipore, Temecula, CA) in 1% goat serum and 0.2% triton PBS. They were then washed an additional 3X with PBS for 5 minutes each. Mounted and coverslipped using Vectashield fluorescent hardset™ mounting medium with DAPI (Vector Laboratories, Burlingame, CA). The slides were imaged with a Leica TCS SP2 scanning confocal microscope (Leica Microsystems, Germany) and quantified using the Leica imaging software.

3.4. Measurement of Staining Intensity 3-Nitrotyrosine (Adapted from Gazzaley et al., 1996)

From each slide at least 4 out of 6 tissues were selected for imaging with the Leica scanning confocal (Leica Microsystems, Germany). All tissues selected must have a visible needle tract to ensure that the effect observed is due to the injected solution. Per tissue, 4 areas were chosen within each hemisphere. The regions chosen were adjacent to each side of the needle tip but avoiding the visible needle damage. They were then scanned only once to prevent quenching, which would affect the results. The images were all scanned at 63X with preset parameters that give the most resolved image in the baseline condition. Said parameters were set per individual study to account for slight variations that may occur during the staining process from one individual study to another. All tissue for a study was sectioned and processed simultaneously. Hardware and software settings were maintained the same for all images scanned thereafter. The overall settings were as follows: area scanned was 56889.33 mm^2, line

average of 1, zoom factor of 1.00, and a scan speed of 400 Hz. The digitized image is 512x512 pixels and an 8 bit grey resolution with a range in intensity of 0-255. The settings for the pinhole, frame average, gain, and offset were adjusted per study to accommodate for expected minor staining differences. The image includes both the cells as well as the neuropil. Analysis of intensity was done using the Leica confocal software. Background produced by nonspecific binding was removed using the baseline correction feature (eliminate autofluorescence) in the image process option. Then using the histogram feature under the quantify tab we were able to get the mean energy of each image, which represents the staining intensity of the image. The average of the mean energy of the animal was obtained and then statistically analyzed.

3.5. Tyrosine Hydroxylase Western Blot

Using a brain blocker on ice a 2 mm thick coronal section of the striatum was removed at the site of the injection. The samples were homogenized in approximately 150 µl of lysis buffer (40 mM Tris-HCL, 274 mM NaCL, 2.0 mM EGTA, 20% glycerol, 1 mM Na3VO4, 1 mM PMSF, 1 mM ß-glycerophosphate, 2.5 Na4P2O7, 50 mM NaF, 1% NP40, and protease inhibitor cocktail: 1.0 mM AEBSF, 0.8 µM aprotinin, 0.02 mM leupeptin, 0.04 mM bestatin, 0.015 mM pepstatin A, and 0.014 mM E-64) with a QSonica Sonicator 3000 cup horn at 7 cycles of 30 seconds of sonication and 60 seconds of cooling. Centrifuged at 4°C first at 3000 rpm for 5 minutes and then the supernatant is centrifuged at 5000 rpm for 5 minutes. The supernatant was removed once more and centrifuged for one final cycle of 6000 rpm for 10 minutes. The protein content was assayed by the Bradford method (Bio-Rad, Hercules, CA). Ten µg of protein were loaded on a 10% Tris-HCL (Invitrogen, Carlsbad, CA) SDS-PAGE and transferred to an iBlot stack membrane (Invitrogen, Carlsbad, CA). After blocking nonspecific binding using Odyssey

blocking buffer (Li-Cor Biosciences, Lincoln, NE) for 1 hour at room temperature, membranes were probed overnight with polyclonal rabbit anti-TH (1:5000, Millipore, Temecula, CA) antibody and monoclonal mouse anti-β-actin antibody (1:20,000, Sigma, St. Louis, MO) in Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE) with 0.2% tween 20 at 4◦C. The next day the membranes were rinsed with 0.1% tween 20 PBS followed by 3 washes at 5 minutes each. They were then incubated in a mixture of Odyssey's IRDye® secondary antibodies donkey anti-rabbit 800CW (1:15,000) and donkey anti-mouse 680LT (1:30,000) within Odyssey's blocking buffer for 1 hour at room temperature. After an additional 3 washes at 5 minutes each with 0.1% tween 20 PBS as well as a final 15 minute wash with PBS alone, the proteins bands were then detected via the Odyssey infrared imager. Bands were quantified using the Odyssey Imager analysis software and normalized against β-actin.

3.6. Fluoro-Jade-C Stain

Tissue was collected and stored as described in section 3.3. To stain first the tissue was rinsed with dH_20 then mounted on Superfrost® Plus slides (VWR, West Chester, PA) and left to air dry. Once dried a border was drawn around the tissue using an ImmEdge™ pen (Vector Laboratories, Burlingame, CA). The tissue then went through washes in ethanol. First for 3 minutes in 100% percent ethanol followed by a 2 minute wash in 70% percent ethanol finally a 2 minute wash with dH_2O. The slides were then exposed to 0.06% potassium permanganate for 10 minutes, the mixture was then removed and the slides allowed to air dry overnight at room temperature. The next day the slides were rinsed 2X with dH_2O for 2 minutes per wash. The tissue was then immersed in 0.00005% Fluoro-Jade C (Millipore, Temecula, CA) in 0.1% acetic acid for 10 minutes. They were then washed 3X with dH_2O for 1 minute per wash. The slides

were then air dried at room temperature and the ImmEdge™ was removed using xylene. The slides were then coverslipped using Vectashield fluorescent hardset™ mounting medium with DAPI (Vector Laboratories, Burlingame, CA) and no. 1.5 coverslips.

3.7. Fluoro-Jade C Cell Counts

Cell counts were performed by two individuals that were blind to the experimental conditions using the AxioVision Rel. 4.8 (Imaging Associates Ltd, Carl Zeiss Group, Jena, Germany) unbiased stereology software for PC. Hardware components of the AxioVision system included a Zeiss microscope (Carl Zeiss Group, Germany) attached to a mechanical stage 75x50 mot; CAN (D) (Carl Zeiss Group, Germany), a high resolution AxioCam MRm Rev. 3 (Carl Zeiss Group, Germany), and a PC computer. Software parameters were set before the commencement of all counts and included a sampling frame area of 3873.904 μm^2; the frame moved in steps automatically set at (x-step =900 µm, y-step=750 µm; 675000 μm^2). A grid size was chosen so that an average of 10 or more probes per section was counted. Twenty-five micrometers was defined as the z-dimension in which cells were counted, giving a 2.5µm window for error on the top and bottom surface of the tissue. This area was excluded to account for the damage incurred by the tissue during the sectioning and staining process, which can lead to counting false positives. For all groups, AxioVison Rel. 4.8 Gundersen coefficient of error was less than or equal to 0.1. We counted four sections per animal; each section had to have a visible needle tract so that the affect seen was due to the respective treatment.

In brief, a cross section of the striatum from one hemisphere per tissue sample was outlined in 10X magnification to derive an estimate of the structure. The estimated striatal volume was automatically calculated by the software. The immediate area around the needle

tract was removed from the outline ensuring that the resulting damage caused by the needle's entry is not included in the data. Actual cell counts were done at 100X magnification using the dissector probe in AxioVison Rel. 4.8 with the optical fractionator. All Fluoro-Jade C positive cells displaying the morphological features of an apoptotic cell had to be within the boundaries of the inclusion lines without touching the exclusion lines to be counted. Cells had to fulfill specific morphological criteria to be classified as striatal cells undergoing apoptosis, said criteria was established during the design stage and was strictly adhered to in the course of all counts. Criteria used were as follow: only cell bodies were counted to exclude degenerating DA terminals. In addition, the soma had to completely display a bright yellow-green color (Schmued et al., 1997); cell bodies that were only partially stained or that the stain was localized within the dendrites were excluded. Cells with a disrupted cell membrane were counted since dying neurons do not solely display a rounded shape but can have what is referred to as blebbling (interspersed bulging) of the membrane due to cytoskeletal decoupling as well as membrane disintegration in the final stages (Kroemer et al., 2009). Finally, cells with a densely stained nucleus were also counted since it indicates the degradation of the genetic material contained therein as expected in a cell undergoing apoptosis (Kroemer et al., 2009). Once counts were complete and the software automatically generated the results, the data was compiled and analyzed by a separate individual that did not participate in the cell counts.

3.8. Statistical Analysis

Statistical analysis of the data was conducted using GraphPad prism (GraphPad Software, Inc., La Jolla, CA) statistical analysis software. The differences between groups were determined utilizing one way ANOVA mean±SEM. The analysis was followed by Tukey's post-hoc test.

Differences between two groups were analyzed by Student's *t*-test. All statistical analysis were conducted with a significance criterion value set at $p<0.05$.

Chapter 4 Role of Substance P in Glutamate-Induced Striatal Lesions and Nitric Oxide Production

METH causes pronounced and sustained toxic effects in the striatum. Our lab has provided substantial evidence implicating SP as pro-toxic; blocking the NK-1R results in marked protection from striatal injury. However, we have only started to gleam the possible mechanism by which SP contributes to METH toxicity. Histological evidence from our lab has implied that METH causes activation of the NK-1R on the SST/NPY/nNOS interneuron as well as contributes to NO synthesis (Wang et al., 2008; Wang and Angulo, 2011a; Wang and Angulo, 2011b). It is the purpose of specific aim 1 to provide a stronger foundation for a causative link between SP signaling and potentiation of the NMDA/nNOS cascade. To do so first we wanted to demonstrate that blocking SP signaling through the NK-1R could modulate NMDA-induced striatal injury as measured by striatal cell loss. Followed by testing whether SP signaling can also modulate NMDA-induced NO synthesis. Finally, attempt to elucidate the source of NO via teasing out whether the bulk of the glutamate-induced NO being produced is coming from iNOS or nNOS; or perhaps a combination of both as there are sources for each found within the striatum.

4.1. Role of Substance P in Glutamate-Induced Striatal Lesions

Glutamate excitotoxicity has been established as integral to METH-induced striatal injury. It was our hypothesis that SP signaling through the NK-1R can potentiate glutamate-mediated excitotoxicity. There has been evidence in other structures and regions of the CNS that glutamate and SP can work in a synergistic fashion. It has already been shown in the cortex that

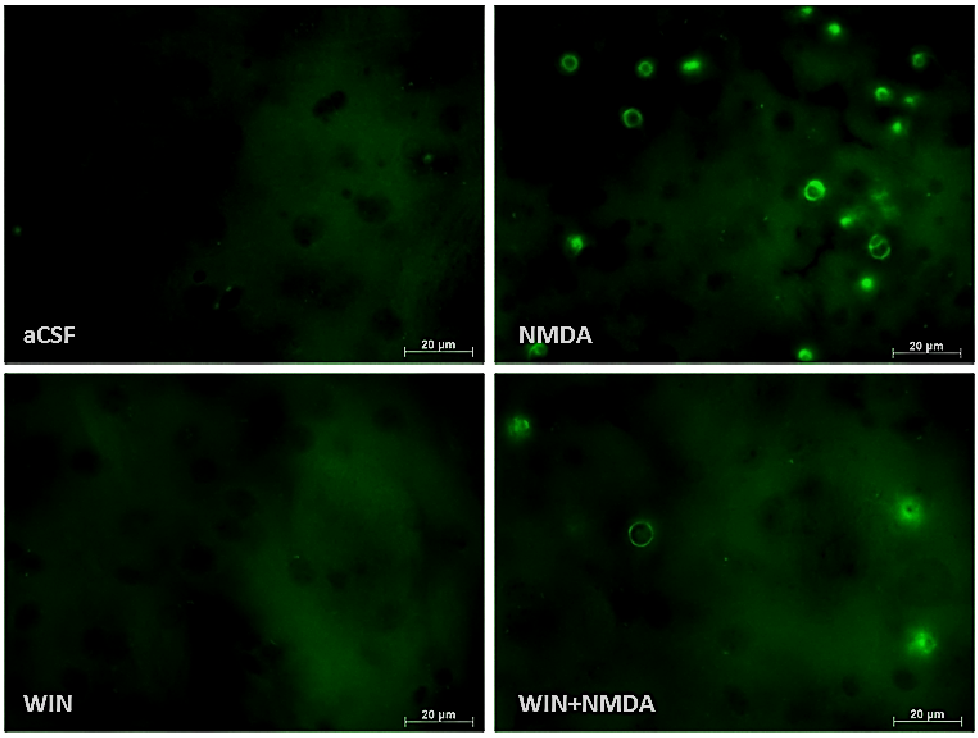
glutamate signaling particularly through the NMDA subtype receptor can increase SP release (Beal et al., 1991; Garside and Mazurek, 1997) as well as in the spinal cord (Liu et al., 1997). In addition, signaling via NMDA receptors located on dorsal horn neurons can cause SP receptor activation (Liu et al., 1997); SP can in turn modulate glutamate as infusion of SP within the substantia nigra was seen to increase the release of glutamate within the striatum (Reid et al., 1990). SP can also modulate glutamatergic signaling. For instance it has been demonstrated in the hippocampus (Lierberman and Mody, 1998) and spinal cord (Wu et al., 2004) that SP signaling via the NK-1R enhanced NMDA receptor function. As I mentioned above it was our intention to further substantiate a link within the striatum between SP and glutamate signaling, specifically in regards to their contribution to METH-induced toxicity. However, in order to explicate this plausible synergistic relationship we removed the extraneous complexities inherent in utilizing METH by narrowing our focus solely on glutamate signaling via the NMDA receptor. Therefore, inhibition of the NK-1R using the selective murine antagonist WIN-51,708 should in essence attenuate NMDA-induced cytotoxicity.

To test our hypothesis we treated two groups (6-7/per condition) of animals. The first group (control) received an i.p. injection of vehicle; the second group the antagonist WIN-51,708 (5 mg/kg). The dosage was previously established in our lab as the minimally effective dose. Within thirty minutes both groups are given an intrastriatal infusion of 1 µl of NMDA (20 nM) at a rate of 0.1 µl per minute to one hemisphere. In the contralateral hemisphere an equivalent infusion of aCSF. The procedure for microinjection was adapted from Ayata et al., (1997) and is more thoroughly described in the Methods section 3.2. The animals were then sacrificed 24 hours after injection via perfusion, the brains were then dissected and the tissue processed for

Fluoro-Jade C staining. Conditions (NMDA dosage & time point) had been previously optimized to translate to our METH paradigm in regards to parameters of striatal injury (unpublished data). So as to assess striatal injury we utilized Fluoro-Jade C staining and stereological cell counts as described in section 3.6-7. Fluoro-Jade C was chosen as an apoptotic marker for its ability to exclusively label neurons undergoing degeneration and eventually apoptosis (Schmued et al., 1997; Eisch and Marchall, 1998; Poirier et al., 2000). To exclude degenerating dopamine terminals, strict exclusion parameters were established prior to the commencement of cell counts as outlined in the methods section 3.7. In brief, only cell bodies that were positive for Fluoro-Jade C were counted and they had to conform to the morphology of cells undergoing apoptosis.

As demonstrated in the data (Figure 4-1) below, infusion of NMDA into the striatum resulted in a remarkable increase in Fluoro-Jade C positive cells. However, the addition of the NK1-R antagonist significantly attenuated the NMDA-induced neuronal cell loss within the striatum. WIN-51,708 by itself was equivalent to baseline control levels.

(A)


aCSF
20 µm
NMDA
20 µm
WIN
20 µm
WIN+NMDA
20 µm

(B)

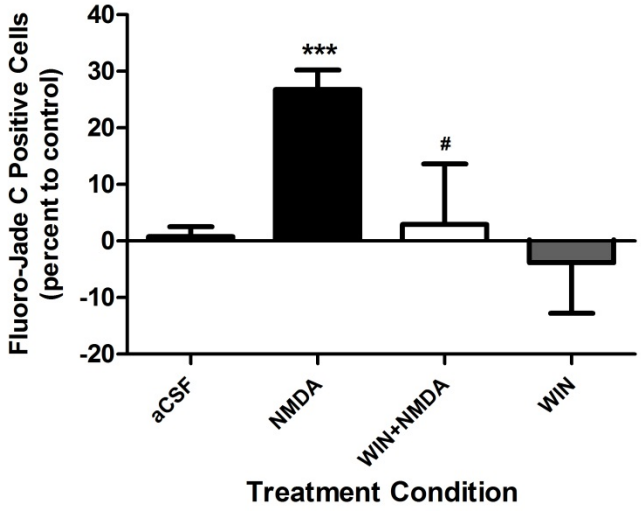

Fluoro-Jade C Positive Cells
(percent to control)
40
30
20
10
0
-10
-20
***
#
aCSF
NMDA
WIN+NMDA
WIN
Treatment Condition

Figure 4-1

Figure 4-1: WIN-51,708 attenuation of NMDA-mediated cell loss. Pretreatment with the neurokinin-1 receptor antagonist WIN-51,708 caused a significant reduction in the NMDA-induced striatal cell loss as measured by Fluoro-Jade C staining. The mice (n=7) received an injection of WIN-51,708 (WIN, 5 mg/kg, i.p.) 30 minutes prior to intrastriatal infusion of NMDA (20 nM); animals were sacrificed 24 hours post-NMDA treatment. Fluoro-Jade C positive cells were counted using an unbiased stereology system. (A) Upright fluorescent images taken at 63X magnification within the striatum. (B) Quantification of Fluoro-Jade C positive cells in the striatum. Inhibition of substance P signaling through the neurokinin-1 receptor significantly attenuated the substantial increase in Fluoro-Jade C (neuronal degeneration marker) positive striatal cells. The data were analyzed by ANOVA. Data were normalized to control group (vehicle & aCSF). ***$p<0.001$ (as compared to the aCSF group), $^{\#}p<0.05$ (as compared to the NMDA group).

4.2. NK-1R Potentiation of NMDA-Induced Nitric Oxide Production

We postulated that SP signaling though the NK-1R on the SST/NPY/nNOS interneuron is able to augment NMDA activation of nNOS resulting in the formation of NO to supraphysiological levels. There is experimental evidence in support of this hypothesis; we have shown that treatment with a systemic bolus injection of METH (30 mg/kg) can result in internalization of the NK-1R on the SST/NPY/nNOS within minutes of treatment (Wang and Angulo, 2011b). Also there is a substantial increase in 3-NT as measured by HPLC and immunohistochemistry, however pretreatment with the selective NK-1R antagonist WIN-51,708 resulted in significant attenuation of the METH-induced NO production (Zhu et al., 2009; Wang and Angulo, 2011a). More so, intrastriatal microinjection of the selective SP agonist GR-73632

in absence of METH, resulted in a significant increase of 3-NT however systemic administration of either WIN-51,708 or 7-NI (selective inhibitor of nNOS) reduced the SP-induced 3-NT production (Wang and Angulo, 2011a). Thus in theory, application of the NK-1R antagonist should reduce the striatal levels of 3-NT produced by NMDA.

Before we investigate the effect of SP on NO production via glutamate signaling we first had to determine the peak of NO production after NMDA infusion by performing a time course. Animals underwent intrastriatal infusion of NMDA (20 nM) as described in Methods (3.2). The mice were then sacrificed via perfusion at the following time points: 6, 12, and 24 hours (6-7 mice/time point). These time points were chosen based on Ayata et al. (1997) and work done previously in our lab that demonstrated that peak NO production after METH is an early event that occurs within the first 24 hours (Zhu et al., 2009). Furthermore, if the assumption is that NO mediated oxidative stress is in large part responsible for cell loss than the bulk of NO synthesis should occur by the peak of striatal cell death, which occurs at 24 hours (Zhu et al., 2005). Therefore to capture this window we focused our efforts within that timeframe. The brains were then collected and processed for 3-NT immunohistochemistry (section 3.3). The tissue was then quantified using a Leica scanning confocal and its associated software to measure 3-NT staining intensity as described in section 3.4.

As shown below (Figure 4-2) 3-NT staining increased over time. Although the most significant increase in staining was at the 24 hour time point thereafter all NMDA experiments will be performed utilizing this time point.

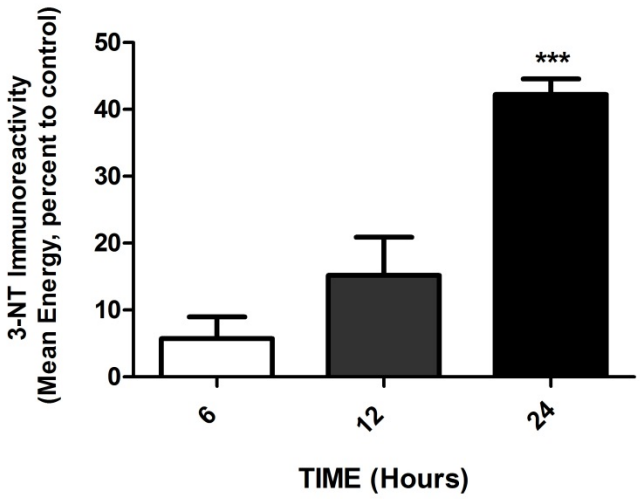
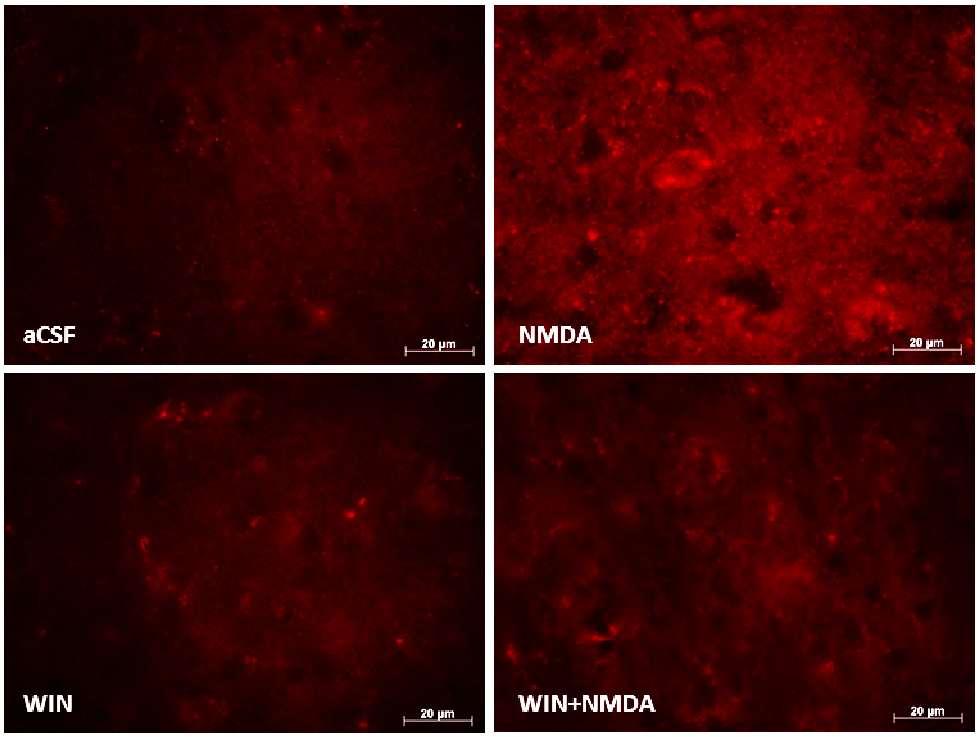


Figure 4-2: NMDA-mediated striatal NO synthesis time course. Infusion of NMDA into the striatum resulted in a time-dependent increase in striatal 3-nitrotyrosine production. Mice (n=7) were treated with an intrastriatal infusion of NMDA (20 nM); then sacrificed at 6, 12, and 24 hours after NMDA. 3-NT levels were measured using a Leica scanning confocal microscope and Leica imaging software. NMDA receptor agonist caused a significant augmentation in striatal 3-NT (indirect index of nitric oxide synthesis) production at 24 hours (***$p<0.001$ as compared to 6 and 12 hours).

Upon determination of an effective time point then an additional set of mice were treated with the NK-1R antagonist WIN-51,708 (5 mg/kg, i.p.) followed within 30 minutes by intrastriatal infusion in one hemisphere with NMDA (20 nM) and of aCSF in the contralateral hemisphere. The mice were sacrificed at 24 hours after NMDA treatment as previously determined. The collected tissue was prepared and quantified in the same manner. As previously shown there was a significant and obvious increase in 3-NT staining for the NMDA group which was significantly decreased in the antagonist plus NMDA group. The antagonist by itself had a slight although not statically significant increase in staining (Figure 4-3).

(A)


aCSF
20 µm
NMDA
20 µm
WIN
20 µm
WIN+NMDA
20 µm

(B)

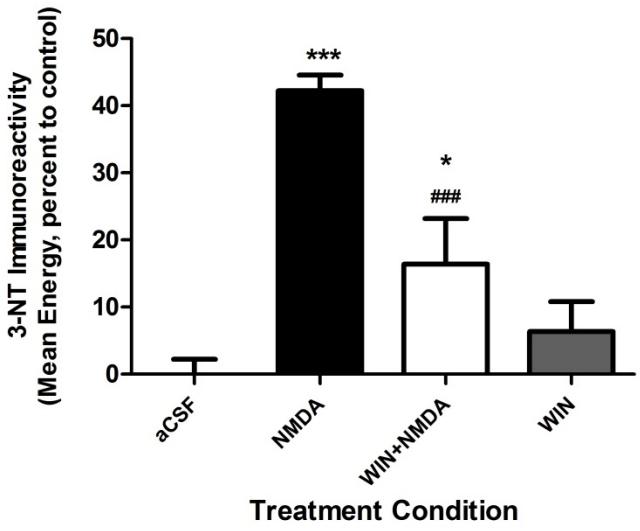

3-NT Immunoreactivity
(Mean Energy, percent to control)
50
40
30
20
10
0
***
*
###
aCSF
NMDA
WIN+NMDA
WIN
Treatment Condition

Figure 4-3

Figure 4-3: WIN-51,708 attenuation of NMDA-induced NO. Pretreatment with the neurokinin-1 receptor antagonist WIN-51,708 attenuated the NMDA-induced increase of striatal 3-nitrotyrosine (3-NT). The mice (n=7) received an injection of WIN-51,708 (WIN, 5 mg/kg, i.p.) 30 minutes prior to NMDA microinjection (20 nM) and the animals were sacrificed 24 hours post-NMDA. 3-NT levels were determined measuring intensity of 3-NT immunohistochemistry via confocal microscopy and associated imaging software. (A) Upright fluorescent images (63X) representative of each group of 3-NT immunoreactivity within the striatum (B) Quantification of 3-NT intensity. Note that the neurokinin-1 receptor antagonist significantly attenuated the NMDA-induced increase in striatal 3-NT synthesis. Statistical analysis performed using a One-way ANOVA and Tukey's multiple comparison test of significant differences (*$p<0.05$, ***$p<0.001$ as compared to the aCSF group, $^{###}p<0.001$ as compared to the NMDA group).

4.3. Contribution of nNOS and iNOS to NMDA-Induced NO Synthesis

Microglia and astrocytes are activated in response to a toxic injury (Almeida et al., 1998). Over activation can be harmful since glia can release many toxic substances including ROS (Krasnova and Cadet, 2009). Only activated glia express iNOS and are able to release NO (Dawson et al., 1996; Almeida, 1998; Boje, 2004). Although there is growing evidence that nNOS is the predominant source of NO in excitotoxicity there is still a deficit in knowledge as to the possible contribution of iNOS to NO synthesis. Furthermore, microglia have been shown to express NK-1 receptor and therefore may present an alternative source of NO production that is also downstream of SP release (Rasley et al., 2002). Here we tested whether this isoform contributes to NMDA-induced NO synthesis.

To determine the contribution of nNOS the mice (7 per condition) were treated with a systemic injection of the nNOS antagonist 7-NI (25 mg/kg). This dosage was obtained from Itzhak et al. (2000) because it adequately blocks nNOS activity (~75%) without the confounding effects on core body temperature. Control animals received an equivalent dosage of saline instead, ensuring that the antagonist itself was nontoxic or exhibited any other confounding affects when comparing the aCSF treated hemispheres in both treatment conditions. Thirty minutes after injection of 7-NI, all the groups underwent intrastriatal infusion of NMDA (20 nM) as well as aCSF as described in the Methods section (3.2). Animals were sacrificed at 24 hours post NMDA treatment and the samples collected, prepared, and quantified in the same manner as in section 4.2. Figure 4-4 shows that intrastriatal infusion of NMDA caused a significant increase in NO synthesis that almost reached 40% in comparison to the baseline condition (aCSF). A systemic injection of 7-NI prior to NMDA resulted in a minor although statistically insignificant decrement in the NO levels produced by NMDA injection. Treatment solely with the inhibitor and an intrastriatal microinjection of aCSF produced 3-NT levels comparable to baseline levels (intrastriatal aCSF, i.p. saline).

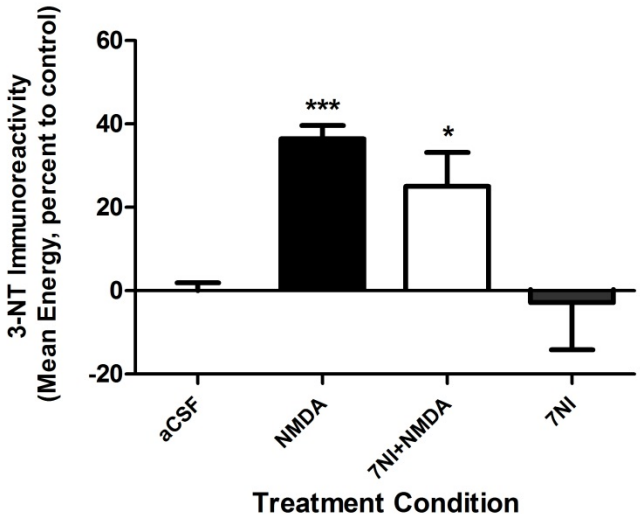


Figure 4-4: Source of NMDA-induced NO: nNOS? Pretreatment with the selective neuronal nitric oxide synthase inhibitor (nNOS) 7-nitroindazole (7-NI) has no significant effect on NMDA-induced synthesis of striatal 3-nitrotyrosine. A group of mice (n=6) were injected with 7-NI (25 mg/kg, i.p.) 30 minutes prior to intrastriatal infusion of NMDA (20 nM) and then sacrificed 24 hours post-NMDA. 3-NT immunoreactivity was measured using confocal microscopy. Administration of 7-NI did not significantly reduce the NMDA-induced striatal production of 3-NT (*$p<0.05$, ***$p<0.001$ as compared to aCSF group).

For iNOS animals were treated with a simultaneous intrastriatal infusion of the selective antagonist 1400W (100 µM) with NMDA (20 nM) in one hemisphere whereas in the contralateral hemisphere they only received the antagonist. Animals were sacrificed and the tissue processed and quantified in the same manner as nNOS animals. Intrastriatal infusion of NMDA caused a significant and expected increase in NO synthesis as compared to control. However, co-infusion of an iNOS inhibitor completely eliminated the NMDA-induced NO. Infusion of the inhibitor by itself had no discernible effect on NO synthesis (Figure 4-5).

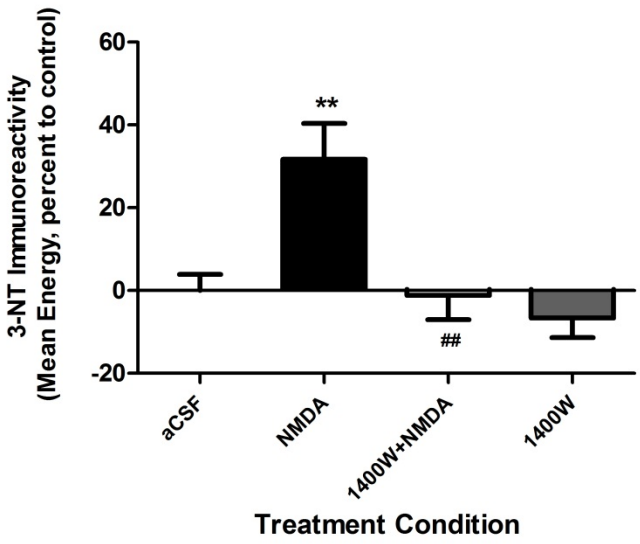


Figure 4-5: Source of NMDA-induced NO: iNOS? Simultaneous administration of the selective inducible nitric oxide synthase inhibitor (iNOS) 1400W and NMDA results in abrogation of the NMDA-induced synthesis of striatal 3-nitrotyrosine. Male ICR mice (n=6) were treated with an intrastriatal co-infusion of 1400W (100 µM) and NMDA (20 nM) and then sacrificed 24 hours after microinjection. 3-NT immunoreactivity was measured using confocal microscopy. Administration of 1400W reduced the NMDA-induced striatal production of 3-NT to basal levels (**$p<0.01$ as compared to aCSF group, $^{\#\#}p<0.01$ as compared to NMDA group).

4.4. Discussion

There is a body of evidence that substantiates SP as playing an exacerbating role in METH-induced toxicity. As previously mentioned, pharmacological inhibition of signaling through its receptor resulted in impressive levels of protection from dopamine terminal degeneration as well as striatal cell loss (Yu et al., 2004; Zhu et al., 2006). Furthermore, complete elimination of all striatal NK-1Rs resulted in protection from striatal cell loss (Zhu et al., 2009). However, our understanding of the mechanisms by which SP could act in a toxic capacity is still nascent. There is however compelling experimental evidence from our lab that implies SP's mode of action as being pro-oxidant through an influence on NO synthesis within the nNOS containing interneuron. Especially in light of the fact that SP's receptor (NK-1R) is located on the SST/NPY/nNOS interneuron (Kaneko et al., 1993).

We have recently shown that immediately after treatment with METH there is robust trafficking (indicative of receptor activation) of the SP receptor in the SST/NPY/nNOS interneuron (Wang and Angulo, 2008; Wang and Angulo, 2011b). Also, use of the SP antagonist WIN-51,708 resulted in a significant reduction in 3-NT immunostaining (Zhu et al., 2009; Wang and Angulo, 2011a). SP may in some fashion contribute or have a modulatory role on the overproduction of NO, which is considered one of the effector's of METH-induced toxicity through its contribution to oxidative stress. A conceivable speculation since it has been suggested that SP has a modulatory influence on NO in other regions of the CNS (Radhakrishnan and Henry, 1995; Radhakrishnan et al., 1995; Jeon et al., 1999). SP's role may be through potentiation of the NMDA/nNOS signaling cascade within the SST/NPY/nNOS interneuron.

Glutamate release and signaling is a necessary component for toxicity to occur after METH, as demonstrated by many studies in which pharmacological modulation of glutamate signaling via the NMDA receptor resulted in a proportional effect on the severity of METH toxicity (Sonsalla et al., 1990; O'Dell et al, 1992; Stephans and Yamamoto, 1994). Of particular relevance is the presence of the NMDA receptor on the SST/NPY/nNOS interneuron (Kawagushi, 1997). An ionotropic subtype of glutamate receptors, NMDA receptors are typically known to modulate the entrance of extracellular calcium into neurons, which is a necessary component for nNOS activation and thus the subsequent production of NO (Strijbos et al., 1996; Almeida et al., 1998). The NK-1R, also located on this interneuron, is a GPCR (Saria, 1999) that has been implicated in the potentiation of the NMDA receptor through one of its intracellular signaling pathways (Wu et al., 2004). In the spinal cord and other structures in the CNS, there exist a relationship between glutamate signaling via the NMDA receptor and SP transmission (Beal et al., 1991; Garside and Mazurek, 1997). In dorsal horn neurons of the spinal cord, it has been shown that NMDA receptor activation resulted in the release of SP (Liu et al., 1997).

In our study we sought to further demonstrate that SP may be augmenting glutamate signaling within the striatum thus contributing to METH-induced toxicity. As expected, infusion of NMDA into the striatum caused a substantial amount of damage as measured by striatal cell loss. Automated stereological cell counts of Fluoro-Jade C positive cells showed that NMDA alone resulted in approximately 27% increase as compared to the baseline condition (aCSF). However, pretreatment with WIN-51,708 30 minutes prior to NMDA infusion brought that number back down close to control levels. The antagonist by itself had no effect and was close to baseline levels in Fluoro-Jade C positive cells. The results provide further corroboration that SP

signaling through the NK-1R may in some fashion modulate glutamate signaling via the NMDA receptor during exposure to METH and thus contribute to its neurotoxic effects. Furthermore, that SP's pro-toxic influence is mediated through the potentiation of NO synthesis.

As we sought to demonstrate in the proceeding study in which we attempted to clarify the influence of SP on NMDA-induced NO synthesis. Although when employing METH we already have an optimal temporal range for NO production, in this study we were only utilizing NMDA to mimic METH damage and translate the results to a METH paradigm. In particular, to highlight the involvement of glutamate and SP's modulation thereof during METH. As a result we had to find the optimal time point for NMDA-induced 3-NT synthesis.

As such we tested three different times, which were 6, 12, and 24 hours post-NMDA. The resulting time point with a statistically significant level of 3-NT was 24 hours. However, as can be seen in figure 4-2 there was a visible trend over time with a clear increase commencing at least at 12 hours (15%). By 24 hours there is an approximate three-fold increase in 3-NT staining as compared to 12 hours. Thereafter we utilized the 24 hour time point to elucidate SP's role in NMDA-mediated striatal NO synthesis. An additional set of animals were treated with WIN-51,708 prior to NMDA, however this time we measured 3-NT immunoreactivity at the time point previously determined. Whereas NMDA alone caused a 42% percent increase (compared to aCSF) in NO as measured by 3-NT. Application of WIN-51,308 before infusion of NMDA dropped that level to 18% as compared to baseline. The antagonist alone did not demonstrate a significant increase in 3-NT.

Attenuation of NMDA mediated 3-NT staining due to inhibition of SP signaling hints at the possible mechanism by which SP is contributing to METH-toxicity. Lending support to our working hypothesis that METH-induced DA overflow will initiate the excessive release of SP. SP will in turn signal through the NK-1R localized on SST/NPY/nNOS and as a result potentiate NMDA mediated activation of nNOS and thus superfluous synthesis of NO. SP could accomplish this through three mechanisms. The first is through an increase in corticostriatal glutamate release, as demonstrated by Marti et al. (2005); SP can induce secondary glutamate release in the striatum. The second is through indirect manipulation of the NMDA receptor (Lieberman and Mody, 1998; Wu et al., 2004), which maintains the receptor open for a longer period of time thus amplifying intracellular calcium influx. The third is by activating the DAG/IP3 pathway (Wu et al., 2004), which mobilizes calcium from intracellular stores within the endoplasmic reticulum. All three mechanisms either directly or indirectly through modulation of glutamatergic activity elevate the levels of intracellular calcium thus increasing the catalytic activity of the calcium-dependent nNOS enzyme.

Although we have observed and documented that METH causes the excessive production of NO within the striatum, follow up studies demonstrated that it was not through an increase in the expression of nNOS (Wang and Angulo, 2011a). Leading to the conclusion that the NO being produced was a not through manipulation of protein expression but rather through an increase in the enzymatic activity of nNOS already present in the striatum. This observation coincides with the above proposed mechanisms of SP's action, as each would provide the necessary components needed for the excessive activation of nNOS. The abundant and sustained influx of calcium will increase the availability of the active form of calmodulin (CaM), which

binds with nNOS acting like a molecular switch that allows for the production of NO (Duncan and Heales, 2005; Chang and Reynolds, 2006). Moreover, when we infused the SP agonist GR-73632 directly into the striatum there was a visible and significant increase in the density of 3-NT staining around the injection (Wang and Angulo, 2011a). However, simultaneous injection of the SP agonist with a selective inhibitor of CaM (calmidazolium chloride) resulted in a significant attenuation in the observed 3-NT staining, providing further evidence that SP's contribution to METH-induced NO synthesis is via a calcium dependent mechanism.

Although there exist three isoforms of NOS the enzyme responsible for NO synthesis, most of the evidence links nNOS as the primary source of NO during pathological conditions (Dawson and Dawson, 1996). A study by Deng and Cadet (1999) showed that METH increased the expression of striatal nNOS positive neurons but they did not see any iNOS positive neurons in any of the brain areas analyzed. However, in mice lacking the gene for iNOS there was a reduction in METH's effect on dopaminergic striatal markers (Itzhak et al., 1999). The protective affect was attributed to the accompanying decrease in METH-induced hyperthermia. Thermal deregulation is another element of METH-toxicity and has been attributed with playing some undefined role in its damaging effects (Eisch and Marshall, 1998; Riddle et al., 2006).

In the brain iNOS is expressed in large quantities primarily by activated glia as part of an immunological response (Boje, 2004; Duncan and Heales, 2005). Furthermore, iNOS activation unlike nNOS is calcium-independent and relies on transcriptional activation (Lecanu et al., 1998; Duncan and Heales, 2005) thus it is considered a more sustained synthesis and subsequent release of NO. Glutamate can activate microglia through the NMDA receptor (Thomas and Kuhn, 2005). Reactive gliosis is an element of METH; however whether it is a response to the

associated neuronal damage or a contributing factor to the pathology is still uncertain (Krasnova and Cadet, 2009). There is however data that supports the latter assumption, LaVoie et al. (2004) demonstrated that there were activated microglia in many brain regions where there was METH toxicity and most importantly in the striatum microgliosis preceded DA terminal degeneration. Furthermore, activation was most pronounced in the striatal subregions that presented the greatest depletion in DAT binding and TH levels. Using an NMDA receptor antagonist, Thomas and Kuhn (2005) blocked microglial activation and showed protection from METH-induced toxicity to DA terminals. Also microglia have been shown to release a number of harmful substances including oxidants and NO, which would exacerbate toxicity (Riddle et al., 2006; Block et al., 2007; Krasnova and Cadet, 2009). Current research has pointed to over activation of microglia as playing a central role in the pathology of neurodegenerative diseases such as Parkinson's (Liu, 2006; Block et al., 2007).

In this study we sought to determine the NOS isomer contributing to the NMDA-mediated NO. We utilized 7-NI to inhibit nNOS and 1400W for iNOS; we measured NO at the previously determined time point (24 hours post-NMDA). Inhibiting nNOS surprisingly did not significantly reduce NO synthesis (~11%); however inhibition of iNOS via 1400W completely abrogated the NMDA-mediated increase in 3-NT. The result can be considered unexpected in light of the evidence I have been presenting thus far, however there are two important considerations to keep in mind when interpreting this data. First, it is that up to this point evidence in support of our working hypothesis has been gathered in studies using METH as the agent of toxicity. Whereas in the preceding studies discussed we only used NMDA in order to isolate the involvement of glutamate within a METH paradigm but ultimately the translation will

not be an exact one. For instance one glaring difference is the time course for NO production and the resulting levels. When employing METH and measuring 3-NT production via HPLC, significant levels of 3-NT were detected as early as 1 hour post-METH and continued to be present past 24 hours at which point it was 6 fold greater than the control (Zhu et al., 2009). Measuring via 3-NT immunoreactivity, there was a substantial increase as compared to baseline as early as 4 hours (Wang and Angulo, 2011a). However, when only treating with NMDA we begin to see a significant increase only at 24 hours. Said differences are going to be present since METH causes the sustained and excessive release of many neurotransmitters and neuropeptides, such as DA, glutamate, GABA, SP as well as other factors such as hyperthermia and reactive gliosis that can compound NO synthesis (Yoshihara et al., 1996; Riddle et al., 2006; Frankel et al., 2007; Krasnova and Cadet, 2009).

There is however one factor that could explain the divergence of our result in our NMDA model with all the data we have provided thus far from our METH studies and that is the time course of microglial activation. Regarding NMDA and microglial reactivity there are a very limited set of comparable studies however they seem to imply that microglial activation would most likely precede the time point for our studies (24 hours). In a study using a mix culture of rat spinal cord neurons and glia, microglia activation was seen beginning 4 hours after NMDA treatment (Tikka and Koistanaho, 2001). More relevant is an *in vivo* rat excitotoxicity model in which treatment was similar to our own, NMDA (50 nM) was directly infused into the cortex and microglial activation was measured at several time points after treatment (Acarin et al., 1996). Investigators observed a pronounced microglial activation commence 10 hours after cortical infusion of NMDA and remain active for several days post-NMDA. If that is so, then we

could expect that for our particular time point the NO being produced would be from a glial source and thus from iNOS as seen in the data.

On the other hand, in all of our METH studies we saw an early induction of NO synthesis that began within 2 hours and remained past 32 hours (Zhu et al., 2009). Based on a temporal analysis of microglia activation, there was significant striatal activation starting at 24 hours reaching a peak 48 hours after METH (LaVoie et al., 2004). This may explain why Deng and Cadet (1999) did not see any iNOS immunoreactivity since their study only extended to 24 hours. Therefore if iNOS is indeed primarily expressed by activated glia then it is possible that their study preceded the timeframe of expression. Importantly, that the 3-NT immunoreactivity present in our METH studies was most likely attributed to nNOS activation thus coinciding with Deng and Cadet (1999). Additionally, since peak apoptosis is at 24 hours and markers of DA terminal damage appear to reach their peak at least an additional 24 hours later (Zhu et al., 2005), then iNOS mediated NO formation may instead play a role in DA terminal injury as well as be a large contributor to the NO present after 24 hours.

Chapter 5 Homeostatic Role of Striatal Neuropeptides During METH-Induced Toxicity

5.1. Neuropeptide Y

Thus far we have presented evidence to the effect that the striatal neuropeptide SP plays a role in METH-induced toxicity. More specifically that SP signaling through the NK-1R is potentiating the effects of METH on the striatum thus enhancing its destructive capacity within this structure (Zhu et al., 2009). Furthermore, that it may do so by enhancing glutamate signaling (section 4.1.) and its subsequent activation of NO producing enzymes thus promoting an oxidant state (section 4.2.). In light of the importance of striatal neuropeptides in modulating the function of the striatum as well as the presence and apparent release of several peptides after METH treatment (Yoshihara et al., 1996; Frankel et al., 2007) we questioned whether any of these could then serve as a counterbalance to SP. Based on a plethora of literature classifying NPY and SST as protective in glutamate-mediated toxicity, they seemed like adequate candidates of further investigation. Especially since it is widely known that glutamate excitotoxicity is an integral element to METH neurotoxicity. Therefore in the proceeding sections I will present the results of the studies conducted on NPY and SST and their role in METH-induced toxicity.

5.1.1. NPY-Y1R & NPY-Y2R METH Tyrosine Hydroxylase and Fluoro-Jade C

In several *in vitro* and *in vivo* studies of excitotoxicity, NPY has acted in a neuroprotective capacity. Using primary mouse hippocampal and cortical neuronal cultures Śmialowska et al. (2009) demonstrated that administration of NPY protected cells from kainite-induced cell death even when administered hours after the toxic insult. In addition, application in

rats of an NPY-Y2R agonist 30 minutes after treatment with kainate resulted in a potent reduction in the size of the lesion as compared to kainate alone. Also, in organotypic cultures of rat hippocampal slices, Silva et al. (2003) found that agonist for several subtypes of the NPY receptors conferred significant protection from AMPA and kainate induced neuronal degeneration. NPY's neuroprotective role appears to extend to 3,4-methylenedioxymethamphetamine toxicity (MDMA), the recreational drug commonly as ecstasy (Álvaro et al., 2008) and METH-induced toxicity as well (Thiriet et al., 2005). However the mechanism by which it mediates this affect has been hinted at but requires more thorough investigation. It is primarily thought to do so through modulation of glutamatergic transmission via depression of neuronal excitability (Silva et al., 2005; Wang, 2005; Álvaro et al., 2008).

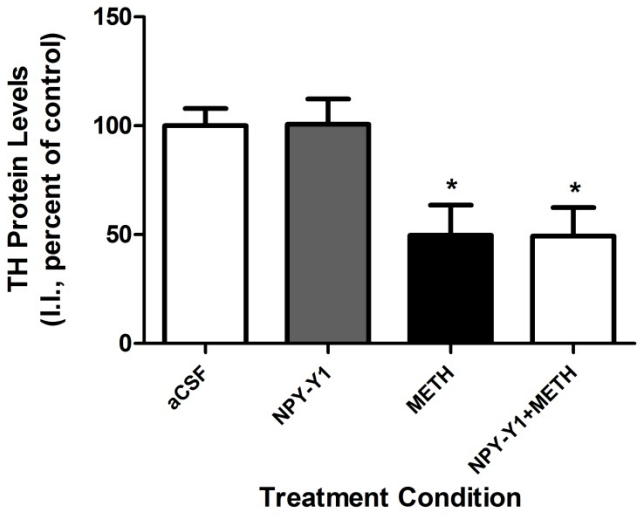
We intended to surmise whether NPY's neuroprotective action is through the indirect attenuation of the NMDA/NO cascade thus serving as a counter to SP's influence. In the striatum NPY is synthesized within the SST/NPY/nNOS interneuron, which also produces NO (Partridge et al., 2009). Said interneuron has been shown to synapse with corticostriatal terminals and the dendrites of striatal projection neuron (Kawagushi et al., 1995; Tepper and Bolam, 2004). Both factors lend credence to the hypothesis that NPY can play a central role in the mitigation of excessive NO production whether in an autocrine or paracrine fashion. Work in our lab has provided proof to that effect as it has shown both NPY receptor agonists (NPY-Y1R and NPY-Y2R) to attenuate 3-NT levels in a dose-dependent manner (unpublished data). It is from these studies that we have derived the optimal dose (20 μM) so as to conduct the next series of experiments. Foremost, can NPY protect from METH-induced injury to the striatum in addition to reducing NO formation? Does that protection extend to dopamine terminals in addition to

striatal cells? Then, if NPY can protect, does it do so through modulation of glutamate signaling via the NMDA subtype receptor? The next series of experiments sought to answer these questions.

To investigate this we used a nonpeptide selective agonist for the NPY-Y1 and Y2 receptor since literature indicates that NPY exerts its protective effects within the striatum through these subtypes (Thiriet et al., 2005). We administered the NPY-Y1R agonist (Leu^{31}, Pro34) neuropeptide Y (20 µM) or the NPY-Y2R agonist neuropeptide Y (3-36) (20 µM) via microinjection directly into the striatum whereas aCSF was injected into the contralateral hemisphere as described in Methods section 3.2. The dosage was determined through a dose response curve (unpublished results) using 3-NT production as an assessment of striatal injury. Fifteen minutes after injection the mice were given a systemic bolus toxic injection of METH (30 mg/kg); another set of animals were given an i.p. injection of saline and served as the baseline. Animals used to measure TH to determine dopamine terminal viability were sacrificed 72 hours after METH treatment by decapitation, their brains snap frozen and stored in -80ºC until ready for analysis. TH protein levels were determined via western blot analysis (Methods section 3.5). Seventy-hours were chosen based on Zhu et al (2005), which shows that TH depletion has reached its peak at this point. Damage to neurons intrinsic to the striatum was determined by counting cells positive for Fluoro-Jade C staining as performed in section 4.1 (Methods section 3.7). In brief, animals used for that purpose were sacrificed 24 hours after treatment via perfusion. The brains were post-fixed, placed in 30% sucrose until they dropped in the solution, then stored into -80°C until processed for Fluoro-Jade C staining as described in Methods section 3.6.

Figure 5-1A demonstrates that as expected METH caused a significant decrease in the striatal protein levels of TH. However, intrastriatal infusion of the NPY-Y1R agonist (Leu31,Pro34)-NPY before METH treatment. TH levels remained the same as the METH alone group thus NPY signaling via the NPY-Y1 receptor does not confer protection on dopamine terminals. The agonist alone did not have a detrimental effect, TH levels remained equivalent to baseline levels. Like the NPY-Y2R agonist, the NPY-Y2 receptor agonist (Neuropeptide Y 13-36) also leaves TH levels unchanged and comparable to baseline levels (Figure 5-1B). Conversely, pretreatment with the NPY-Y2 receptor agonist attenuated the METH-induced reduction in TH levels thus implying that dopamine terminals were protected.

(A)



(B)

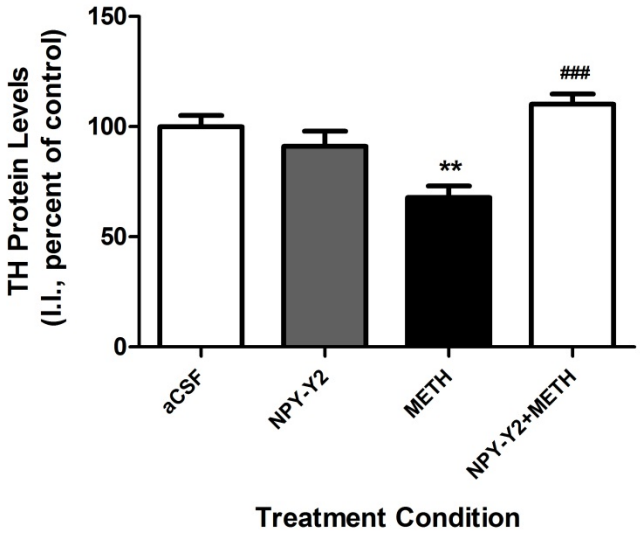
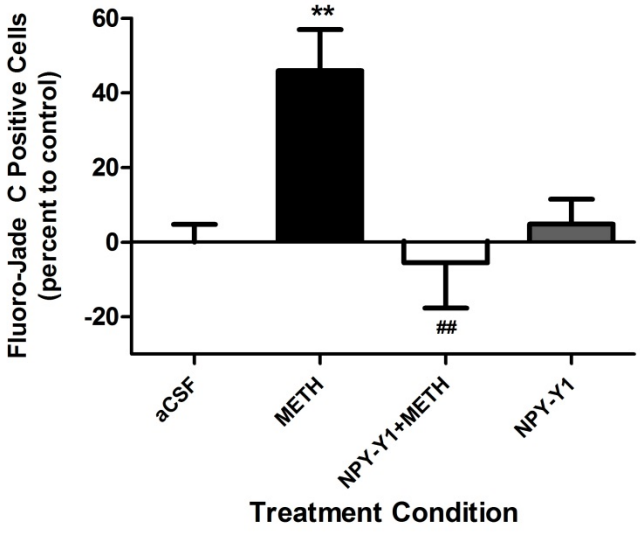


Figure 5-1: Effect of NPY agonists on striatal TH levels after METH. NPY-Y1R ([Leu31,Pro34]-Neuropeptide Y) and NPY-Y2R (Neuropeptide Y 13-36) agonists effect on the METH-induced reduction in the striatal levels of tyrosine hydroxylase (TH). Mice (n=6) received intrastriatal infusions of aCSF (right striatum) or NPY receptor agonist (left striatum); followed 15 minutes later by an injection of METH (30 mg/kg, i.p.) or saline. Animals were sacrificed at 72 hours after METH treatment. TH protein levels were measured via western blot analysis. **(A)** Treatment with an NPY-Y1 receptor agonist had no effect on the METH-induced drop in striatal TH levels. **(B)** However, microinjection of a selective NPY-Y2 receptor agonist attenuated the METH-induced significant reduction in striatal TH levels (* $p<0.05$, ** $p<0.01$ as compared to the aCSF group, $^{###}p<0.001$ as compared to the METH group).

In regards to striatal neurons (Figure 5-2), NPY signaling through both receptors conferred protection that was equivalent to baseline levels. As expected, treatment with METH alone caused a significant amount of cell loss; said loss was approximately 35-40%. Treatment

with either NPY agonist on its own did not result in a significant increase of apoptosis relative to baseline levels (aCSF intrastriatal and saline i.p.).

(A)



(B)

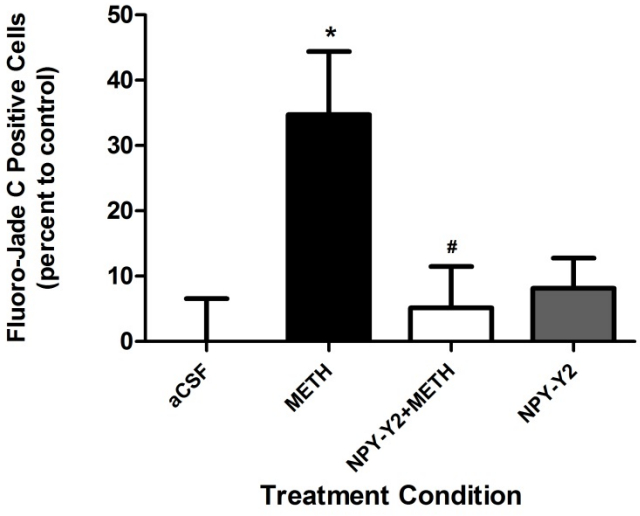


Figure 5-2: NPY agonists protect striatal cells from METH. Pretreatment with agonists for the NPY-Y1 and Y2 receptor had a significant protective effect on METH-induced striatal cell loss as measured by Fluoro-Jade C staining. Mice (n=6) received intrastriatal infusions of an NPY receptor agonist; followed

15 minutes later by an injection of METH (30 mg/kg, i.p.) or saline. Animals were sacrificed at 24 hours after METH treatment. Striatal cell loss was measured using automated stereological cell counts. **(A)** Treatment with an NPY-Y1 receptor agonist protected from METH-induced cell loss. **(B)** As did microinjection of a selective NPY-Y2 receptor agonist (* $p<0.05$, ** $p<0.01$ as compared to the aCSF group, $^{\#}p<0.05$ as compared to the METH group).

5.1.2. NPY-Y1R & NPY-Y2R NMDA 3-Nitrotyrosine and Fluoro-Jade C

As mentioned in section 5.1.1., many of the studies that demonstrated NPY's neuroprotective capacity have primarily been done utilizing models of glutamate excitotoxicity (Silva et al., 2003; Śmialowska et al., 2009). This is because one of NPY's functions is the modulation of neurotransmitter release (Sun et al., 2001; Adewale et al., 2007), included on that list is the modulation of glutamatergic transmission (Lynch et al., 1989; Van den Pol, 1996; Álvaro et al., 2008). NPY is thought to perform its function through the dampening of calcium channel influx either at the dendrite/somatic targets or at the axon terminal (Chen and Van den Pol, 1996; Quian et al., 1997; Rhim et al., 1997). Like SP, NPY's receptors are GPCRs that can in addition to modulating voltage-gated ion channels have been implicated in modulating ligand-gated channels, specifically glutamatergic receptors (van den Pol et al., 1996).

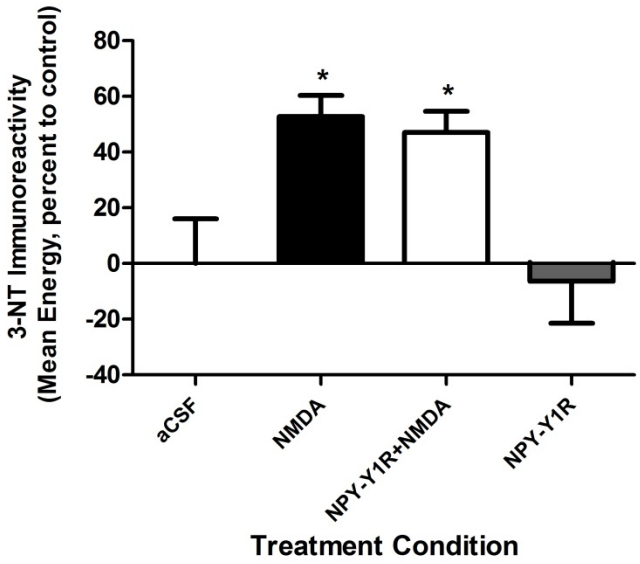
Therefore it is reasonable to consider that NPY could serve as a counteragent to SP's influence on the NMDA/NO cascade through its inhibitory effect on excitatory signaling. In the next series of experiments we sought to parse out whether it is through glutamatergic transmission that NPY was able to a) reduce the levels of METH-induced NO and b) serve as a neuroprotectant during METH. Thus if it this were its mode of action then it should have an

equivalent effect on NMDA-induced 3-NT production as well as translate to a protective effect on NMDA-induced striatal cell loss.

We will have two sets of animals, in the control condition we will infuse the NPY-Y1R agonist (Leu^{31}, Pro34) NPY (20 µM) or the NPY-Y2R agonist NPY (3-36) (20 µM) via microinjection directly into the left hemisphere and aCSF into the right hemisphere as described in Methods section 3.2. For the experimental condition, a mixture of the agonist and NMDA will be injected into the right hemisphere. NMDA (20 nM) will be infused by itself into the left hemisphere. For both 3-NT and Fluoro-Jade C staining the animals are sacrificed 24 hours after treatment via perfusion. The brains are dissected, post-fixed, cry-protected, and stored (-80°C) in the same manner. Procedures for both stains and the method of quantification for each are further discussed in Methods sections 3.3-3.4 (3-NT) and sections 3.6-3.7 (Fluoro-Jade C).

Figure 5-3 shows that as expected NMDA alone caused a substantial production of NO as compared to baseline. Surprisingly, treatment with either an NPY-Y1 or Y2 receptor agonist had no effect on NMDA-induced striatal NO synthesis. Neither agonist alone caused any significant production of 3-NT.

(A)



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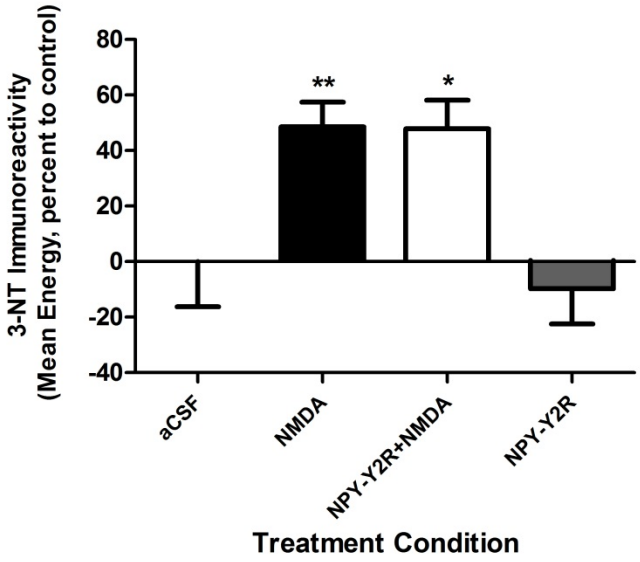
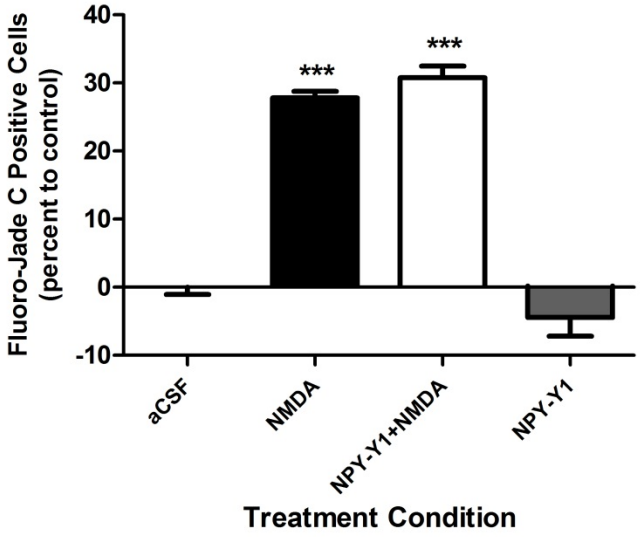


Figure 5-3: Assessment of NPY agonists' modulation of NMDA-induced NO. Both the NPY-Y1 and Y2 receptor agonists had no effect on the NMDA-induced increase in 3-nitrotyrosine (3-NT) striatal levels. Mice (n=6) received co-infusion of an NPY receptor agonist and NMDA. Animals were then sacrificed at 24 hours after NMDA treatment. 3-NT immunoreactivity was measured using a Leica

scanning confocal microscope and the accompanying Leica imaging software. **(A)** Treatment with an NPY-Y1 receptor agonist had no effect on the NMDA-induced increase in 3-NT striatal level. **(B)** Neither did infusion of an NPY-Y2 receptor agonist. (* $p<0.05$, ** $p<0.01$ as compared to the aCSF group).

In regards to striatal neurons (Figure 5-4), NPY signaling through both receptors did not protect striatal injury caused by NMDA treatment, cell loss was comparable to NMDA levels (approximately 30%-40%). Treatment with either NPY agonist on its own did not result in a significant increase of apoptosis relative to baseline levels (aCSF intrastriatal and saline i.p.).

(A)



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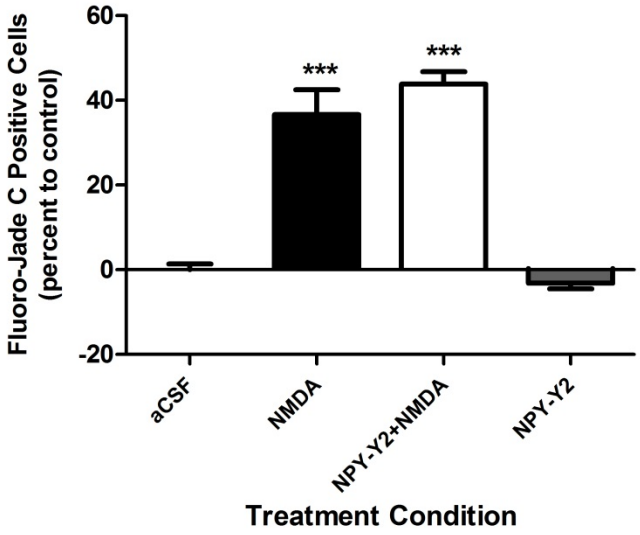

Figure 5-4: NPY and excitotoxicity in the striatum. Intrastriatal infusion of either NPY receptor agonist with NMDA had no effect on the NMDA-induced striatal cell death. Mice (n=6) received intrastriatal infusions of aCSF (right striatum) or an NPY receptor agonist (left striatum). Animals were sacrificed at 24 hours after NMDA treatment. TH protein levels were measured via western blot analysis. **(A)** Treatment with an NPY-Y1 receptor agonist had no effect on the METH-induced drop in striatal TH levels. **(B)** However, microinjection of a selective NPY-Y2 receptor agonist prevented the significant reduction in striatal TH levels (***$p<0.001$ as compared to the aCSF group).

5.2. Somatostatin

SST like NPY has been shown to serve a neuroprotective function in glutamate-mediated excitotoxicity. However, there seems to be a dearth of literature as to its role in METH-induced toxicity. Studies indicate that SST's modulatory action is akin to NPY, in that it exerts control over voltage-gated calcium channels primarily in an inhibitory fashion through activation of GPCR-mediated intracellular pathways (Forloni et al., 1997; Rodriguez-Sanchez et al., 1997).

We postulated that SST would indirectly attenuate activation of the NMDA/NO pathway through the SST_2 receptor as well as minimize METH- and NMDA-induced striatal injury.

5.2.1. SST METH 3-Nitrotyrosine

If SST is serving as a counterbalance to the pro-oxidant influence of SP than it stands to reason that it would do so by counteracting the METH-induced increase in NO synthesis. In doing so it would pull the system back in a homeostatic fashion from the push that results from SP signaling. Furthermore, as stated previously, SST is produced in the very same interneuron that has the NK-1 and NMDA receptor as well as nNOS (Kaneko et al., 1993; Kawagushi et al., 1995; Kumar, 2008), so it is in a downstream position to become active and have an interceptive role during the ongoing cascade that leads to toxicity. Therefore it is our hypothesis that introduction of an SST analogue during METH treatment will modulate NO synthesis, particularly to reduce its production.

To accomplish this we treated the animals with an intrastriatal infusion of the SST analogue OCT, 15 minutes before a bolus i.p. injection of METH (30 mg/kg) or saline. Octreotide has the highest affinity for the SST_2 receptor followed by the SST_5 receptor (Cervia et al., 2008), which in the striatum is primarily localized on the projection neurons (Allen et al., 2003). It has been confirmed that SST signaling through the SST_2 receptor found on the projection neurons can adjust the firing rate of these neurons and consequently the output of the striatum (Galarraga et al., 2007) therefore it would have the highest probability of having a protective influence. In addition, in many of models in which SST has been found to be protective it is through SST receptors subtype 2 and 5 (Rauca et al., 1997; Cervia et al., 2008;

Kiagiadaki and Thermos, 2008; Kiagiadaki et al., 2010). Since we didn't have an established dose for our particular model of toxicity we chose three different dosages to test (0.1 nM, 1.0 nM, and 10 nM) based on Rauca et al. (1997). They tested the very same dosages in a model of excitotoxicity. For the agonist alone condition we tested both 0.1 nM and the 10 nM dosage.

The SST analogue octreotide was chosen on a number of criteria, first that it has the highest affinity for the $SSTR_2$, which is the most abundant in the striatum as current experimental data implicates it is located on the striatal projection neurons (Galarraga et al., 2007). Therefore it would be the most plausible receptor to have an impact on METH-induced toxicity. Second, that it demonstrated neuroprotection in other models of damage within the CNS such as ischaemia (Cervia et al., 2008). Especially models that were testing glutamate based neurotoxicity. The mice were then perfused 6 hours after METH treatment. The time point was based on previous work in our lab that determined the time course for 3-NT production after METH treatment (Zhu et al., 2009). The tissue was then prepared for 3-NT fluorescent staining and quantification.

As seen in the data graph (Figure 5-5), METH caused a potent and significant increase in 3-NT staining. However, the addition of OCT resulted in a significant dose dependent decrease in the METH-induced 3-NT staining, specifically the 1.0 nM and the 10 nM dosages. The 10 nM dosage was the most effective and was chosen as the dosage for all proceeding SST studies. The agonist alone condition matched aCSF in staining intensity. Another set of animals were used to test the 10 nM OCT dosage by itself, which did not have significant increase on 3-NT staining compared to the baseline condition (data not shown).

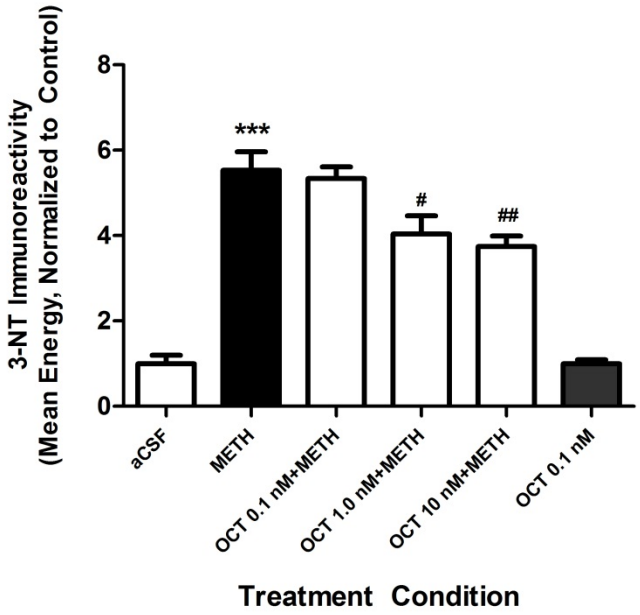

Figure 5-5: SST analogue (octreotide) modulation of METH-induced 3-NT. Pretreatment with the SST analogue octreotide (OCT) caused a dose dependent decrease in METH-induced striatal 3-nitrotyrosine levels (3-NT). Mice (n=6) received intrastriatal infusions of aCSF (right striatum) or OCT (left striatum); followed 15 minutes later by an injection of METH (30 mg/kg, i.p.) or saline. Animals were sacrificed 6 hours after METH treatment. 3-NT levels were determined utilizing confocal microscopy and Leica imaging software. Treatment with an SST analogue attenuated METH-induced NO synthesis as measured by 3-NT immunohistochemistry (***$p<0.001$ as compared to the aCSF group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ as compared to the METH group).

5.2.2. SST METH Tyrosine Hydroxylase and Fluoro-Jade C

Will the significant decrease in 3-NT staining translate to a visible and significant protective effect as measured by well-established parameters of METH toxicity? Namely dopamine terminal damage and striatal cell loss. It is the purpose of the following two

experiments to determine that very question. Here we were interested in examining whether SST could confer protection on the METH-induced toxicity on dopamine terminals as measured by striatal TH protein levels and from the apoptosis of neurons native to the striatum (Fluoro-Jade C)

To determine the answer to our query, animals were treated in a similar fashion as in 5.2.1. In brief, both the control and experimental condition were treated with intrastriatal infusions of the SST analogue OCT (10 nM) in one hemisphere and aCSF in the other hemisphere. Fifteen minutes after the surgery the control group was i.p. injected with saline and the experimental condition with METH (30 mg/kg). Animals used for TH analysis were sacrificed 72 hours later via decapitation. Their brains were snap frozen and then stored in -80°C until used for western blot analysis and quantification (Methods section 3.5). Animals used for Fluoro-Jade C analysis were intracardially perfused and fixed 24 hours after treatment and processed for Fluoro-Jade staining and cell counts (Methods sections 3.6-3.7).

As seen in the figure (Figure 5-6) below, animals treated with METH showed the expected and significant reduction in TH levels. Animals pretreated with OCT did not demonstrated protection of dopamine terminals, as seen below TH levels remained almost equivalent to METH levels. Although the OCT alone group's TH levels were comparable to baseline levels.

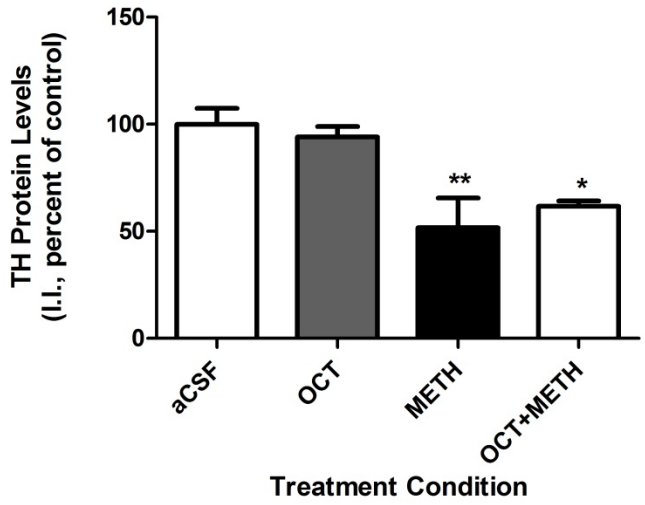

Figure 5-6: Effect of octreotide of striatal TH levels post METH. Pretreatment with the SST analogue octreotide (OCT) did not protect from the METH-induced reduction in the striatal levels of tyrosine hydroxylase (TH). Mice (n=6) received intrastriatal infusions of aCSF (right striatum) or OCT (left striatum); followed 15 minutes later by an injection of METH (30 mg/kg, i.p.) or saline. Animals were sacrificed at 72 hours after METH treatment. TH protein levels were assessed by western blot analysis. Treatment with an SST analogue did not protect from METH-induced dopamine terminal toxicity (* $p<0.05$, ** $p<0.01$ as compared to the aCSF group).

However, in regards to cell loss, pretreatment with OCT showed a significant protection that almost reached control baseline levels. The agonist by itself showed no effect whereas METH as expected had a substantial and significant increase in cell loss (Figure 5-7).

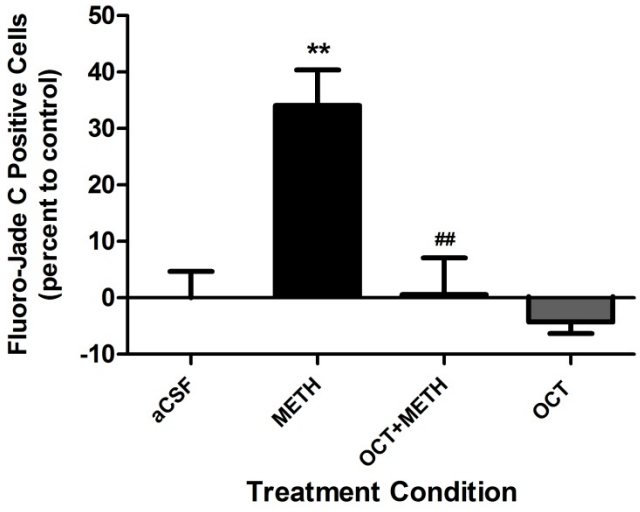

Figure 5-7: Protection from METH-induced cell death by SST analogue. Pretreatment with an SST analogue (OCT) had a significant protective effect on METH-induced striatal cell loss as measured by Fluoro-Jade C staining. Mice (n=6) received intrastriatal infusions the SST analogue OCT; followed 15 minutes later by an injection of METH (30 mg/kg, i.p.) or saline. Animals were sacrificed at 24 hours after METH treatment. Striatal cell loss was measured using automated stereological cell counts of Fluoro-Jade C positive cells. Treatment with OCT protected from METH-induced cell loss. (**$p<0.01$ as compared to the aCSF group, $^{\#\#}p<0.01$ as compared to the METH group).

5.2.3. SST NMDA 3-Nitrotyrosine and Fluoro-Jade C

As with NPY, one of the main reasons for investigating SST as a neuroprotectant agent during METH neurotoxicity is that it has been observed to serve said role in glutamatergic-induced toxicity (Forloni et al., 1997; Kumar, 2008). SST's endogenous function as a modulator of glutamate transmission has been often cited as the means by which it can protect from excitotoxicity (Momiyama and Zaborszky, 2006; Tallent and Qiu, 2008). Although in our

analysis SST may have failed to protect dopamine terminals; it did have a strong protective effect in regards to striatal cell loss (5.3.2.). In addition, we demonstrated that it had a mitigating influence on NO synthesis thus reducing the oxidant state caused by METH (5.3.1.). Glutamate is an important and intrinsic component of METH-induced toxicity and in NO production. Activation of the ionotropic NMDA receptor located on SST/NPY/nNOS interneuron allows for the entrance of extracellular calcium, which is a necessary ingredient for the activation of nNOS and thus the synthesis of NO. Therefore, testing SST's modulation of glutamate signaling appears as a reasonable and logical first step in trying to elucidate the mechanism by which SST was able to modulate 3-NT immunoreactivity and confer protection on striatal neurons from METH-induced apoptosis.

We will have two sets of animals, in the control condition we will administer OCT (10 nM) via microinjection directly into the left hemisphere and aCSF into the right hemisphere as described in Methods (section 3-2). For the experimental condition, NMDA (20 nM) was infused into one hemisphere whereas OCT together with NMDA was infused into the contralateral hemisphere. The animals were sacrificed 24 hours later. Since the time point is equivalent for 3-NT as it is for Fluoro-Jade C in regards to NMDA, then tissue from the same brain could be used for the different analysis and quantification.

Treatment with the SST analogue did not have any effect on NMDA-induced NO production. The agonist by itself matched baseline levels in 3-NT immunoreactivity (Figure 5-8).

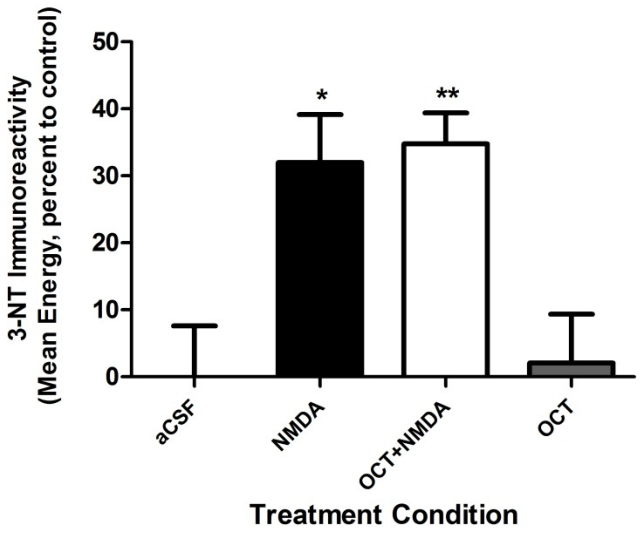

Figure 5-8: SST and NMDA-induced striatal NO production. Co-infusion of NMDA with OCT (SST analogue) had no effect on the NMDA-induced increase in 3-nitrotyrosine (3-NT) striatal levels. Mice (n=6) received an intrastriatal microinjection of a mixture of OCT and NMDA. Animals were then sacrificed at 24 hours after NMDA treatment. 3-NT immunoreactivity was measured using a Leica scanning confocal microscope and the accompanying Leica imaging software. Treatment with OCT had no effect on the NMDA-induced increase in 3-NT striatal levels. (* $p<0.05$, ** $p<0.01$ as compared to the aCSF group).

Treatment with OCT did not have any significant effect on NMDA-induced apoptosis. The agonist by itself matched baseline levels of Fluoro-Jade C positive cells (Figure 5-9)

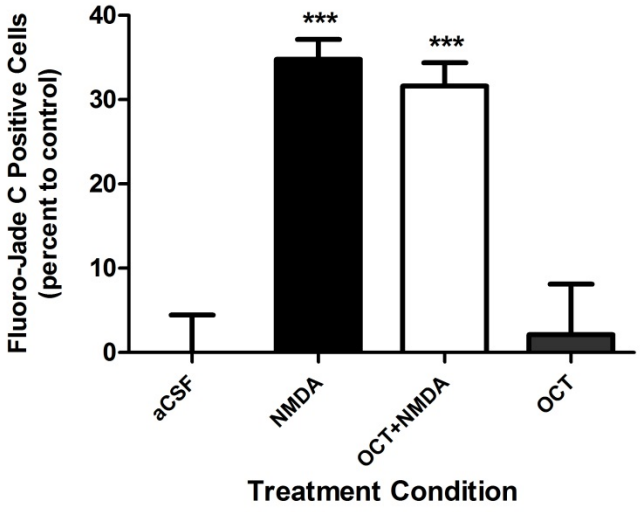

Figure 5-9: SST and NMDA-mediated striatal injury. SST does not confer protection to striatal cells from the NMDA-induced striatal cell death. Twelve-week old male ICR mice (n=6) received co-infusion of an SST receptor agonist (OCT) and NMDA. Animals were then sacrificed 24 hours after NMDA treatment. Cell death was measured via unbiased stereological cell counts of Fluoro-Jade C positive cells. Treatment with OCT had no effect on the NMDA-induced striatal cell death whereas OCT on its own did not cause any significant increase in cell loss (***$p<0.001$ as compared to the aCSF group).

5.3. Discussion

In chapter 4 we provided compelling experimental evidence as well as references to a body of literature that substantiates the striatal neuropeptide SP of being functionally capable of exacerbating METH toxicity. Specifically through potentiation of NMDA signaling and in effect of NO synthesis therefore contributing to the excessive oxidative stress that serves as the effector of the toxic cascade triggered by METH. However, SP is not the sole neuropeptide synthesized within the striatum and studies have indicated that in the wake of METH there are a number of neuropeptides that are released in the striatum including SP, NPY, and SST (Rodriguez-Sanchez et al., 1997; Thiriet et al., 2005; Horner et al., 2006; Frankel et al., 2007). Neuropeptides have a central role in modulating the function and output of the striatum leading to the thought that there must be an endogenous balance present to counteract SP's influence. NPY and SST may serve as an opposing protective force that can counteract SP's detrimental effects on the striatum. As modulators of striatal activity and many of its transmitters primarily through inhibition, SST and NPY have the capability to play a pivotal role in the events following METH.

Both are synthesized and stored in the very same interneuron that has nNOS (Thiriet et al., 2005; Rajput et al., 2011a). The SST/NPY/nNOS may only comprise a miniscule percentage (~0.8%-2%) of the neurons that compose the striatum (Cicchetti et al., 2000; Tepper and Bolam, 2004) but they are neuroanatomically poised to have a critical role in the modulation of the structure since they are localized throughout the striatum (Allen et al., 2003). SST/NPY/nNOS interneurons are characterized as the second largest in size of the striatal neurons as well as having long axons that extend throughout the structure (Kawagushi et al., 1995). They synapse with projection neurons and with the terminals of glutamate releasing neurons from the cortex

(Hathway et al., 2001; Galarraga et al., 2007), both are neuronal populations that are important players in the events following METH. Finally, said interneurons are refractory to METH-induced toxicity and have proven to also survive during the course of progressive neurodegenerative diseases that affect the basal ganglia such as Huntington's (Forloni et al., 1997; Silva 2005; Horner et al., 2006; Zhu et al., 2006b; Rajput et al., 2011a). Their resistance to METH and excitotoxicity has been attributed to several factors, first their elevated levels of the mitochondrial radical scavenger MnSOD (Gonzalez-Zulueta et al., 1998). Second, the presence of the neuropeptides NPY (Thiriet et al., 2005) and SST (Kumar, 2008) have been shown to be intrinsic elements to the nNOS's interneuron survival. Thiriet et al. (2005) demonstrated that animals engineered not to express NPY were much more susceptible to METH-induced cell death than the wildtype mice. Using an antisense oligonucleotide Kumar (2008) was able to block SST in cultured rat striatal neurons after which the SST/NPY/nNOS interneurons were no longer resistant to glutamate excitotoxicity (NMDA and quinolinic acid mediated). The preceding provide two important considerations 1) SST/NPY/nNOS interneurons are present throughout METH exposure and thus able to exert their influence through the peptides they package and 2) SST and NPY are critical elements to the survival of their interneuron despite its production of pathological quantities of NO during a toxicological insult. Thus already one can imply their capacity to serve as neuroprotectants.

NPY has already been shown to protect from excitotoxic insults to the CNS especially in vulnerable areas like the hippocampus (Silva et al., 2003; Śmialowska et al., 2003; Śmialowska et al., 2009). In both *in vitro* as well as *in vivo* models of excitotoxicity, NPY has been able to mitigate the cell loss of structures exposed to toxic levels of glutamate (Silva et al., 2005). Of

particular clinical relevance, is NPY's ability to protect primary neuronal cultures when applied hours after the insult (Śmialowska et al., 2009) Also, exogenous application of NPY 30 minutes post kainate infusion resulted in a significant reduction in the hippocampal lesion volume of rats (Śmialowska et al., 2003). NPY has also been protective from toxicological insults such as from MDMA a recreational drug from the amphetamine family. Using a mixed rat retinal neuronal culture, Álvaro et al. (2008) was able to substantially mitigate MDMA-induced cell death both necrotic and apoptotic through the administration of exogenous NPY. Like in METH-toxicity it is thought that one of the significant factors to MDMA-induced neuronal degeneration is the production oxygen- and nitrogen-derived species (Álvaro et al., 2008) as well as the involvement of glutamate.

Therefore, it is not surprising that NPY's protective role also extended to METH-induced toxicity in the striatum of an *in vivo* rodent model (Thiriet et al., 2005). Of particular interest is the observation that in the wake of various types of insults to the brain such as METH administration (Horner et al., 2006), excitotoxic lesions (Silva et al., 2005), or seizure like activity (Patrylo et al., 1999); NPY immunoreactivity increases including the number of neurons expressing NPY mRNA and even altered NPY receptor expression. We have observed a similar phenomenon after treatment with a toxic dose of METH (30 mg/kg), employing RT-PCR we saw a significant elevation in striatal NPY mRNA (16 hours post-METH) indicative of an attempt to replenish a depleted supply of NPY after release (unpublished result). Overall these observations implicate that the brain is mobilizing an endogenously existing protective or counter response to a particular type of insult. Moreover, that said insult typically involves an element of excitotoxicity and oxidative stress. We have also seen that intrastriatal application of agonists for

the NPY-Y1 and Y2 receptor results in the dose-dependent decrease of excessive NO production thus dampening the formation of oxidants, inversely application of a Y1 or Y2 antagonist abolished the attenuation (unpublished results). NPY's influence on NO levels after METH treatment corroborates the above mentioned studies, which indicate that NPY functions as a neuroprotectant during excitotoxicity and METH-induced toxicity. However, in regards to METH, work done so far has only focused on METH-induced apoptosis of striatal cells (Thiriet et al., 2005) it would be novel to test whether that protection extended to the DA terminals.

We sought to determine whether NPY could confer protection to dopamine terminals via the Y1 and Y2 receptor. Utilizing the selective NPY-Y1 receptor agonist ([Leu31,Pro34]-Neuropeptide Y) we microinjected said agonist directly into the striatum 15 minutes prior to a systemic bolus toxic injection of METH. Treatment with the Y1 receptor agonist failed to protect dopamine terminals from METH as measured by TH protein levels. On the other hand, similar treatment with a Y2 receptor (Neuropeptide Y 13-36) agonist did confer protection since TH levels remained close to baseline levels in spite of treatment with METH.

The differing result can be attributed to the relationship that appears to exist between DA and NPY within the striatum (Aoki and Pickel, 1989; Adewale et al., 2005; Adewale et al., 2007). Studies in several regions and models have shown that the endogenous release of NPY may be DA receptor mediated (Bina and Cincotta, 2000; Gruber et al., 2002; Cao et al., 2007). This includes a study measuring NPY release in the rat ventral striatum after application of d-amphetamine, which concluded NPY efflux was mediated by both classes of DA receptors (Gruber et al., 2002). This may also extend to NPY biosynthesis; Bina and Cincotta (2000) showed that application of a D_1/D_2 agonist depressed elevated levels of NPY mRNA in the

mouse hypothalamus. Horner et al. (2006) found that post-METH application there was an elevation in NPY mRNA-expressing striatal neurons via a D_1 receptor-mediated mechanism. Reciprocally, NPY can modulate the synthesis of DA depending upon the NPY receptor it binds to (Aoki and Pickel, 1989; Adewale et al., 2005). The Y1 receptor thought to be located on the soma of the DA producing neuron appears to attenuate DA synthesis whereas the Y2 receptor may be present on the terminal of said neuron and can induce synthesis (Adewale et al., 2005). TH is the rate-limiting enzyme necessary for the production of catecholamines such as DA (Fibiger and McGeer, 1971; Adewale et al., 2007). The presence of TH in the striatum is used as an indicator of DA terminal viability.

Perhaps in addition to upregulating DA production in the aftermath of METH injury it may also exert an additional protective affect during METH via signaling through the Y2 receptor. During METH exposure the DA terminal is a highly toxic milieu that includes excessive levels of cytosolic DA, the harmful products of DA autoxidation, NO, ROS, and RNS (Krasnova and Cadet, 2009). Through its well characterized mode of action in dampening calcium influx, NPY may reduce the excessive calcium influx in a circumstance that is already perilous for the survival of the DA terminal. In addition, there is evidence that NPY via the Y1 or Y2 receptor is capable of activating extracellular signal-regulated kinase 1 (ERK1) and ERK2, which have been shown to protect from neuronal death (Thiriet et al., 2005). It is hypothesized that ERKs may confer protection via stabilization of mitochondrial function (Thiriet et al., 2005). As I have mentioned previously disruption of mitochondrial function can be detrimental to neuronal survival since it can lead to several harmful consequences such as the fatal disruption of

the cells energetics, unbalancing the delicate homeostatic control of intracellular calcium levels, and oxidative species production (Chang and Reynolds, 2006).

In concordance with Thiriet et al. (2005), we also found NPY to serve a neuroprotective function in regards to METH-induced cell death. Despite differences in our model that included dosing and treatment regimen as well as time point (we did 24 hours, they did 72 hours) our results were similar in nature, both agonists conferred substantial protection from METH-induced striatal cell death. Although they found the Y2R agonist to be more potent, we however saw an equivalent protective effect from both agonists. Both agonists were effective in protecting striatal cells from injury.

It has been theorized that the mechanism by which NPY confers protection is by dampening neuronal hyperexcitability through the modulation of calcium signaling pathways both pre- and postsynaptic (Aoki and Pickel, 1989; Álvaro et al., 2008). There is evidence that it is a G-protein-coupled mechanism in which NPY receptors are directly coupled to calcium channels thus inhibiting calcium influx (Van den Pol et al., 1996; Sun et al., 2001, said mechanism can apparently be abrogated by protein kinase C (Silva et al., 2005; Wang, 2005). Consequently NPY can mediate the release, usually in an inhibitory manner, of a number of neurotransmitters and neuropeptides as well as their transmission within the target neuron (Lynch et al., 1989; Aoki and Pickel, 1989; Wang, 2005). Highlighted often is its effect on glutamatergic transmission, which would indeed serve as an effective means to protect from excitotoxicity.

Van den Pol et al. (1996) observed that in neuronal cultures derived from the suprachiasmatic nucleus of rats; exogenously applied NPY caused a striking depression of glutamate-evoked rises in cytosolic calcium. Furthermore, that NPY-mediated depression of cytosolic calcium was dependent on the concomitant application of glutamate; in the absence of glutamate NPY had no effect on calcium. Leading to the conclusion that NPY was modulating the postsynaptic response to glutamate signaling. An additional purpose of this aim was to conduct an initial investigation into the mechanisms underlying NPY's protective role by testing whether NPY can protect from NMDA-mediated striatal injury. Especially in light of all the studies that showed NPY was protective in various excitotoxic models in addition to our own observation in which NPY reduced METH-induced NO production. We postulated that perhaps NPY is able to mediate this in an autocrine manner as it is located on the same interneuron that possesses nNOS; by exerting an inhibiting influence on the NMDA receptors located therein. In doing so, it would modulate NO synthesis and counteract SP's signaling via the NK-1R and its subsequent augmentation of the NMDA receptor.

As expected intrastriatal infusion of NMDA resulted in a significant elevation of 3-NT immunoreactivity. However, simultaneous administration of either a NPY-Y1 or Y2 receptor agonist with NMDA did not have an effect on the NMDA-induced increase on 3-NT. In addition, neither NPY receptor agonist afforded any protection from NMDA mediated striatal injury (cell death).

So then what alternative mechanisms could be utilized by NPY that would explain the data? Thus far NPY has attenuated METH-induced NO synthesis while also protecting striatal cells from METH-induced apoptosis, which can be attributed to an increasingly toxic oxidative

state. There are at least three different mechanisms by which NPY could serve in a protective fashion that would also result in modulation of NO synthesis.

The first possibility is a monosynaptic paracrine mechanism in which NPY would inhibit glutamate release from corticostriatal terminals. The SST/NPY/nNOS has been shown to synapse with corticostriatal neurons (Hathway et al., 2001) and therefore NPY can interact with the terminals as a retrograde messenger since NPY is also stored in the soma (Silva et al., 2005). NPY has been shown to depress glutamate release presynaptically in multiple regions of the brain within rodent models (Bacci et al., 2002; Silva et al., 2003; Silva et al., 2005). Wang (2005) demonstrated that NPY acting through the Y1 receptor was able to inhibit the release of glutamate from cerebrocortical terminals via calcium channel depression. Van den Pol et al. (1996) found that long-term depression of postsynaptic glutamate-mediated excitatory activity depended on an NPY-dependent inhibition of presynaptic glutamate release. Inhibition of glutamate release from corticostriatal neurons onto the SST/NPY/nNOS would reduce the influx of calcium and thus minimize the activation of nNOS subsequently NO production.

A second additional paracrine mechanism manner would be within the striatum. NPY has been shown to modulate the release of GABA and its subsequent transmission (Chen and Van den Pol, 1996; Bacci et al., 2002; Wang, 2005). Although primarily attributed to depress GABA release through a calcium dependent presynaptic mechanism, Sun et al. (2001) demonstrated that in the rat thalamus NPY can utilize both a presynaptic and a postsynaptic mechanism to suppress GABA release. By modulating potassium (K^+) current via G-protein-activated inwardly rectifying K^+ (GIRK) channels, NPY was able to reduce the membrane excitability of the postsynaptic neuron and thus inhibit GABA release. In the striatum SST/NPY/nNOS

interneurons have been shown to synapse with projection neurons (Westwood and Hanson, 1999; Partridge et al., 2009). In addition, employing immunohistochemistry we have observed that all striatal cell types expressed both NPY-Y1 and Y2 receptors (unpublished result).

Projection neurons as their name implies project outside of the striatum as components of an extrastriatal polysynaptic loop in which striatal projection neurons synapse onto neurons in the substantia nigra pars reticulata in the direct pathway. These neurons in the substantia nigra extend to the thalamus and maintain a tonic inhibition on thalamic neurons, preventing them from exciting corticostriatal neurons that release glutamate into the striatum. Therefore, when striatal projection neurons release GABA they neutralize the inhibition of thalamic neurons resulting in the release of glutamate back into the striatum. The loop then continues in a feedforward manner, however reducing the release of GABA from striatal projection neurons synapsing onto the substantia nigra neurons will maintain the inhibition of thalamic neurons thus mitigating the dumping of glutamate from the cortex. In essence, NPY would indirectly reduce the continual introduction of glutamate into the striatum via a polysynaptic extrastriatal mechanism by attenuating the postsynaptic release of GABA from striatal projection neurons.

Lastly, in an autocrine intrastriatal mechanism through modulation of the glutamate AMPA receptor subtype. The AMPA receptor like the NMDA receptor is an ionotropic channel capable of allowing the influx of calcium (Chen et al. 1996). Although there were some histological studies indicating that AMPA receptors were not present on the SST/NPY/nNOS interneuron (Chen et al, 1996; Bernard et al., 1997), Partridge et al. (2009) recorded excitatory activity on this interneuron in response to AMPA stimulation. The presence of AMPA receptors would provide an additional means to elevate intracellular calcium and subsequently nNOS

catalytic activity. NPY has been shown to modulate glutamate signaling via this receptor. In fact, the NPY-mediated depression of calcium demonstrated by Van den Pol et al. (1996) was concluded to be an effect of a reduction in sensitivity to glutamate signaling in the target neuron. It had already been previously shown that glutamate transmission in these neurons was through all glutamate ionotropic subtype receptors including AMPA. Bacci et al. (2002) showed that NPY has a depressive effect on AMPA mediated glutamate transmission although they could not conclude definitively whether it was a pre- or postsynaptic mechanism.

SST was chosen for investigation for many of the same reasons as NPY since they share several characteristics and modes of action that make them excellent candidates as endogenous agents of neuroprotection during pathological states. SST is found throughout the mammalian CNS and its receptors as well; in the striatum it is colocalized with NPY and nNOS (Rajput et al., 2011a). SST like NPY has been shown in multiple studies to serve as a neuroprotectant in several paradigms of excitotoxicity, this has been attributed to its ability to dampen neuronal hyperexcitability (Allen et al., 2003; Mastrodimou et al., 2008). Some of these models include middle cerebral artery occlusion (Rauca et al., 1999), retinal ischaemia (Cervia et al., 2008; Kigiadaki and Thermos, 2008; Kigiadaki et al., 2010), and epilepsy (Tallent and Qiu, 2008). Intravitreal administration of either an SST_2 or an SST_5 receptor analogue dramatically reduced AMPA-mediated cell death within the rat retina (Kigiadaki and Thermos, 2008; Kigiadaki et al., 2010).

A neurochemical analysis of SSTR 1 and 5 knockout mice revealed that they shared many similarities to a transgenic Huntington's murine model (Rajput et al., 2011a). One such similarity was the critical loss of a large percentage of striatal projection neurons thus

implicating SST signaling as playing a central role in the regulation of neurodegeneration. In fact, one neurochemical index of an Alzheimer's brain is the depletion of cortical SST (Forloni et al., 1997). The selective loss of SST containing neurons is also a hallmark of an epileptic hippocampus in rodents as well as in humans suffering from temporal lobe epilepsy (Tallent and Qiu, 2008). All of the above mentioned neurological diseases share certain similarities, one of which is aberrant glutamatergic transmission as either a primary or secondary factor in their pathology. Additionally, excessive NO synthesis whether by a calcium-dependent pathway or as part of a hyperactive inflammatory response has also been implicated as contributing to disease progression (Boje, 2004; Duncan and Heales, 2005).

A number of studies have suggested that a link exist between SST and NO, Priego et al. (2005) showed that induction of NO production in response to endotoxin administration resulted in a definite increase in SST mRNA expression in the hypothalamus. In addition, NO can also stimulate SST release (Priego et al., 2005). Overall, multiple lines of study indicate that SST has a biphasic relationship with NO that is most likely mediated by either different subtypes of its receptors or perhaps in a tissue specific manner (Vasilaki et al., 2004). In a model of retinal ischaemia, Mastrodimou et al. (2008) found that an SST_2 receptor agonist was protective via a cGMP mediated pathway that may involve NO release. This study was a follow up from previous work in a rat retinal explant model that showed an increase in nNOS produced NO as well as cGMP levels after SST administration (Mastrodimou et al., 2006).

Whereas research investigating tumoral angiogenesis in an *in vitro* model using Chinese hamster ovary (CHO) cells, showed that SST inhibited NO synthesis via several intracellular pathways (Arena et al., 2005). Of particular interest, was that the SST receptors 2 and 3 mediated

the inhibition of nNOS activation primarily through the depression of intracellular calcium mobilization. Lastly, it was theorized that in certain circumstances such as in the retina, SST's regulation of NO levels may be an indirect function of its modulation of glutamatergic activity (Vasilaki and Thermos, 2009). Note, that regardless whether SST increased or decreased NO production, in all of the listed models its application served a protective function. We were interested in exploring whether SST like NPY, could attenuate the METH-induced excessive synthesis of NO and further along whether it could also serve a protective function during the course of METH toxicity.

In our study we utilized the SST analogue OCT, which has the highest affinity for the SST receptors 2 and 5 because in multiple lines of excitotoxicity research in murine models most of SST's protective effects are observed to be a function of the SST_2 receptor (Rauca et al., 1999; Cervia et al., 2008; Mastrodimou et al., 2008). In addition several receptor expression studies imply that SST_2 or perhaps SST_5 are the predominant subtype expressed within the rodent striatum (Allen et al., 2003; Galarraga et al., 2007; Rajput et al., 2011a; Rajput et al, 2011b). Akin to our studies with NPY, OCT was infused directly into the striatum prior to the systemic administration of a toxic dosage of METH. NO synthesis was assessed via 3-NT immunoreactivity. As demonstrated in previous studies treatment with METH caused a significantly high production of striatal NO that was at least fourfold from baseline levels. However, pretreatment with an SST agonist resulted in a dose-dependent reduction in METH-induced 3-NT staining, with the more potent dosage (10 nM) causing a 40% drop. The data suggested that SST had the potential to play a neuroprotective role in METH toxicity. OCT alone was equivalent to the baseline in 3-NT immunoreactivity.

Does SST's modulation of NO synthesis result in protection from METH-induced DA terminal injury as well as striatal cell loss? In of itself it would be quite novel as there doesn't seem to be any studies related to the role of SST in METH toxicity. The above studies provide a substantial foundation for considering SST as being neuroprotective from the toxic effects of METH on the striatum. Utilizing western blot we measured the levels of striatal TH as a determinant of DA terminal viability. Direct infusion of the SST analogue, OCT by itself followed by an i.p. injection of saline had no effect on TH levels (equivalent to baseline levels). As expected, the METH (30 mg/kg, i.p.) alone condition had a visible and detrimental effect on TH showing an almost 50% drop from baseline. Infusion of OCT followed by METH did not confer protection on DA terminals, TH levels remained equivalent to the METH alone group.

However, when investigating striatal cell loss there was a clear and astounding protective effect. Once again, the agonist alone group saw no clear difference from the control condition. METH however had a significant and clear rise in cell death. Pretreatment with OCT however almost completely abolished the METH-induced cell death. Our own studies have implicated that DA terminal degeneration is a separate and distinct mechanism from those underlying striatal cell loss (Zhu et al., 2006a) which is further strengthened when one considers that their time course differs as well. Peak cell loss is reached 24 hours after METH administration whereas DA terminal degeneration reaches its peak at least another 24 hours later (Zhu et al., 2005) implying that either an additional set of events must occur for the initiation of DA terminal degeneration or cell loss must precede terminal degeneration. There have been some indicators that SST receptors can become rapidly downregulated in response to endogenous SST (Vasilaki et al., 2004; Tallent and Qiu, 2008). Additionally, they may also become desensitized to SST

agonists whether through internalization or uncoupling of the cell surface receptor to the intracellular machinery necessary second messenger signaling (Cervia et al., 2008). It is possible that SST's protective influence is better suited for the early events following METH and thus why it was ineffective in protecting from DA terminal damage.

To further dissect the underlying mechanisms of SST's protective actions we were interested in exploring whether it retains its protective effect in NMDA-mediated striatal injury. In its function as a neuromodulator, SST has been shown to play an intrinsic role in the release of several key neurotransmitters (Hathway et al., 1999; Lopez-Huerta et al., 2008) thus ultimately controlling the overall output and thus function of several structures such as the hippocampus (Tallent and Qiu, 2008) and striatum (Galarraga et al., 2007; Santis et al., 2009). Mice genetically engineered to express an inactivated form of the SST_2 receptor showed severely compromised fine motor control (Allen et al., 2003). Furthermore, several studies have indicated that there is a relationship between glutamate and SST within the striatum, in murine striatal neuronal cultures application of NMDA caused the release of SST (Forloni et al., 1997; Kumar, 2008). In an *in vivo* model, the infusion of either AMPA or NMDA resulted in striatal SST release in rats (Hathway et al., 2001). In an animal model of aberrant glutamatergic transmission such as in a kindling rodent model of epilepsy, the investigators found a higher baseline level of SST released in the hippocampus than in the naïve animals (Marti et al., 2000). Generally, in these models of hyperexcitability an altered biosynthesis of SST has been found and is theorized as a compensatory mechanism attempting to establish homeostatic balance in the affected region (Tallent and Qiu, 2008).

As I delineated above there are multiple studies that demonstrate the efficacy of SST in protecting from excessive glutamate transmission, would the same hold true within the striatum? As discussed throughout, glutamate excitotoxicity is an integral part of METH toxicity, thus the aim was to try to tease out whether it is modulation of glutamatergic transmission that underlies SST's protection of METH-induced striatal cell loss. First, if SST was a homeostatic opposing force to SP's influence then could it negate the augmentation of NMDA signaling by dropping NMDA-mediated NO synthesis? If it is serving its protective function by modulation of glutamatergic transmission then it should also mitigate NMDA striatal injury. However, like NPY co-infusion of the SST analogue with NMDA failed to have any effect on NO synthesis or cell death. The NMDA alone condition showed an expected and significant increase in NO synthesis that was about 30% above baseline. Infusion of OCT with NMDA had no effect on the NMDA-mediated NO. Comparably, NMDA alone also caused a significant amount of striatal cell loss whereas addition of OCT did not mitigate the cell loss.

The above data indicates that SST's protective actions do not include modulation of striatal NMDA receptor signaling. However, that does not preclude an inhibitory action on glutamate; akin to NPY it can depress the cortical release of glutamate into the striatum. There is experimental evidence indicating that there are SST_2 receptors present on the terminal of murine corticostriatal neurons (Hathway et al., 2001). There are studies that indicate that SST has the ability to modulate presynaptic calcium currents (Selmer et al., 2000). SST's ability to influence calcium current influx suggests that it is functioning in an inhibitory manner on transmitter release. It is thought that one of its actions in protecting from excitotoxicity is through inhibition on glutamate release from its terminal (Tallent and Qiu, 2008) There is corroborating data that

shows a decrease in glutamate release after application of OCT to the retina of mice (Cervia et al., 2008). Momiyama and Zaborszky (2006) showed that exogenous application of SST to slices of the basal forebrain of rats reduced glutamate release in a calcium dependent manner. Moreover, its inhibitory action could serve as a means to stem the secondary or rather continual loop of glutamate release that occurs with METH thus reducing activation of the NMDA/nNOS cascade.

One additional possibility is an autocrine mechanism in which SST depresses nNOS catalytic activity through intracellular second messenger signaling. As I mentioned previously there have been several studies indicating that SST has a relationship to NO synthesis. Said relationship has been demonstrated to be both facilitating and inhibiting in nature (Lopez et al., 2001; Vasilaki et al., 2004; Arena et al., 2005; Cordelier et al., 2006). The differences have been attributed to SST receptor subtype as well as cellular strain and or tissue differences, which would provide different intracellular substrates for second messenger signaling (Arena et al., 2005). However, in two independent groups utilizing the same cell lines (CHO-K1) expressing rat SST receptors they found that SST analogues were able to depress nNOS catalytic activity through the activation of second messenger pathways (Arena et al., 2005; Cordelier et al., 2006). Cordelier et al. (2006) demonstrated that an analogue for SST_5 receptor depressed NO synthesis by inactivation of nNOS via phosphorylation. Whereas Arena et al. (2005) showed that activation of $SST_{2/3}$ receptors blocked intracellular signaling for the mobilization of calcium from intracellular stores (endoplasmic reticulum).

Although the exact receptor subtype expressed by individual murine striatal neuronal populations is still uncertain, the overall consensus is that the striatum expresses a heterogeneous

mixture and individual neurons as well (Selmer et al., 2000). In a histological analysis, Rajput et al. (2011b) showed that markers for all SST receptor subtypes colocalized with a marker for rat striatal projection neurons. In addition, Allen et al. (2003) found that in the mouse striatum the SST_2 receptor was localized on cholinergic interneurons and on projection neurons that release SP. Therefore it is quite feasible that the SST/NPY/nNOS interneuron may possess the SST receptor necessary to activate the above discussed autocrine mechanisms and depress nNOS catalytic activity in a more direct fashion.

Chapter 6: Conclusion

Past work in our lab as well as current results lend strength to the hypothesis that SP signaling in the striatum is exacerbating METH-induced neurotoxicity. Moreover, that SP is doing so by contributing to the harmful oxidative environment created by METH. SP may either act in a parallel or serial pathway that ultimately strengthens glutamate signaling. We conducted a series of experiments to further explore the link between SP signaling via the striatal NK-1R, NMDA signaling, and NO synthesis. We had speculated that during METH SP can augment or supplement NMDA mediated glutamate transmission and thus calcium-dependent NO production. To dissect this possible relationship we eliminated METH and narrowed in on NMDA transmission in a model that can be applied to METH toxicity. An immunohistochemical assessment of striatal cell loss showed that inhibition of SP signaling resulted in a decrease in the NMDA-mediated cell death, suggesting that SP can potentiate excitotoxicity. In addition, an analysis of NMDA-mediated NO synthesis demonstrated that SP signaling was contributing to NO production.

However, in this particular model, NO was being produced from the calcium-independent iNOS. Our result could be attributed to idiosyncrasies of our model such as the time course of NMDA-mediated microglial activation and the time point we chose to measure NO. SP could very well contribute to iNOS mediated NO synthesis as it has been shown that microglia express functional NK-1R's that can activate iNOS transcription (Rasley et al., 2002; Block et al., 2006). Our result does not invalidate our working hypothesis but rather adds a new level to consider, one that implies that iNOS can contribute NO most likely to the events taking place 24 hours after METH. At which point the longer lasting NOS isomer can supply a prolonged production

of NO. Previous experimental evidence from our lab provides compelling proof that SP can also activate the catalytic activity of the calcium-dependent nNOS found on the SST/NPY/nNOS interneuron. Histological observation showed that the NK-1R is activated early on after METH administration (Wang and Angulo, 2011b). Moreover, that infusion of an SP agonist into the striatum results in 3-NT immunoreactivity whereas coinfusion of a CaM inhibitor caused a substantial reduction in 3-NT (Wang and Angulo, 2011a). All of which lends credence to the postulation that during the early events following METH, SP augments NMDA-mediated activation of the calcium-dependent nNOS. Once microglia become active, it can also participate in a parallel pathway that contributes to activating iNOS.

Thus far we had seen one striatal neuropeptide that was potentiating NMDA signaling in a pro-toxic capacity. As a result we were interested in exploring whether NPY or SST could serve as counterbalances by functioning as inhibitory forces to SP's augmentation. Both NPY and SST had proven protective in several lines of research utilizing excitotoxic models. Therefore we sought to test whether 1) SST could depress NO synthesis and 2) could they both protect from METH-induced striatal injury. We had already observed that utilizing an agonist for the NPY-Y1 and Y2 receptor had attenuated METH-induced NO in a dose-dependent manner. Using a similar paradigm we tried an SST analogue and found the same effect. Measurement of two parameters of METH striatal injury demonstrated that NPY and SST could serve as potent neuroprotectants of cell death. Whereas only NPY via the Y2 receptor was able to confer significant protection of DA terminals as measured by TH protein levels. This is most likely due to its presence on the DA terminal and its ability to abrogate calcium influx. The DA terminal is

already a highly unstable environment during METH, the addition of calcium ions would add an additional element that could push matters further into a more toxic range.

The final sets of experiments were meant as a preliminary investigation into the possible mechanisms underlying NPY and SST's protective actions during METH. Based on several studies on the neuroprotective capacity of NPY and SST, it seemed probable that their mode of action was through an influence on glutamate signaling. It is for this reason that we utilized NMDA to induce striatal injury so as to elucidate whether NPY and SST could still remain protective. Interestingly, neither NPY nor SST had any effect on NMDA-induced NO or cell death. As shown in the diagram below (A), it seems more probable that NPY and SST could have enacted their protective effects by modulation of glutamate release from corticostriatal neurons via a monosynaptic presynaptic mechanism. An additional possibility (B), are separate autocrine mechanisms that involve second messenger signaling within the SST/NPY/nNOS interneuron. NPY could depress signaling through the glutamate AMPA receptor on the SST/NPY/nNOS interneuron. As to SST, there are indicators that it can directly depress nNOS' catalytic activity either through phosphorylation or by preventing the release of intracellular calcium. Finally (C), as part of its function as a neuromodulator NPY can inhibit the postsynaptic release of the transmitter GABA. All projection neurons in the striatum utilize GABA as their main transmitter. Depression of GABA release in the direct pathway (striatonigral) can maintain the inhibition of the substantia nigra on the thalamus thus preventing excitation of corticostriatal neurons and their subsequent release of glutamate into the striatum.

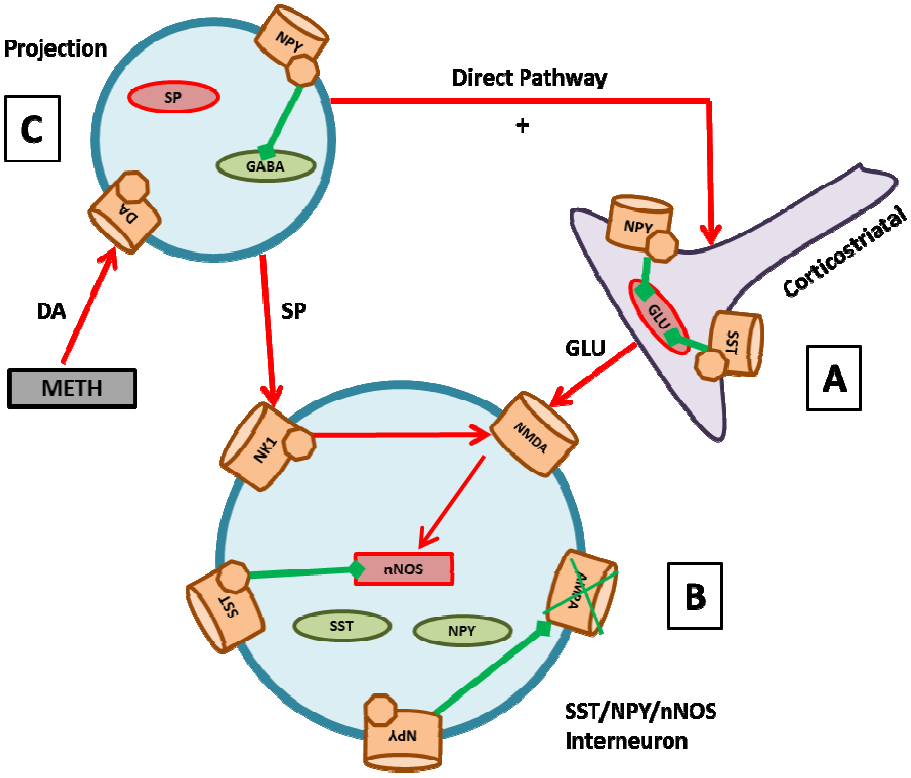

Figure 6-1: Schematic of hypothesized protective mechanisms of NPY and SST. METH-induced DA overflow stimulates the release of SP and glutamate. Glutamate released from corticostriatal neurons will promote the influx of calcium (NMDA & AMPA) into the SST/NPY/nNOS interneuron whereas SP (NK-1R) will augment NMDA receptor function. Causing excessive NO synthesis by activating the calcium-dependent nNOS. However, NPY and SST can act as balancing forces in the following possible mechanisms. (A) Paracrine: both can presynaptically inhibit the release of glutamate from corticostriatal axon collaterals. (B) Autocrine: NPY can depress AMPA function thus attenuating a source of calcium. SST can depress nNOS activation by phosphorylation or preventing calcium release from intracellular stores. (C) Paracrine: NPY can suppress activation of the direct pathway by attenuating GABA release. This prevents the disinhibition of the thalamus and its subsequent excitation of cortical glutamate neurons.

As neuromodulators, neuropeptides exert significant influence on the function of the areas of the brain they govern. Understanding the role striatal neuropeptides may play during METH toxicity could illuminate new pathways and mechanisms that can be used in the development of novel therapeutic agents. Especially in light of the fact that METH affects similar areas and pathways as several neurodegenerative diseases that currently lack any cure.

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