On the Mechanism of Neurotoxicity from Methamphetamine: The Role of Neuropeptide Y

By

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Abstract

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The purpose of this study is to describe the potential neuroprotective effect of neuropeptide Y in response to striatal nitric oxide overproduction after methamphetamine (METH) use. Our lab has established a time course and optimal dosage for modeling acute METH neurotoxicity in the murine brain. A 30 mg/kg systemic injection of METH depletes intracellular dopamine and serotonin levels, and induces dopamine receptor endocytosis as well cell loss of approximately 30% of striatal neurons.

Dopamine overflow from presynaptic terminals paired with glutamate signaling from the cerebral cortex commences a neuronal cascade, which leads to the overproduction of nitric oxide, inflammatory cytokines and reactive oxidative/nitrogen byproducts. We observe that when neuropeptide Y (Y1R, Y2R) agonists are administered prior to METH, the appearance of apoptotic cells and neurodegeneration markers are attenuated. Additionally, there is an endogenous upregulation of striatal neuropeptide Y mRNA stores during the early hours after METH administration.

The following study establishes an optimal dose and time course for neuropeptide Y induction, and suggests that neuropeptides are at play to establish homeostasis after acute METH toxicity. We characterize the cellular response to METH-induced nitric oxide production through fluorescent co-label, and demonstrate that the effect of neuropeptide Y on these cell types is modulated by specific neuropeptide Y receptors. The neuroprotective effect of NPY persists even in the presence of substance P agonists, associated with exacerbation of methamphetamine-induced neurotoxicity.

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LIST OF ABBREVIATIONS

3-NT: 3-Nitrotyrosine

5-HT: Serotonin

AMPH: Amphetamine

APAF-1: Apoptotic Protease Activating Factor

Ca: Calcium

cAMP : Cyclic Adenine Monophosphate

cGMP: Cyclic Guanine Monophosphate

ChAT: Acetyl Cholinergic Transferase

CNS : Central Nervous System

COMT: catechol-o-methyl transferases

Cy3: Cyanine 3

Cyto C: Cytochrome C

D1R: Dopamine-1 Receptor

D2R: Dopamine-2 Receptor

DA: Dopamine

DARPP-32: Dopamine and Adenosine 3',5'-monophosphate-regulated phosphoprotein (32 kilodaltons)

DAT: Dopamine Transporter

Dk: Donkey

ER: Endoplasmic Reticulum

eNOS: Endothelial Nitric Oxide Synthase

FITC: Fluorescein Isothiocyanate

GABA: Gamma-aminobutyric acid

GC: Guanylyl Cyclase

GLU: Glutamate

Gt: Goat

GTP: Guanine Nucleotide Triphosphate

iNOS: Inducible Nitric Oxide Synthase

IF: Immunofluorescence

IP: Intraperitoneal

L-DOPA : L-dihydroxyphenylalanine

MAO: monoamine oxidase

METH: Methamphetamine

NK-1R: Neurokinin-1 Receptor

NMDA: N-methyl-D-Aspartate Receptor

nNOS: Neuronal Nitric Oxide Synthase

NOS: Nitric Oxide Synthase

NO: Nitric Oxide

NPY: Neuropeptide Y

NPY-1R : Neuropeptide Y – 1 Receptor

NPY-2R : Neuropeptide Y – 2 Receptor

NPY-5R : Neuropeptide Y – 5 Receptor

PARP: Poly (ADP-ribose) polymerase

PC-12 cells: Pheochromocytoma cells

PD: Parkinson's disease

PFA: Paraformaldehyde

PFC: Prefrontal Cortex

Rb: Rabbit

ROS: Reactive Oxygen Species

RNS: Reactive Nitrogen Species

SERT : Serotonin Receptors

SP : Substance P

SST: Somatostatin

TH : Tyrosine Hydroxylase

TUNEL: Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling

Tx-PBS : Triton-X Phosphate Buffered Saline

VMAT2 : Vesicular Monoamine Transporter

VTA: Ventral Tegmental Area

SPECIFIC AIMS

On the Mechanism of Methamphetamine-Induced Nitric Oxide Production in the Mouse Striatum: Neurotoxicity and Protection

Specific Aim 1. ***To investigate the role of the striatal neuropeptide Y in the METH-induced production of nitric oxide.***

a) To test the hypothesis that NPY receptor agonists will attenuate and antagonists will potentiate the METH-induced production of striatal 3-NT.

Approach: Assess 3-NT production 4 hours after systemic injection of METH (30 mg/kg) and intrastriatal infusion of NPY-1R and NP-Y2R agonists and antagonists. Use software to assess immunofluorescence intensity of anti-3-NT antibody binding to target. Three doses for each drug are used to establish an optimal dose for NPY use.

b) To test the hypothesis that striatal SST/NPY/NOS interneurons express NPY-1R and NPY-2R receptors.

Approach: Use immunofluorescent label to calculate the percentage of striatal neurons that express NPY-1R and NPY-2R receptors to characterize the presence of NPY receptors in the murine striatum. Stain untreated tissue for NPY-1R and NPY-2R with each cell phenotype

c) To test the hypothesis that METH impacts the utilization of striatal NPY.

Approach: Assess NPY mRNA levels in striatal extracts with RT-PCR at 4 and 16 hours after systemic injection of METH (30 mg/kg)

<u>Specific Aim 2.</u> To examine the impact of METH-induced nitric oxide production on striatal neurons.

a) To assess the neuronal targets of the METH-induced nitric oxide: Colocalization of cGMP with select markers of striatal projection and interneurons.

> Approach: Use immunohistochemical stain to calculate the percentage of cGMP-expressing cells in striatal cell populations at 4 and 8 hours after systemic injection of METH (30 mg/kg). DARPP-32, ChAT, Parvalbumin and NOS antibodies are used to label cell phenotype

b) To test the hypothesis that the METH-induced cGMP colocalizes with caspase-3 activation in striatal cells.

> Approach: Calculate the percentage of NO-responding cells that also contain activated caspase-3 at 8 hours after systemic METH administration. Determine if cGMP-expressing cells show apoptotic markers at this early time point using immunofluorescent techniques.

c) To test the hypothesis that an NPY-2R agonist can attenuate the METH-induced activation of striatal caspase-3 and cGMP production.

> Approach: Determine the effect of exogenous NPY2R agonist administration on mitochondrial apoptotic markers at 8 hours post systemic METH administration. Immunofluorescent localization of cGMP and active-caspase 3-expressing cells.

CHAPTER 1

I. Background Significance

A. DRUGS OF ABUSE – CLASSIFICATION AND HISTORY

METH was first synthesized in 1919 by Akira Ogata by reduction from ephedrine (Tamura 1989). Other reports suggest it was also being synthesized during the same period in Germany (Grinspoon and Hedblom, 1975). METH was initially sought as a therapeutic treatment for a range of ailments from narcolepsy and obesity to schizophrenia (Grollman, 1954). By 1930, amphetamines were marketed at local drug stores as an anti-asthmatic inhalers and were later sold as pills.

Following the second world war, amphetamines were legally manufactured. American soldiers had been using this drug overseas and its appeal quickly extended to students and shift workers in tablet and intravenous form. Japan's Stimulant Control Law was enacted in 1951 to " control the import, export, possession, manufacture, transfer, and use of materials of psychostimulants and its salts." By the late 1960's, physicians began to take notice of the detrimental and addictive consequenses of METH use (Hawks et al., 1969). It was not until the Controlled Substances Act of 1970 that methamphetamine was scheduled as a class II drug, and its use became severely limited in the United States.

In 1971, physicians at San Francisco General Hospital reported neurological deficits and unusual pulsation in cerebral arteries (Margolis and Newton 1971) that were later attributed to drug abuse. There were reports of dyskinesia in monkeys (Eibergen and Carlson, 1976) and sudden death in an 18-year-old girl (Ross et al., 1987) that were both linked to METH use. We now have a clearer picture of the scope of METH damage in users.

Drug addiction is both a physiological and psychological disease that affects innate motivation for necessities and prioritizes drug seeking behavior. While motivation is often driven by the desire for hedonic reward such as hunger, sleep, and sex, internal and external cues like drugs can also stimulate this response. When drug-seeking behavior becomes "persistent and compulsive" despite knowledge of its harmful effects, it is termed an addiction. Importantly, the drug use and seeking behavior persists in the "face of adverse consequences" (Everitt and

Robbins, 2005) so that a user will have “a great deal of time spent in activities necessary to obtain the substance” (American Psychiatric Association, 2000). The “incentive salience hypothesis” describes this relentless desire to obtain a drug, which is sometimes detached from a user’s desire to feel the effects of the drug (Berridge and Robinson 1998). This is the foundation for research on conditioned reinforcement and drug-related stimuli since there is also a physiological basis for drug craving (Robbins 1976, Hill 1970). As the “high” of addictive drugs subside, a user may feel an anxiety to administer the substance.

Drug craving and reinforcement is most directly studied by intracranial self-stimulation. Rodents and primates are allowed to freely choose drug administration (by lever press, beak peck or otherwise), and they do so time and time again despite being faced with negative consequences or irregular reward (McGregor and Roberts, 1993). The addicted state is further classified by the appearance of tolerance as drug use continues and withdrawal when drug use is ceased (Kandel et al., 2000).

There is substantial evidence that the mesolimbic dopamine system modulates both reward and drug-seeking behavior. Both lesion studies and blocking dopamine receptors in this brain circuit decreases feeding and drinking behavior in animals (Kandel et al., 2000). Neuronal patch clamp recordings demonstrate that dopamine neurons are excited when presented with the same stimuli (Schulz et al., 1985). Even before the presentation of cocaine, mesolimbic dopamine concentrations in rats will increase in response to a cue for this reward (Wheeler et al., 2011).

The United States Drug Enforcement Association classifies drugs of abuse into categories termed Schedule I – Schedule IV, which define the potential of the substance for abuse and its use for medical benefit. Schedule I and II drugs have the strongest abuse potential and each category proceeds to be less severe (http://www.justice.gov/dea/pubs/scheduling.html - accessed 6/29/11). Drugs of abuse are also classified by their effect on the CNS. CNS depressants include ethanol, barbiturates, and opiates. These drugs slow heart rate, breathing, and act on gamma aminobutyric acid (GABA)-ergic and cholinergic receptors. Heroin, one of the

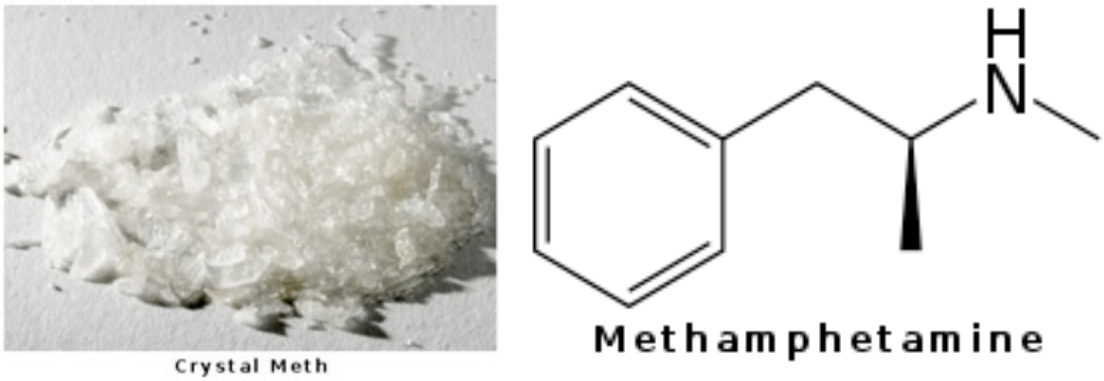
most well-known schedule I drugs, is an opiate and CNS depressant. Like other CNS depressants, heroin creates a drowsy, analgesic effect in its users. Opiates have been used to treat diarrhea by decreasing intestinal motility, and are found in cough suppressants. Opioid receptors have three endogenous ligands – endorphins, dynorphins and enkephalins and three receptor subtypes – mu, delta and kappa. Opiates and their receptors are often used to study pain mediation. The mesolimbic dopamine system is tightly linked to the rewarding effects of psychostimulant drugs, but may not be as crucial to the pleasurable feelings from heroin or alcohol administration (Koob and LeMoal, 2006).

Cocaine is a schedule II drug that belongs to the stimulant class, along with nicotine and amphetamines, which increase heart rate and DA signaling. Cocaine was first extracted from coca leaves in the late 1800s, and was prescribed as an analgesic and for indigestion or depression. Nicotine, the addictive agent found in cigarettes, cigars, and smokeless tobacco was reported to have 70.9 million users 12 and older in the United States in 2007 (Substance Abuse and Mental Health Services Administration, 2008). Nicotine binds to nicotinic and muscarinic receptors – receptors for acetylcholine. It enhances release of dopamine through presynaptic cholinergic receptors rather than the dopamine transporter (DAT). Nicotine self-administration is prevented by drug lesion of the mesocorticolimbic DA system, tying its pleasurable reward to the same neural system as other drugs of abuse (Corrigall et al., 1992).

Our research focuses on the class of stimulants known as amphetamines, specifically methamphetamine (METH), a manufactured psychostimulant known for its euphoric effects (Figure 1). In its purest form METH is an odorless crystalline solid, and it can be inhaled, smoked, and produced as an injectable liquid. (NIDA 2006). METH is the most potent amphetamine, followed by d,l-amphetamine and l-amphetamine successively. METH is the N-methyl derivative of alpha-methylphenethylamine.

On the street, METH is also known as “ice,” “speed,” “crystal,” “crank,” and “blue amp,” and has a half-life of approximately 12 hours that leaves users on sleepless benders, though it may remain in the blood for approximately 36 hours after use. METH is metabolized to less potent

amphetamines in the liver, and may be in urine for up to 7 days (Cruickshank and Dyer 2009).



http://toxicopoeia.com/?get=compounds&compound=Methamphetamine&type=psychoactive

Figure 1. Crystal appearance and chemical structure of Methamphetamine

METH use has a high potential for abuse and addiction, but its withdrawal symptoms manifest differently than other drugs of abuse. Users report anxiety, agitation, cognitive impairment, depression and disturbed sleep drug withdrawal. Addicts usually experience extreme depression without somatic effects. (Gossop et al., 1982, Kalechstein et al., 2003, McGregor et al, 2005).

METH induces cell death by neurotoxicity and also induces significant behavioral and physiological changes in its users (Deng et al., 1999, 2002; Cadet et al., 2003). At low doses (5-30mg in humans), amphetamines can increase alertness and wakefulness as observed in medications for attention deficit disorders. At more potent dosages (>50 mg in humans), users experience decreased appetite, hyperlocomotion, anxiety, hyperthermia and sometimes psychosis. In a study of high-dose intravenous METH users, subjects reported aggression, hypertension, paranoia, psychosis, and headaches. Researchers reported continuous talking during the study (Bell 1973). Stereotyped behavior can be seen in human users and is observed in rodents by persistent tail tapping or circling. Amphetamines exhibit behavioral effects almost immediately after dose (Cruickshank and Dyer 2009).

METH's short and long-term neurochemical effects are well documented (Cadet and Krasnova, 2009). Monoamine transporter density is depleted, neurotransmitter signaling is

impaired, and there is abnormal glucose metabolism in the brains of METH addicts (Ernst and Chang, 2008, Volkow et al., 2001, Sekine et al., 2006). DA depletion can last up to 4 years after completing a toxic dose regimen (Woolverton et al., 1989) and a loss of DAT persists for 3 years (Johanson et al., 2006, McCann et al., 1998). Degeneration of brain tissue by METH is often seen in the nigrostriatal tract - including the caudate, putamen and globus pallidus. In addition to the damage to dopamine receptors and neurons METH causes substantial long-term changes to the serotonergic system. In abstinent METH addicts, researchers observe more aggressive behavior and lower 5-HT transporter density (Sekine et al., 2006). Decreased levels of 5-HT and its metabolite (5-hydroxyindoleacetic acid) 5-HIAA are observed in the caudate of baboons by autoradiography after METH exposure (Villemagne et al., 1998).

The behavioral effects of METH, including habituation, withdrawal, sensitization, and self-administration, are also studied in both human and animal models. Stimulant withdrawal is also characterized by anhedonia and dysphoria, which is likely explained by the downregulation of DA receptor (D2R) expression and DA release after discontinued drug use (Volkow et al., 1997). After a period of sustained abstinence, METH abusers have been reported to experience decreased motor speed and verbal learning (Volkow et al., 2001).

B. SOCIETAL COST OF METHAMPHETAMINE

METH has been a growing and expensive problem for the world's healthcare and law enforcement systems during the last decade. The United Nations Office on Drugs and Crime world drug report categorizes methamphetamine with other Amphetamine-Type Stimulants as the second most abused drug type in the world after marijuana. Methamphetamine use is more popular among men and residents of western states. The most recent survey from UNODC estimates that in 2009, 0.3%-1.3% of the world's population used amphetamines. In the United States, there are an estimated 3.5 million users, or 1.1% of the population (UNODC, 2011). Approximately 1% of 12th graders used METH in the last year (Johnston et. al, 2011).

The Justice Department's 2010 Methamphetamine Threat Assessment (USDOJ, 2010)

further highlights the burden of METH use on society. The report states that there were 215 METH laboratory seizures in 2009, but that this number should decline as Mexico-based labs grow in number and production capacity. METH is easily accessed and produced by the reduction of ephedrine and pseudoephedrine - found in cough syrups. The production and distribution of METH in the United States is dominated by organized crime units, and its users often reside in low socioeconomic communities. The drug can be dissolved alone in a form called "freebasing", which chemically removes Cl^- from the molecule for more potency. A new manufacturing method termed "shake and bake" is now being used for at-home METH production, and has caught the attention of medical professionals in burn units across the country. The use of a single container with multiple explosive chemicals has resulted in several accidents and even deaths.

METH use is medically costly as well. Prenatal exposure to METH is associated with preterm delivery, pre-eclampsia, and low birth weight. If survivable, infants usually experience withdrawal and/or birth defects (Golub et al., 2005). Much research is now being dedicated to the concomitant use of METH in HIV-positive individuals-either before or after diagnosis. It appears that these patients experience are more vulnerable to toxicity and dementia than the population at large. HIV encephalitis and HIV p24 presence is also noted post mortem in more drug abusers than non-drug abusing HIV-infected patients (Bell et al., 1998). Reactive microgliosis, excess dopamine, and HIV proteins, including gp120, gp41, Tat, Vpr and Nef, are implicated in HIV-associated dementia (HAD) and HIV-associated neurocognitive disorders (HAND). The presence of these proteins, lipid peroxidation and reactive microglia are also noted in oxidative stress and METH toxicity (Banerjee et al., 2010, Cadet and Krasnova, 2007, Purohit et al., 2011). When pretreated with gp120, Tat and METH, animals have significantly decreased glutathione levels and significantly increased lipid peroxidation levels as measured by malodialdehyde (Banerjee et al., 2010). This is strong neurochemical evidence that METH use can advance HAD and HAND.

C. STRIATAL INFRASTRUCTURE

The dorsal nuclei of the basal ganglia are comprised, from dorsal to ventral location, of the striatum, globus pallidus, subthalamic nucleus and substantia nigra (Figure 2). The striatum is largely involved in the neurological system for motor control. These nuclei send outputs to the brain stem and thalamus while receiving input from the cerebral cortex, with no direct connection to the spine. Disorders of the basal ganglia are associated with tremor and either excessive or diminished movement, such as Parkinson's Disease and Huntington's Disease. While damage to the basal ganglia is largely correlated with neurodegenerative disorders, it is also implicated in cognitive and behavioral dysfunction.

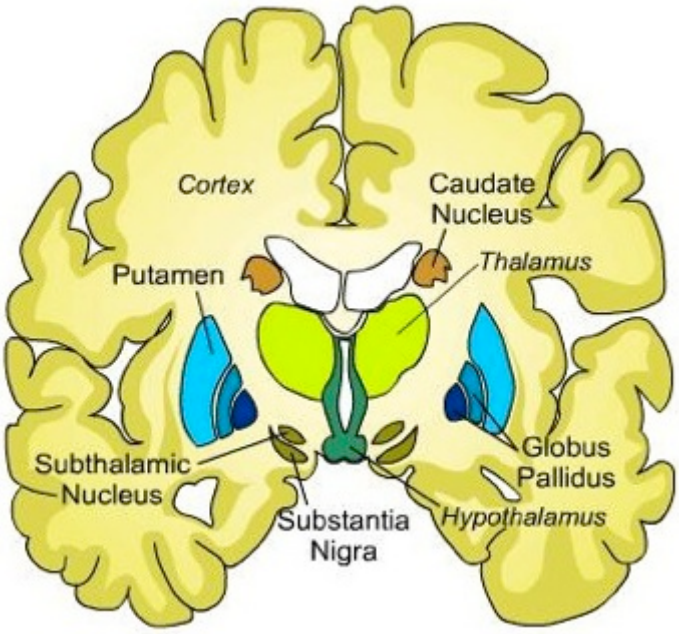


Figure 2. Coronal View of Striatum. Basal ganglia sit at the base of the forebrain. (Parkinson's Disease: National Clinical Guideline for Diagnosis and Management in Primary and Secondary Care: 2006)

The striatum is comprised of the caudate, putamen and nucleus accumbens, and appears as striped (striated) gray matter. There are both matrix and striosomes that appear as vastly different structures with histochemical staining. The matrix is striated as discussed while

striosomes are blotch-like circles, larger than cells, and project to the substantia nigra. Within the striatum, the internal capsule divides the caudate from the putamen.

The striatum regulates inhibitory output from the internal pallidal segment and the substantia nigra pars compacta by direct (from subthalamic nucleus) and indirect (through external pallidal segment) mechanisms.

D. DOPAMINERGIC, GLUTAMATERGIC AND GABAERGIC TRANSMISSION

Dopamine signaling is crucial to both normal striatal activity and METH-induced striatal toxicity. There are four major dopaminergic tracts in the brain – mesolimbic, mesocortical, nigrostriatal and tuberoinfundibular (Figure 3). The mesocortical pathway connects the ventral tegmentum to the cerebral cortex and is involved in frontal lobe function of the dorsolateral prefrontal cortex (DLPFC). This pathway's function is associated with planning and decision-making as well as learning reinforcement. Neurons of the tuberoinfundibular or hypothalamic pathway extend from the arcuate nucleus to the median eminance. This projection regulates prolactin secretion from the anterior pituitary (Kandel et al., 2000).

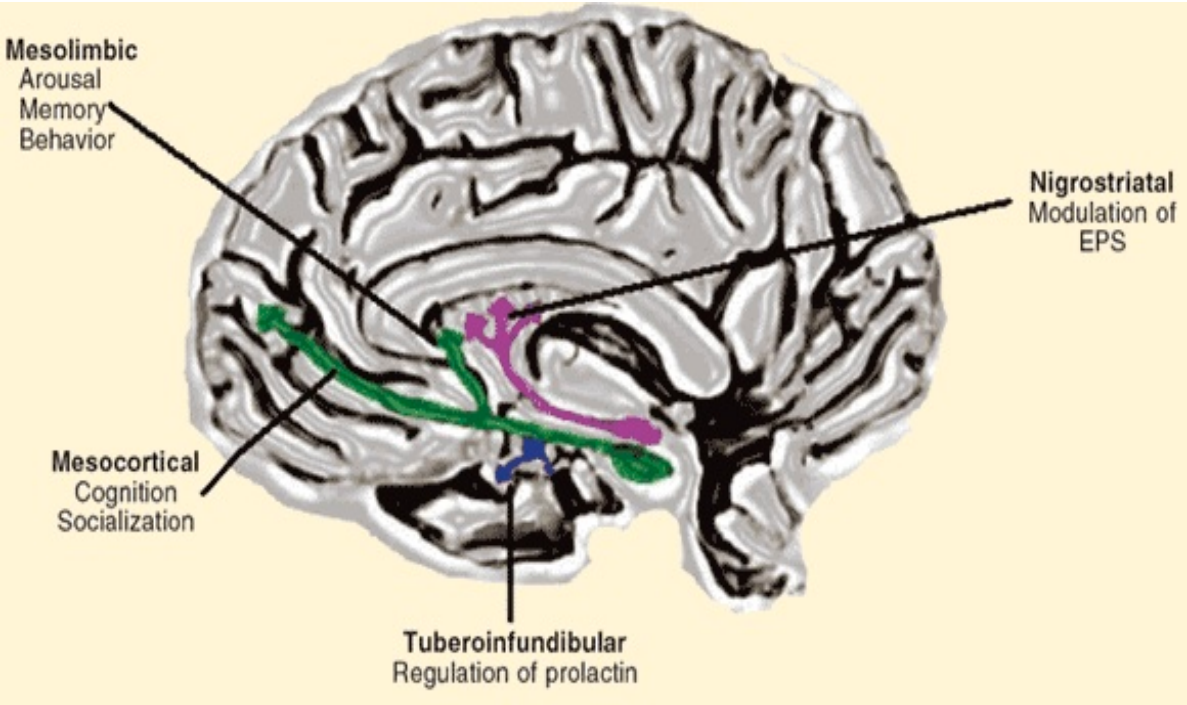


Figure 3: Dopaminergic Tracts. (The Brain from Top to Bottom. Canadian Institutes of Health Research: Institute of Neuroscience, Mental Health and Addiction)

Our studies focus on the remaining two dopaminergic tracts – the nigrostriatal and mesolimbic pathways. The nigrostriatal pathway, which projects from the substantia nigra of the midbrain up through to the striatum, largely regulates motor control. One of the hallmark characteristics of Parkinson's disease and schizophrenia is the loss of this group of neurons.

The mesolimbic tract runs from the ventral tegmental area (VTA) of the midbrain to nucleus accumbens and the limbic system. The striatum receives DA inputs from this region to its ventral areas and from the substantia nigra to its dorsal region. The mesolimbic tract is largely associated with drug abuse and reward systems, memory and behavior. Most drugs of abuse enhance neurotransmitter release from DA neurons of the mesolimic DA system (Gardner 2005, Wise 1994, Wise and Bozarth, 1984). Electrophysological study reveals that METH neurotoxicity decreases phasic, but not tonic, DA signaling (Howard et al., 2011). 6-OHDA lesions reduce elicit similar electrical signaling in the rat striatum (Garris et al., 1997, Bergstrom and Garris 2003).

DA ($C_6H_3(OH)_2$-CH_2-CH_2-$NH_{2)}$, a catecholamine amine transmitter synthesized by a

decarboxylation of L-dihydroxyphenylalanine (L-DOPA), is the precursor for norepinephrine and epinephrine production. Like other small molecule transmitters, DA is a low molecular weight substance and is released from docking vesicles at the presynaptic cell (Kandel et al., 2000). Tyrosine hydroxylase (TH), present in all catecholamine-producing cells, must be available to create L-DOPA (Figure 4). When the gene for TH is knocked out in mice, animals are unable to synthesize DA. They can survive for just two weeks (Kobayashi 1995). Not only is DA production necessary for survival and homeostasis, but it also mediates the rewarding effects of METH in the striatum. When DA antagonists are applied in this brain region, amphetamine self-administration routines are altered (Wilson and Schuster, 1972).

During normal removal of DA and catecholamines from the synaptic cleft, molecules can undergo diffusion, re-uptake and degradation by enzymes. After exerting activity on a postsynaptic neural cell, DA is often taken up into the presynaptic cell. A significant number of the molecules are repackaged into vesicles, but DA can also be broken down by monoamine oxidase-B (MAO-B), the most common form of MAO in the striatum (~80% of DA breakdown). This is also the enzyme responsible for DA oxidation since it produces H_2O_2 as a byproduct. About 10% of the time, DA can also be catabolized by catechol-o-methyl transferases (COMT) to form 3-Methoxytyramine (3MT) (Kandel et al. 2000) (Figure 4). Without repackaging in to vesicles, DA is exposed for autoxidation or metabolism in to toxic byproducts.

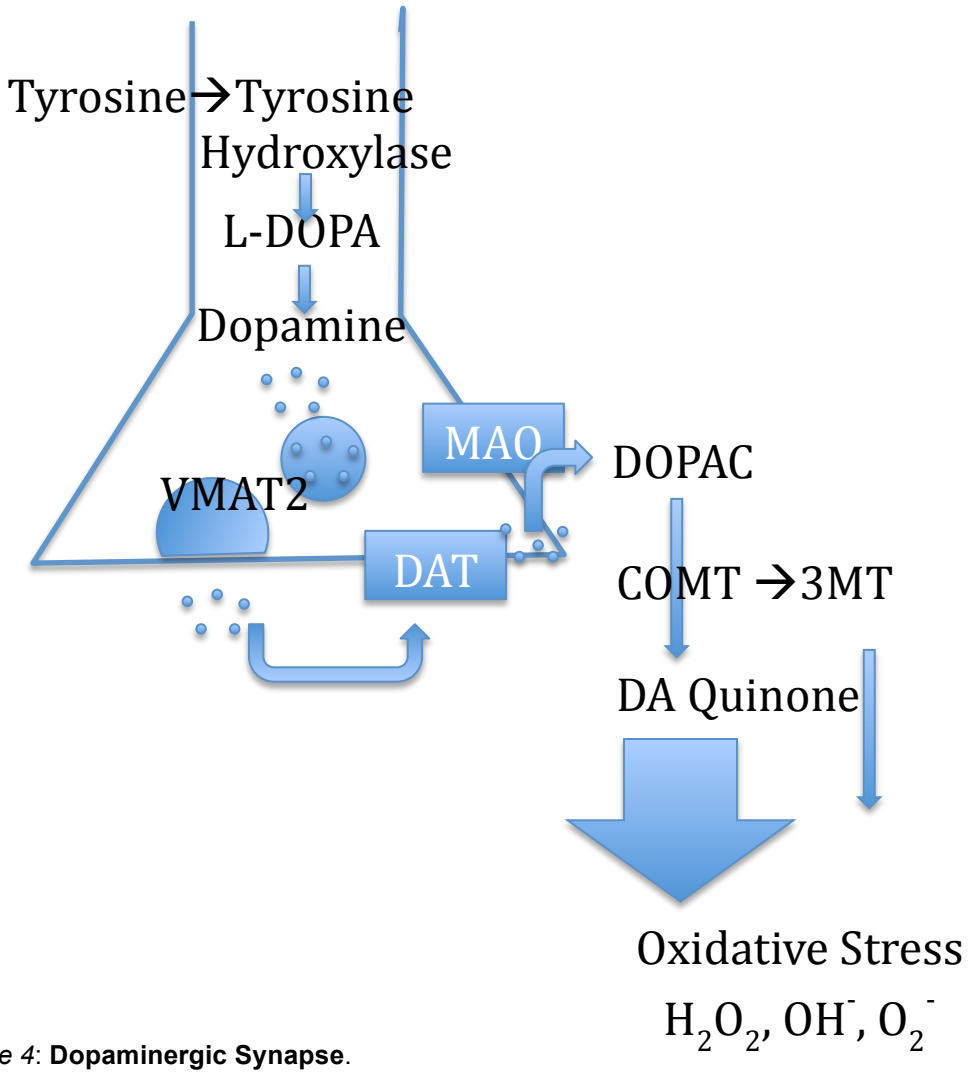


Figure 4: **Dopaminergic Synapse**.

DA can exert activity at autoreceptor sites on DA-producing neurons as well as on postsynaptic cells with DA receptors. 90% of striatal neurons are medium-sized spiny projection neurons that receive inputs from the VTA, and are differentiated based on their primary dopamine receptor expression and anatomical projection (Everitt and Robbins, 2005, Chuhma et al., 2009). Dopamine 1-receptor (D1R)/GABA-ergic cells project the substantia nigra pars reticulata and primarily express dynorphin and SP proteins. This tract is regarded as a direct striatonigral pathway, whereas Dopamine 2-receptor(D2R)/GABA-ergic cells project to the lateral globus pallidus, an indirect striatopallidal pathway, and produce the peptide enkephalin (Matemales et al., 2009). There are approximately equal numbers of each projection neuron population in the striatum.

Of the five subtypes of dopamine receptors (D1R, D2R, D3R, D4R and D5R), each receptor is characterized by their likeness to either of the first two receptors, such that the D1 class is comprised of D1R and D5R. The D2 class is composed of D2R, D3R and D4R. These distinctions are made due to gene structure and pharmacological properties. D1-like receptors are largely regarded as stimulatory and activate adenylyl cyclase. This intracellular cascade activates both cAMP-dependent protein kinase and protein phosphatase-1 inhibitor DARPP-32. In turn, ATP is converted to cyclic AMP that will bind protein kinase A. D1R signaling can also signal via phospholipase-C, which is independent of cAMP and uses intracellular calcium instead. The D2 subclass participates in negative feedback by inhibiting this second messenger system. DA receptors are metabotropic G-protein coupled protein receptors (GPCR) and signal through ligand (neurotransmitter) binding (Kandel et al., 2000). They contain seven transmembrane domains and can both open and close channels based on ion transmission, a slower mechanism than ionotropic transmission.

D1Rs are essential to metabolic function. D1R knockouts die prematurely (Drago et al., 1994) while D2R receptor-deficient mice can survive to adulthood with severe motor control deficits (Baik et al., 1995). D1R agonists trigger cocaine and cue-associated relapse, and the effect can be blocked with receptor antagonists (Koob and Volkow, 2010).

D4R have been implicated in increased exploratory behavioral traits. These receptors are largely expressed in the hypothalamus and limbic systems (Kandel et al., 2000). Investigation of D3R in the drug-addicted striatum is new, but shows promise for the target of therapeutic treatment. D3R antagonists can inhibit intrastriatal self-administration of stimulants in rodents, while ablating the receptor does not have the same effect (Song et al., 2011). D3Rs are also associated with cytogenesis in the striatum and substantia nigra pars compacta (Van Kampen et al., 2004, Van Kampen and Eckman, 2006).

While DA production and autoxidation contribute to striatal neurotoxicity in the presence of METH, downstream regulation of glutamate (GLU) and GABA transmission is crucial to this process. The striatum receives excitatory GLU input from the cortex and thalamus (Walaas 1981,

Zaborszky et al., 1985). This transmitter signals through 3 types of receptors-NMDA, AMPA and GPCRs, all of which may be involved in toxicity signaling.

Both NMDA and AMPA receptors are ionotropic, opening channels for sodium, potassium, and calcium. When METH enhances GLU from the cortex via D1R-dependent mechanism, GLU signals back to AMPA and NMDA receptors located on striatal dopaminergic cells. Signaling through NMDA receptors increases intracellular calcium levels via calcium/calmodulin binding and leads to toxicity by mitochondrial damage (Takano et al., 2003, Mark et al., 2007). As described later, NPY may exhibit protective effects during toxicity by modulating GLU-induced intracellular calcium.

GLU also induces the formation of superoxide radicals and DA overflow (Gunasekar et al., 1995). GLU receptor blockade (mGluR5) attenuates ROS formation and striatal DA terminal toxicity, as well as decreases DA overflow in the striatum (Battaglia et al., 2002, Tokunaga et al., 2009). This receptor subtype is coupled with NMDARs, and mGluR5 signaling enhances both DA and NMDA activity (Kew and Kemp, 2005, Pisani et al., 1997). NMDAR antagonists also block METH-induced DA depletion. Pretreatment with this antagonist MK801 prevents METH-induced hyperthermia as well, while NMDAR agonists can exacerbate a non-toxic dose of METH (Sonsalla et al., 1998, Bowyer et al., 2001).

Glutamate is derived from glutamine and is the precursor for GABA, the brain's major inhibitor transmitter. After signaling, GABA is sequestered and processed through the "GABA shunt" in order to replenish stores of glutamine and GLU, with the help of GABA transaminase and alpha ketoglutarate. GABA also participates in control of DA and GLU release. When the direct D1R dependent pathway is activated by METH, GABAergic cells from the striatum extend to the substantia nigra and globus pallidus-internal, which normally inhibits the thalamus. GABA transmission to the substantia nigra decreases nigrothalamic activity and disinhibits thalamocortical transmission. This increases GLU activity (lowered GABA inhibition) from the cortex to the striatum. Through the indirect (D2R-dependent) pathway, GABA is sent to the globus pallidus-external to act on the subthalamic nucleus, which then stimulates the substantia

nigra and globus pallidus internal. In the presence of METH, substantia nigra and globus pallidus internal stimulation inhibits the thalamus and release of GLU from cortex to the striatum. It is the interaction of these transmitter systems that modulates striatal homeostasis, motor activity, and METH-induced excitotoxicity.

Table 1 Striatal Neurotransmitters

Transmitter	**Dopamine**	**Glutamate**	**GABA**
Signal	**Modulatory and Inhibitory**	**Excitatory**	**Inhibitory**
Synthesis	**Decarbox of L-DOPA** **Derived from Tyrosine** **Hydroxylase**	**Glutamine**	**Derived from Glutamate,** **Glucose, Glutamine**
Receptors	**D1R,2,3,4,5**	**NMDAR,** **AMPA** **GPCRs**	**GABA-A, GABA-B**

Table 1 Striatal Neurotransmitters: Functions and precursors of signaling involved in METH-induced neurotoxicity

E. STRIATAL INTERNEURON POPULATIONS

In addition to the 90% of D1R/GABA and D2R/GABA spiny projection neurons, there are 10% of remaining striatal cells that comprise the interneuron population. These include medium aspiny neurons- parvalbumin/GABA, calretinin/GABA and NPY/NOS/SST and cholinergic (ChAT)-expressing cells (Vincent and von Krosigk 1988).

While there are relatively few interneurons, their synaptic connections are crucial to regulation of striatal output. Interneurons that stain for ChAT are large in size (20-50μm) and receive input from the cortex, thalamus, and collaterals of striatal projection cells (Kawaguchi et al., 1997). They comprise 1-3% of all cells in this region (Kawaguchi et al., 1995). ChAT cells modulate both the feedforward outputs from the striatum and feedback responses within the striatum by exciting other interneurons and inhibiting projection neurons. ChAT cells contain a majority of D2R and muscarinic receptors, but also some D1R, NMDAR, and mGluRs (1,2), and NK-1Rs (Kawaguchi et al., 1997). The widespread activities of ChAT cells are acknowledged in METH toxicity literature and striatal function.

A small GABAergic cell type is known as the calretinin cell, which expresses the enzyme glutamic acid decarboxylase (GAD_{67}). They are cholinergic and a small population also contains PV (Holt et al., 1999, Figueredo-Cardenas et al., 1996). Little is known about the physiological properties of the calretinin cell type, but they are susceptible to METH toxicity within 7 days after treatment (Jayanthi et al., 2004). Calretinin cell function may also be associated with movement regulation as evidenced by models of Huntington's disease (Hamann et al., 2005).

The last two cell types are local inhibitory neurons with long axonal projections. Parvalbumin (PV) interneurons have GABAergic outputs, cortical input, and comprise approximately 0.7% of striatal cells (Tepper et al., 2008). This population has dense axon collaterals and fire in phasic patterns (Kita 1993, Kawaguchi 1997). These cells have most of the mGLUR subtypes and also contain D2Rs (Kawaguchi et al., 1995). The function of PV cells is crucial for sensorimotor integration and MSN regulation (Koos and Tepper,, 1999).

The Somatostatin/neuropeptide Y/Nitric Oxide Synthase (SST/NPY/NOS) subset of interneurons comprises another subclass of GABAergic cells. Less than 5% of cells in the rat

striatum are labeled somatostatin-containing aspiny cells (West et al., 1996). They are smaller in size than ChAT cells and synthesize the enzyme NOS along with the neuropeptides SST and NPY. SST/NPY/NOS cells regulate other striatal transmitters through NOS production, including GLU, GABA and ChAT (Kawaguchi et al., 1997). These cells express NK-1Rs and D1Rs, receiving direct stimulation from projection neurons. Importantly, SST/NPY/NOS cells are resistant to neurodegeneration in Huntington's disease and after doses of NMDA agonists that are usually toxic to striatal cells (Kumar et al., 1997). When cell phenotypes were labeled by TUNEL after toxic METH treatment, 21% were determined to have DARPP-32 phenotype, 45% were parvalbumin cells, and 29% ChAT/calretinin cholinergic cells. SST/NPY/NOS cells show resistance to METH-induced cell death (Zhu et al., 2006). Blockade of NK-1R on these cells can eliminate METH toxicity and demonstrates the critical role of SP signaling in the process (Wang et al., 2008). Additionally, NPY shows protective characteristics in models of epilepsy in the hippocampus (Xapelli et al., 2008). It is clear that this cell type modulates some effects of METH toxicity and warrants further investigation.

F. NEUROPEPTIDE PROCESSING

Neuropeptides are produced in a process that is different from DA and monoamine production. Biologically inactive precursors of neuropeptides are transcribed in the endoplasmic reticulum (ER) near the cell body before they are transported to the golgi apparatus for further processing. In this organelle, glycosylation and additional modifications take place before packaging the polypeptide into vesicles for fast axonal transport down the cell to the nerve terminal. This is where protein processing occurs and can actually take several hours. For example, prepro-substance P become pro-Substance P and eventually the biologically active substance P. Once active, substance P is released from vesicles at the cell terminal without docking at the synaptic membrane. Neuropeptides usually exert their effect on the postsynaptic cell in nanomolar concentrations and have a high affinity for their postsynaptic receptors. These peptides exhibit long lasting effect and can exhibit multidirectional effects by diffusion. Their

activity is terminated by either diffusion or breakdown by proteases (neuroscience.uth.tmc.edu - accessed 6/25/11).

Neuropeptide receptors are also classified as GPCRs. As previously mentioned, GPCRs regulate signals for most of the transmitters in the striatum, including DA, 5-HT, NPY, GABA, GLU, tachykinins and neurokinins. These membrane proteins can be glycosylated at any of their transmembrane domains that may allow the receptor to shift from its inactive to active state or back again.

Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe- Gly-Leu-Met-NH2) is a tachykinin neuropeptide that is present on striatonigral projection neurons, and is of interest to us because the receptors that mediate SP signaling are located in the striatum (ChAT and NPY/NOS cells) (Pascual and Bost 1990). SP is coded for by the preprotachykinin-A gene and exerts an inflammatory effect on the CNS by signal transmission through neurokinin-1 (NK-1) receptors. SP is degraded specifically by NEP (neutral endopeptidase) or by diffusion (Lee et al., 1981). During the inflammatory response, Substance P induces the production of interleukin-1 and cytokines from astrocytic and microglial stores, and has proved to be the target of much pain research to this effect (Martin 1992, 1993). Additional work has discovered its role in psoriasis and eczema-related itching (Hon et al., 2007) as well as gastrointestinal infection (McGowan 1985) and depression (Argyropoulos and Nutt, 2000)

The second neuropeptide that we study is NPY. NPY is abundantly distributed throughout the CNS and is associated with homeostasis maintenance, including energy, food intake, and anxiety. It is overexpressed in human and animal models of epilepsy (Furtinger et al. 2001). NPY-1R mRNA expression is found in the striatum, caudate, putamen and nucleus accumbens, whereas NPY-2R mRNA is expressed in the hippocampus, hypothalamus, thalamus, amygdala and brainstem. NPY-4R and NPY-5R are not detected in the striatum (Silva et al., 2005).

Since its discovery in 1982, researchers have learned how important NPY is to regulation of neural and systemic activity. The generation of knockout and transgenic animals confirmed previous findings about this molecule's orexigenic role in the hypothalamus, antiepileptic role in the hippocampus, and analgesic role in medulla and elsewhere (Bannon et al., 2000). For

example, NPY-2R knockout mice do not present with yohimbine-induced DA increase like wild type mice, suggesting that NPY-2R transmission decreases DA levels in the mPFC (Zambello et al., 2011). NPY-1R knockout mice lack the NPY-induced feeding response, while NPY expression in the hypothalamus is usually increased in stressful metabolic conditions (Pedrazzini 2004). Both NPY knockout and NPY-1R knockout animals exhibit decreased ethanol-induced sedation and elevated ethanol consumption (Thiele et al., 1998, Thiele et al., 2002). This may be related to the role of NPY in anxiety and pain (Wahlestadt et al., 1993).

PTSD subjects have lower baseline plasma NPY levels and exhibit hyper aroused behavior (Rasmusson et al., 2000). It is well documented that there is elevated drug use among PTSD patients, and that stress hormones like corticotropin releasing factor can contribute to this process (Koob and Zorrilla 2010). NPY receptor signaling is also associated with protective mechanisms in epilepsy (Vezzani et al., 2004), and NPY is upregulated in the brains of rats with sustained seizure at 6-12 hours after injury (Bellmann et al., 1991). Taken together, these studies show a modulatory role for NPY and its receptors in neurochemical signaling.

G. METHAMPHETAMINE NEUROTOXICITY AND ABNORMAL STRIATAL PHYSIOLOGY

Endogenous NO is produced from L-arginine by the enzyme NOS. NO has several physiological, and often regulatory, functions throughout the body including vasodilation and cell signaling. There are three known isoforms of NOS – neuronal (nNOS, type 1), inducible (iNOS-type 2), and endothelial (eNOS-type 3) (Nathan and Xie, 1994). Of these isoforms, nNOS and eNOS are activated by intracellular calcium increase and regulate calmodulin binding.

METH-induced neural damage in the striatum is triggered by excess GLU and DA release (Sonsalla et al., 1991, Nash and Yamamoto, 1992, O'Dell et al., 1993), and GLU binding to its striatal receptors (Eisch et al., 1996). At neurotoxic doses, METH induces both GLU release and oxidative stress in the striatum (Yamamoto and Zhu, 1998). GLU signaling through NMDA receptors allow a calcium influx to the post-synaptic cell where calmodulin is allowed to bind to the NO producing enzyme, NOS. In the presence of arginine, both NO and citrulline are released.

The NMDA receptor antagonist, MK-801, decreases striatal DA overflow in the presence of METH, blocks drug-induced mitochondrial damage to complex II, and pretreatment with this agent prevents the depletion of striatal DA terminals by this drug, further evidence for the participation of GLU in excitotoxic signaling (O'Dell et al., 1993, Pu and Vorhees, 1995, Brown et al., 2005). In fact, the amount of GLU release can be correlated with the extent of neural damage in selective brain regions (Stephans and Yamamoto, 1994).

METH-induced damage is dependent on excessive DA levels and concomitant GLU release (Xu et al., 2005). D1R (SCH23390) and D2R (raclopride) selective antagonists prevent METH-induced apoptosis, loss of DAT terminals, TH depletion as well as attenuate GFAP induction post-drug treatment (Sonsalla et al., 1986, Xu et al., 2005). As previously stated, DA causes adenylyl cyclase to convert ATP to cyclic AMP that will bind protein kinase A. This complex can modulate the activity of various downstream proteins. When DA signaling is paired with GLU signaling from the cortex after amphetamine administration, the striatum begins a cascade of what is now well-documented excitotoxic processes. METH triggers calpain-mediated proteolysis that is activated by GLU-induced calcium influx . This results in a strong feed-forward loop that disinhibits the normal overall striatal output, and results in abnormally excessive DA production (Yamamoto and Bankson, 2005).

While striatal DA and GLU release are dramatically increased immediately following METH administration, DA levels are depleted within 24 hours (Seiden et al., 1976). The effect of METH on the loss of 5-HT and DA terminals in the striatum is monitored by loss of striatal tyrosine hydroxylase (McGeer et al., 1971) and tryptophan hydroxylase activity (Hotchkiss and Gibb, 1980), DAT binding, 5-HT levels (Villemagne et al., 1998) and DA levels in the striatum of animals with METH treatment from 1 hour onwards (Truong et al. 2005, Green et al., 1992, Villemagne et al. 1998, Woolverton et al. 1989).

METH and amphetamine analogs are particularly psychoactive because of their structural similarity to dopamine, all of which contain a phenyl ring attached to an amino group. This

chemical design allows it to enter the axon terminals of dopamine neurons (Iversen 2006). METH enters striatal terminals through DAT via the exchange diffusion model discussed later. Raiteri and collegues (1979) established the presence of DATs when the group blocked DA release with a DA reuptake inhibitor, nomifensine. DAT is essential for removing extracellular dopamine, and when absent, extracellular DA does not increase after amphetamine administration (Jones et al. 1998).

DATs are phosphorylated and internalized after METH treatment (Fleckenstein et al., 1997). Protein kinase C activators can trigger DA efflux, and when incubated in protein kinase C inhibitor, METH-induced DAT loss and outward DA transport is not observed in the rat striatum (Kantor et al., 1998, Pristupa et al., 1998). Serotonin and norepinephrine transporters are similarly disturbed (Fleckenstein et al., 1997, Kokoshka et al., 1998, Haughey et al., 2000). When the N terminus is removed from DATs, the area usually phosphorylated in the presence of METH, amphetamine-induced DA efflux is decreased by 80% (Khoshbouei et al., 2004).

Evidence for the participation of DAT in METH toxicity is also seen by the loss of DAT in animal studies (Wagner et al., 1980, Bunswick et al., 1992, Eisch et al., 1992), the brains of chronic METH users (McCann et al., 1998, Volkow et al 2001), and postmortem tissue by autoradiography (Wilson et al., 1996). DAT knockout mice are hyperactive, dwarf-sized, and have increased extracellular dopamine with a lower overall concentration of dopamine throughout their lifetime (Jones et al., 1998). In studies of neurotoxicity in non-human primates, DAT binding detected by PET scanning determined that the transporter decreases in a dose-dependent manner with METH administration (Villemagne et al., 1998).

While METH was originally thought to deplete DA with the loss of DA terminals (Ricaurte et al., 1982), the exchange diffusion model solidified the significance of the DAT in METH-induced toxicity and introduced a mechanism for DA loss. In this system, the introduction of a substrate into a compartment can trigger the release of a second substrate from the same compartment . This "carrier" was later used to explain the transportation of both dopamine and amphetamine analogs across the synaptosomal membrane (Zaczek et al., 1991). DA is

transported down its concentration gradient unless AMPH diffuses in to the cell, whereby DA is released from intracellular stores in a calcium-independent manner (Sulzer et al. 1995, Fischer and Cho, 1979). The idea that DA and amphetamines share a common transport mechanism has been confirmed by measurement of current, and by DA efflux from cells in the presence of AMPH (Sitte et al., 1998). DA is leaked from presynaptic vesicles and is also released to synaptic clefts, which in turn decreases the quantal size of DA (Liang and Rutledge 1982, Sulzer et al. 1995, Jones et al. 1998). The mechanism for DA redistribution can be explained by the action of Vesicular monoamine transporters (VMAT2).

VMAT2s are the membrane-bound proteins that are responsible for the sequestration and repackaging of dopamine into presynaptic vesicles after the catecholamine exhibits its effect on a postsynaptic cell. The presence of VMAT2 enables the drug reward response and METH has an affinity for VMAT2, which rivals that of DA (Eiden and Weihe 2011, Hyman et al., 2006). While heterozygous VMAT2 knockout mice thrive, homozygous knockout mice do not survive longer than their first few postnatal days (Fon et al., 1997, Takahashi et al., 1997, Wang et al., 1997). Mice that express 5% of normal VMAT2 can survive, but experience severe nigrostriatal DA dysfunction and neurodegeneration that resembles Parkinson's disease (Caudle et al., 2007). VMAT2 LO (5% expression) animals show an accelerated age-dependent decline in motor activity and increase in striatal DOPAC, oxidative stress and TH levels. Since METH alters VMAT2 function, it is no surprise that many of neurodegeneration markers are present in pathology of METH toxicity.

Even further, METH induces the redistribution of DA from VMAT-2-associated vesicles as soon as 1 hour after treatment, (Eyerman and Yamamoto, 2005) and VMAT-2 knockout mice are susceptible to the toxic effects of METH and DA buildup (Fumagalli et al., 1999). Both DAT reversal and VMAT2 redistribution are necessary for the volume of DA loss that we observe after METH treatment (Jones et al., 1998). DAT and VMAT2 function are crucial to striatal homeostasis because dysfunction of these transporters displaces DA stores and subsequently alters levels of presynaptic DA-associated markers. In vivo PET scanning in primates shows that METH-reduced DAT binding corresponds with dose-dependent loss of DA, DOPAC, and TH levels (Melega et al.,

2000)

METH is thought to disrupt VMAT2-associated vesicles through the "weak base hypothesis" (Sulzer 2011). Since METH is a lipophilic weak base, it acquires protons in acidic vesicles and is less membrane permeable than its deprotonated form. While the acidic environment is ideal for accumulation of DA against its concentration gradient, METH destroys this natural gradient. Vesicles are unfit to repackage transmitter and DA begins to build up in extracellular space (Sulzer and Rayport, 1990, Sulzer 2011). While METH and amphetamines are VMAT2 substrates that disrupt vesicular function, there is continued leakage in the presence of reuptake inhibitors like reserpine (Volz et al., 2006, Sulzer 2011). The weak base hypothesis does not explain additional methods of DA leakage. It has been suggested that a carrier-mediated exchange mechanism could also release DA to the cytosol (Partilla et al., 2006).

Through either or both mechanisms, the drug-induced release of monoamines from vesicular packaging allows for the auto-oxidation of DA and formation of oxygen radicals (Takahashi et al., 1997, Fumagalli et al., 1999). These toxic byproducts eventually destabilize the mitochondria of striatal cells, and are responsible for the damaged protein and apoptotic effects that are observed (LaVoie and Hastings, 1999).

Reactive species are observed in the normal brain for processes such as mitochondrial respiration, but METH generates superoxide radicals in an excessive manner (Cadet and Brannock, 1998). Of interest to us is that NO production appears to mediate GLU damage in the striatum (Dawson and Dawson, 1996). It is important to recognize that oxidative stress occurs when homeostasis between free radicals and antioxidants are unbalanced. METH generates levels of free radicals that are too potent for scavengers to neutralize.

Key:
METH=Methamphetamine
VMAT2=Vesicular Monoamine Transporter
DAT=Dopamine Transporter
D1R=Dopamine 1 Receptor
D2R=Dopamine 2 Receptor
L-DOPA=L-3,4-dihydroxyphenylalanine

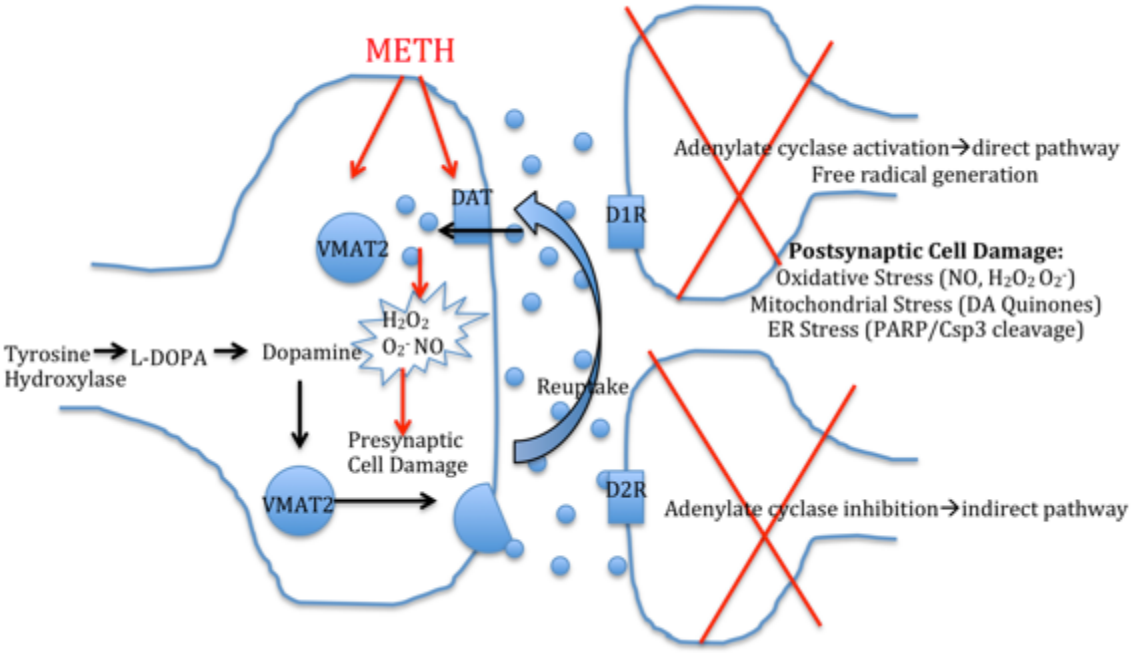


Figure 5. METH-induced neurotoxicity

Nitric oxide (NO) is a reactive free radical that is toxic at high doses but otherwise may exhibit a neutral or protective effect. NO can damage the blood brain barrier, permitting interchange of brain fluids and inducing an inflammatory cascade (Bowyer et al., 2008). Aside from interneurons, NO can be generated by microglia and astrocytes that are activated from neuroinflammation. ROS/RNS such as NO can alter DAT function. Reactive free radicals are especially harmful to DA neurons in part due to their vulnerability to malonate, which disrupts

complex II of the mitochondria and potentiates DA depletion as well as ATP loss (Burrows et al., 2000, Beal et al., 1993).

NO can scavenge oxidative radicals and directly inhibit caspase activity. However, it exacerbates damage in most models of neurodegenerative disease. NO appears in diseases such as Alzheimer's, ALS, Parkinson's Disease and AIDS, displaying similar pathology to the METH-intoxicated brain (Asanuma et al., 2004, Boje 2004, Ferrucci et al., 2008). NO owes its ubiquitous role in systemic biology to its short half-life and ability to diffuse across lipid membranes (Boje 2004).

While NO is unstable and difficult to quantify, byproducts such as 3-NT and cGMP are more easily measured, and can sustain a toxic effect for a longer period of time. Once diffused inside the plasma, NO can activate soluble guanaylyl cyclase (sGC) and generate cGMP. 3-NT is produced a result of the reaction between nitrating oxidants and tyrosine, in the presence of the toxin peroxynitrite (Ischiropoulos and Beckman, 2003, Blanchard-Fillion et al., 2006, Pacher et al., 2007). When metabolized, 3-NT triggers cell death in dopaminergic PC-12 cells (Blanchard-Fillon, et al., 2006). In the following studies, cGMP and 3-NT levels are measured as a substitute for NO activity.

II. Preliminary Studies

Our lab has taken a particular interest in nNOS because nNOS knockout mice show an attenuation of METH-induced neurotoxicity (Itzhak et al. 1998). NOS inhibition also eliminates loss of DAT binding sites (Itzhak and Ali, 1996). METH increases the number of postive nNOS cells, but not iNOS cells at early (1 hour) and late (24 hours) timepoints in the striatum (Deng and Cadet, 1999). Most importantly, nNOS is expressed in our brain region of interest by the interneuron population that coexpresses NPY and we have confirmed by immunohistochemistry the presence of somatostatin and NPY coproduction.

A strong implication for the participation of NO in DA/GLU toxicity lies in the idea that antioxidants attenuate METH-induced damage markers (Imam et al., 2001). Transgenic mice that overexpress superoxide dismutase (CuZnSOD) are less susceptible to METH-induced loss of DA and DOPAC in the cortices and striatum (Cadet et al., 1994). They also have more serotonin

uptake (Hirata et al., 1995). MnSOD transgenic mice show protection against METH neurotoxicity because of their ability to scavenge superoxide radicals in the brain.

Finally, there may be a role for microglia in the protective response to METH toxicity. METH activates microglia as assessed by immunohistochemistry where cells appear with thicker branching, peaking at 2 days after drug treatment (Thomas et al., 2004, LaVoie et al., 2004). Microglial activation has been observed in neurotoxic pathology (LaVoie et al., 2004), but more recent research has begun to describe the role of these cells in the signaling process. Microglial activation is found in association with the inflammatory response and release of cytokines (interleukins (IL-1β, IL-6), tumor necrosis factor (TNF-α), transcription factors (NF-κB), and interferon gamma (IFN-γ)), which in turn potentiate the release of METH-induced GLU release to continue the toxic cycle. Pretreatment with reserpine, L-DOPA, and clorgyline all dramatically potentiate the DA depletion caused by METH while also significantly activating microglia (Kuhn et al., 2008). Rather than acting to promote a neuroprotective or inflammatory response to presynaptic dopamine terminal damage, microglia seem to participate in crosstalk with nearby striatal cells in an ongoing manner. In turn, this continues distress signals and release of damage markers.

Our lab investigates the neurotoxic effects of METH during the first 24 hours of drug administration, and the role of neuropeptides in this process. Eventually, METH induces excessive postsynaptic signaling that is modulated by striatal neurokinin-1 receptors (NK-1R), the specific receptor for the neuropeptide SP (Ayata et al., 1997; Schulz et al., 1995). Along with the release of calcium from GLU signaling NOS produces citrulline and NO at harmful concentrations. Once diffused inside the plasma, NO can activate soluble guanylyl cyclase soluble guanylyl cyclase soluble guanylyl cyclase ,(sGC) and generate cGMP (Figure 6)

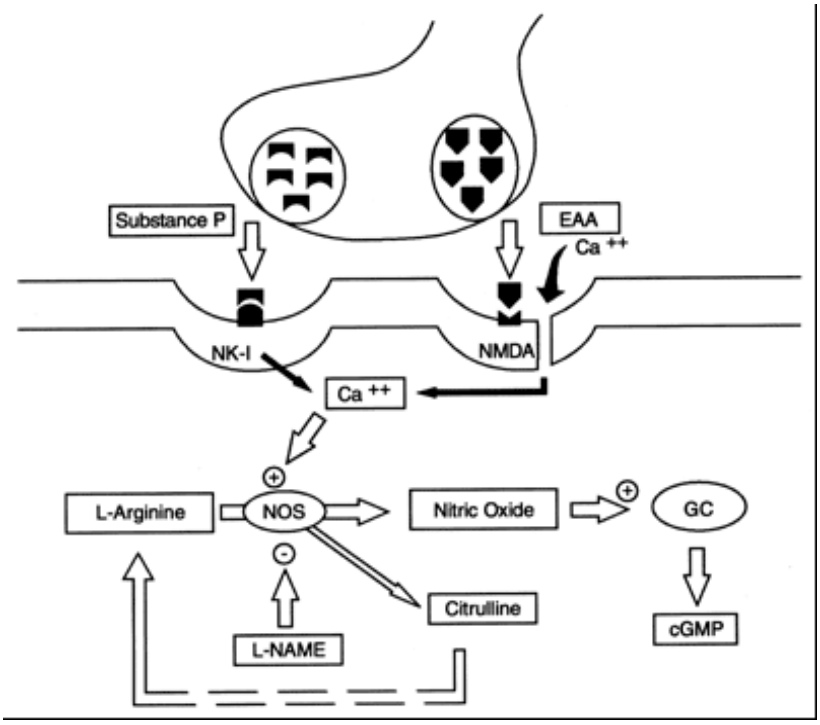


Figure 6. Nitric Oxide release is regulated by the NK-1 and NMDA receptors. (Radhakrishnan and Henry 2005) METH excitotoxicity is modulated by SP and Glutamate. Excessive release of these transmitters culminates in the production of NO and the synthesis of cGMP.

The seminal paper for my work was published in 1994, where our laboratory leader Dr. Jesus Angulo reviewed the action of neuropeptide regulation in the striatal brain regions. Here it was noted that these peptide classes (enkephalin, dynorphin, tachykinins and neurotensin) interact with dopamine signaling to produce a dynamic chorus of regulatory action in this brain system (Angulo and McEwen, 1994). Soon after it was shown that an NMDA receptor antagonist (MK-801) could elevate levels of several neuropeptides in the striatum after continued treatment, but an acute treatment of the same antagonist depleted stores of neuropeptide precursors (Angulo et al., 1995). This effect was reversed when administered with dopamine receptor antagonists (Noailles et al., 1996). In this way, the interaction of neuropeptides with DA and NMDA receptors was recognized.

Histochemical study later revealed that binge dosing of METH induced the production of neuropeptide mRNA (preprotachykinin, preproenkephalin) in a dose dependent manner. This

effect was pronounced in the dorsomedial and ventromedial brain regions (Zhang et al., 1997). In 2001, the Angulo group described drug-induced dopamine overflow through neuropeptide activity.

The link between SP and METH was discovered in 1984 after systemic treatment with binge dosing (15 mg/kg, 5 doses, every 6 hours), when SP-like immunoreactivity was nearly doubled in the striata of rats and also increased in the VTA and mediahabencular nucleus after single and multiple METH doses (Ritter et al., 1984, Hanson et al., 1986). This effect was potentiated with the addition of DA reuptake blockers (Ritter et al., 1985) and completely blocked by the addition of D2 receptor antagonists.

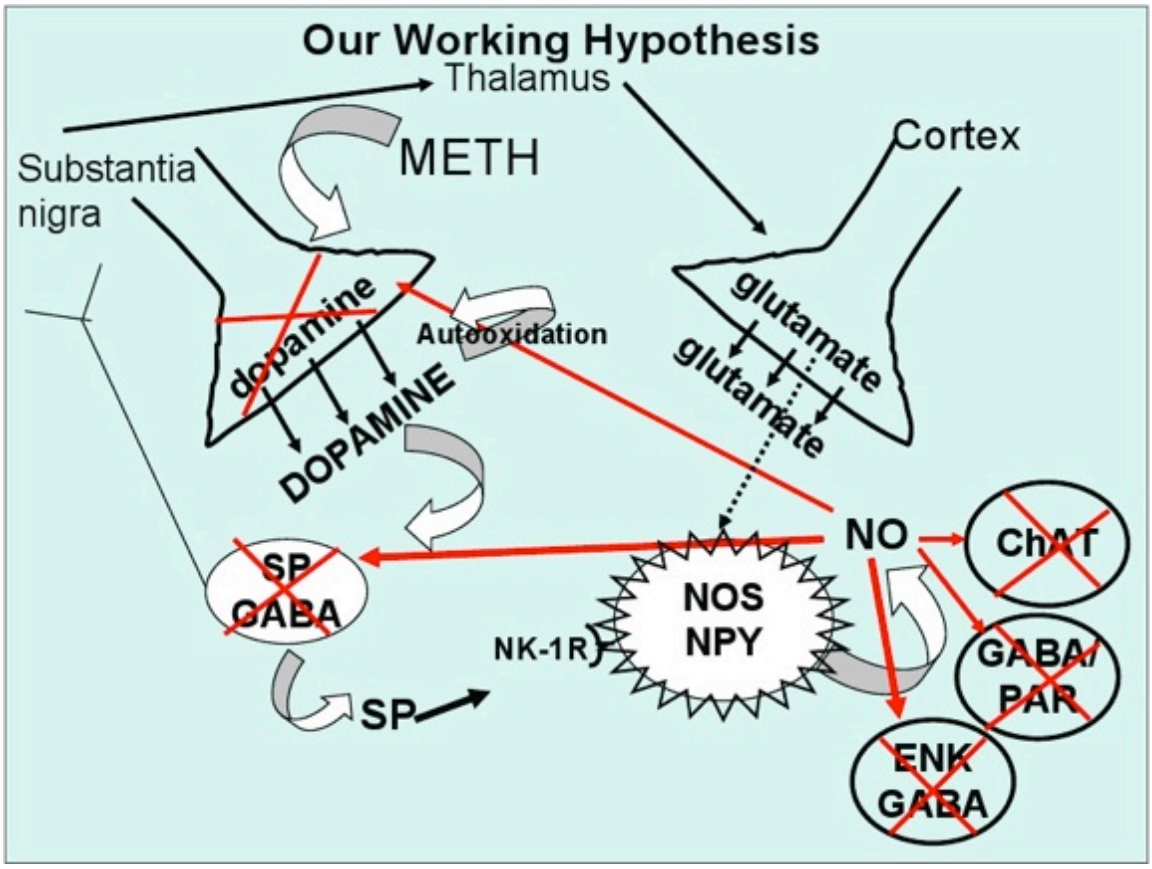
Our *in vivo* data demonstrate that striatal DA-terminal toxicity, reactive astrocyte induction, NO production, 3-NT and l-citrulline production, as well as apoptosis are all dramatically induced by SP signaling via the NK-1R agonist GR-73632 in the presence of METH (Wang and Angulo, 2011, Angulo et al. 2004, Yu et al. 2002,2004). 30 minutes after an IP injection of METH, the NK-1R/SP complex is almost exclusively internalized (endocytosis) into the interneurons expressing NPY, SST and nNOS (Wang et al. 2008). When an NK-1R antagonist (WIN-51,708) is injected 30 minutes before METH, GR-73632-induced 3-NT production is attenuated (Wang and Angulo, 2011). The number of lost DA terminals and apoptotic striatal neurons are also significantly reduced when compared to METH alone (Wang et al., 2008). Finally, the infusion of a calmodulin inhibitor (calmidazolium) attenuates GR,73632-induced 3-NT production (Wang and Angulo, 2011). When taken together, striatal 3-NT production is dependent on nNOS production, calcium signaling and NK-1R signaling. Calcium-calmodulin binding regulates the active state of NO production.

Our current research links the effect of neuropeptides to the production of oxidative and nitrosative byproducts in the presence of METH administration. In order to identify the mechanism by which SP potentiates neural damage in the presence of METH, activation of the NOS enzyme and the production of 3-NT was measured. 3-NT is produced by the nitrosylation of tyrosine in the presence of RNS, and is an indirect marker of NO production. METH does not upregulate the number of NOS-expressing cells in the striatum, but it does increase levels of 3-NT production by

30-fold 4 hours post-METH (Wang and Angulo, 2011) and seven-fold 6 hours post drug-treatment (Wang et al., 2008).

The protective effect of NPY has been investigated in both the hippocampus and the striatum. NPY is thought to be a neuromodulator because of its coexpression in cells that produce transmitters such as GLU, GABA and SST (Silva et. al., 2003). In a model of KCl-evoked GLU release, NPY attenuated transmitter signaling in the hippocampus (Silva et al., 2005). METH-induced microglial activation and motility in hippocampal tissue can be downregulated by NPY administration (Xapelli et al, 2008). Of interest is the fact that NK-1R and NO species are found together on about 95% of SST interneurons, a cell population of the nigrostriatal system that is not apoptotic in response to METH and neurodegenerative disease such as Huntington's Disease (Kumar et. al. 1997, Cicchetti et al., 1996). The coexpression of NK-1Rs and NOS on these neurons suggests that they may have a protective mechanism for avoiding SP and NO-induced apoptosis. (Itzhak and Ali 1996).

Excessive release of DA induced by METH stimulates the release of SP from collaterals of SP-containing projection neurons of the striatum. SP activates the NK-1Rs on the NOS-expressing SST interneurons. It is the NO produced in this way that damages the DA-terminals of the striatum and induces apoptosis of some striatal neurons. The long-term goal of this project is to elucidate the mechanism by which NK-1Rs signal toxicity of the DA-terminals and apoptosis of some striatal neurons (SP/GABA, ENK/GABA, GABA/PAR, Ach), as well as the mechanism in which NPY attenuates the appearance of apoptotic markers.



KEY:

METH – methamphetamine	**SP - substance P**
GABA - gamma butyric acid	**NK-1R - neurokinin 1 receptor**
SOM – somatostatin	**NOS - nitric oxide synthase**
NPY - neuropeptide Y	**NO - nitric oxide**
Ach – acetylcholine	**PAR - parvalbumin**
ENK - enkephalin	

Figure 7. Our Working Hypothesis: METH induces excessive DA release from substantia nigra by direct D1R signaling, and abundant GLU release via a thalamocorticostriatal pathway. DA exerts harmful effects on striatal neurons by autoxidation and by triggering the release of SP. SP release is detrimental to striatal cells because it triggers NOS production of NO via the NK-1R on NOS/NPY interneuron. At high levels, NO is toxic and can trigger apoptotic pathways. Most striatal cell populations are susceptible to cell death by NO-dependent signaling, but the NOS/NPY neuron itself is immune to apoptosis during METH-induced toxicity.

Since both METH neurotoxicity and neurodegenerative disorders involve modulation of DA systems in the midbrain, many of the cellular and molecular outcomes can be compared (Thrash et al., 2009). As mentioned, NPY/NOS cells are not apoptotic in response to METH and neurodegenerative disease (Kumar et. al. 1997, Cicchetti et al., 1996). An animal model of TBI with fluid percussion shows that NPY is involved in a compensatory response to cell damage markers and NO release (McIntosh and Ferriero, 1992). TBI causes brain compression that later leads to neuroinflammation, cell death and the release of ROS. Of note are the altered calcium levels and DNA damage that accompanies the cellular breakdown (Gold et al., 2009).

Apoptosis, DNA damage, and oxidative stress are well established in association with METH-induced neurotoxicity. Cell death is noted via necrotic and apoptotic pathways (Choi et al., 2002, Fumagalli et al., 1999). A single bolus dose of METH (40 mg/kg) activates cleaved caspases (3,9,12) and proteins associated with mitochondrial-dependent cell death (Jayanthi et al., 2004). TBI activates both caspase-dependent and calpain-dependent (calcium-activated proteases) cellular damage, dependent on the αII-spectrin and microtubule associated protein-2 (MAP-τ) (Farkas et al., 2005, Kobeissy et al., 2006).Opii and colleagues (2007) also demonstrated that TBI induces mitochondrial damage through oxidative stress in cortical and hippocampal brain regions.

AD is characterized by the formation of amyloid plaques from amyloid beta protein, which lead to an inflammatory response in the brain by astrocytes and microglia. At this juncture, AD begins to look like METH toxicity. Inflammatory cytokines (IL-1β, NF-κB and TNF-α) form and there is marked depletion of calcium-dependent proteins including calbindin, Fos and Arc (McGeer and McGeer, 2004). These inflammatory cytokines generate NO via signal transduction pathways, and eventually lead to cell death. In an LPS-stimulated cell culture of microglia, NPY shows the ability to inhibit NO production, IL-1β release and NOS expression via NPY-1Rs (Ferrera et al., 2011). These data reiterate the modulatory role of NPY in NO formation and potential therapeutic potential of NPY in inflammatory disease treatment. While current neuropeptide analogs are generally unsuitable for therapeutic treatment due to their metabolic

instability, research is underway to uncover more chemically stable alternatives (Green et al., 2010).

PD motor activity may be modulated, in part, by NO-related activity. Activation of striatopallidal neurons by optogenetic technique induces Parkinsonian like freezing whereas striatonigral activation reversed this effect (Kravitz et al., 2010). Similarly, SP and NMDA antagonists can suppress motor activation by inhibiting D1R activation (Deutsch et al., 1996, Mancuso et al., 1994). When NOS inhibitors are given with L-DOPA in animals, rotorod performance is improved (Padovan Neto et al., 2009) If NPY exerts an effect striatal DA activity and NOS activation, it may play a role in restoring motor deficits and dyskinesia related to PD.

CHAPTER 2

I. MATERIALS AND METHODS

A. ANIMAL CARE AND USE

All procedures regarding animal use were performed in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care Committee at Hunter College of the City University of New York. Male Institute of Cancer Research mice (Taconic, Germantown, NY) were housed individually on a 12 hour light/dark cycle with food and water available *ad libitum* from 10-13 weeks of age.

B. DRUG PREPARATION AND TREATMENT

(+)-Methamphetamine hydrochloride (Sigma, St. Louis, MO) was dissolved in 10mM phosphate-buffered saline, pH 7.4 (PBS). Groups of mice were injected intraperitoneally with either one bolus METH dose of 30 mg/kg of body weight in a volume of 200μl or a matching volume of saline.

NPY-1R agonist Leu31–Pro34 NPY, H-Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Leu-Thr-Arg-Pro-Arg-Tyr-NH_2, (H-8575, Bachem, Torrance, CA) and NPY Y2 agonist NPY (3-36), H-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH_2, (H-8570, Bachem, Torrance, CA) were dissolved in saline and further diluted in vehicle. NPY Y1 antagonist (BIBP3226 (Bachem, Torrance, CA) and NPY-2R antagonist BIIE0246 (Tocris Biosciences, Ellisville, MO) were diluted in aCSF vehicle.

For sacrifice and perfusion, animals were fully anesthestized with ketamine (100mg/kg) and acepromazine (3mg/kg), and perfused through the heart with 20ml of PBS followed by a 20 ml of 4% paraformaldehyde and 0.2% glutaraldehyde. The brains were post-fixed overnight in the fixative at 4ºC followed by 20% sucrose solution over 24 hours at 4ºC for cryo-protection. The brains were frozen at -80ºC until used. Coronal sections 30μm in thickness were cut in a microtome at -20ºC and stored in anti-freezing solution (30% glycerin solution in ethylene glycol) at -20ºC until used. Sections were collected serially from the striatum between bregma 0.02 and

1.4 mm into cryoprotectant solution. 36 sections were collected from each animal. For intrastriatal surgeries (Aim 1a, 2c) brain tissue was nicked in the left dorsal cortex for orientation.

C. INTRASTRIATAL SURGERY

Surgical technique was performed as described in previous literature (Wang and Angulo, 2011). Each animal was anesthetized with isofluorane gas (2.5%) via chamber for 5 minutes prior to surgical preparation. Once sedated, animal was transferred to stereotaxic frame (Model 5000, David Kopf Instruments, CA) with nose cone where an electric razor was used to shave fur over the injection site. The skin was cleaned with iodine on a sterile cotton swab and a 1-inch incision was made with hand held razor blade over injection site. A hole was drilled in the skull and a 25 gage 2µl Hamilton microinjection syringe was lowered into the striatum. Distance of injection sites (bregma, 0.5 mm; lateral, ±2.0mm; ventral, ±2.5mm) was determined using a mouse brain atlas (Franklin and Paxinos, 1997). The microinjection needle was left in position for 5 minutes prior to drug injection. Drugs were injected over a 10 minute period at a rate of 0.1µl/min and the needle was left in place for an additional 5 minutes before removal from the striatum. Animals received bilateral injections: drug (right striatum) or aCSF (left striatum) was administered via intracranial infusion to allow the left striatum to serve as a control. Intraperitoneal injections were given of either methamphetamine or saline at doses listed above immediately following sterotaxic surgery (wound closed with two sutures).

D. IMMUNOHISTOCHEMISTRY FOR 3-NT

For immunostaining of 3-nitrotyrosine (Aim1a), floating sections of striatal tissue were washed 3 times for 5 minutes each in PBS with 0.3% Triton X-100 followed by incubation with M.O.M Blocking buffer (BMK-2202, Vector laboratories, Burlingame, CA) for 1 hour at room temperature. This was followed by incubation in working solution of M.O.M diluents buffer (80µl /ml TX-PBS) for 10 minutes. The sections were incubated with a monoclonal anti-mouse antibody against 3-nitrotyrosine (1:500, SCBT, CA) in diluents buffer at 4 °C overnight. Next day, the sections were rinsed with PBS 3 times for 10 minutes each and stained with a Dk x Ms Cy3

secondary antibody for 1 hour, (Chemicon, Temecula, CA) while protected from light at room temperature. The sections were rinsed with PBS for 5 minutes and mounted onto glass slides with Vector Hard Set Mounting Medium H-1400 (Vector Laboratories, CA).

E. IMMUNOHISTOCHEMISTRY FOR NPY RECEPTORS

6 sections from each of 4 untreated animals were stained with antibodies for cell marker (DARPP-32, ChAT, Parvalbumin, NOS) and NPY receptor subtype. This comprised 8 groups. Floating sections of striatal tissue were washed 3 times for 5 minutes each in PBS with 0.3% Triton X-100 followed by a 1 hour incubation at room temperature with 10% donkey serum (NDS). The sections were incubated 24-48 hours at 4 °C with primary antibodies. For NPY1R label, polyclonal Rb x Y1R antibody (1:250, Novus, R-1015-100) or Rb x NPY2R (1:100, Novus, NBP1-39724) were used for receptor label and Ms x NOS1 (1:250, SCBT cat #5302), Gt x chAT (Millipore AB144p Billerica, CA) 1:500 Gt x DARPP-32 (1:100, SCBT, sc-21601) , Ms x Parvalbumin (1:500, Millipore, MAB1572, Billerica, CA) were used to label cell type. After primary incubation, sections were rinsed with PBS 3 times for 10 minutes each and stained with two secondary antibodies, each for 1 hour, (Dk anti-Gt FITC, Dk x Ms FITC ap192F, Dk x Rb FITC ap182F, Rb x Ms FITC ap160F, Dk x Rb Cy3) (Chemicon, Temecula, CA) Sections were rocked while protected from light at room temperature. The sections were rinsed with PBS for 5 minutes and mounted onto glass slides with Vector Hard Set Mounting Medium H-1400 (Vector Laboratories, CA).

F. IMMUNOHISTOCHEMISTRY FOR cGMP COLABEL

Floating sections of striatal tissue were washed 3 times for 5 minutes each in PBS with 0.3% Triton X-100 and blocked for non-specific binding using 10% Normal Donkey Serum or Mouse-on-Mouse IgG (BMK-2202, Vector laboratories, Burlingame, CA) at room temperature for 1 hr. The sections were then incubated in primary antibody overnight at 4ºC with block serum with the following markers: Rb x cGMP (1:500 09-101 Millipore) and phenotype markers as descrived

above. The next day, sections were washed and incubated for 1-2 hours in secondary antibody (as given above) (Chemicon, Temecula, CA) while protected from light at room temperature. The sections were washed and mounted onto superfrost glass slides, sealed and coverslipped with Vectorshield hard set™ mounting medium for fluorescence (H-1400 Vector Laboratories, Burlingame CA).

G. IMMUNOHISTOCHEMISTRY FOR ACTIVE CASPASE-3

Floating sections of striatal tissue were washed 3 times for 5 minutes each in PBS with 0.3% Triton X-100 and blocked for non-specific binding using 10% Normal Donkey Serum or Mouse-on-Mouse IgG (BMK-2202, Vector laboratories, Burlingame, CA) at room temperature for 1 hr. The sections were then incubated in primary antibody overnight at 4ºC with the following markers: Rb x cGMP 09-101 Millipore, Gt x active caspase-3 The sections were incubated for 1-2 hours in secondary antibody, Dk x Rb Cy3 1:500 and Dk x Gt FITC 1:500 (Chemicon, Temecula, CA) while protected from light at room temperature. The sections were washed and mounted onto superfrost glass slides, sealed and coverslipped with Vectorshield hard set™ mounting medium for fluorescence (H-1400 Vector Laboratories, Burlingame CA).

Table 2 *Summary of Reagents used for histochemistry*

Assay	Antigen Retrieval	Block	Primary AB	Secondary AB
3-NT (Aim 1a)	Citric Acid 3x5min at 65C	M.O.M Blocking buffer (36µl/ml Tx-PBS)	anti-Ms 3-NT 1:500 (SCBT, CA)	Gt x Ms Cy3 1:500 (Chemicon, Temecula, CA)
Y1R,Y2R (Aim 1b)	Citric Acid 3x5min at 65C	10%NDS	Rb x Y1R Rb x Y2R (DARPP-32, ChAT, Parvalbumin or NOS)	Dk anti-Gt FITC, Dk x Ms FITC ap192F, Dk x Rb FITC ap182F, Rb x Ms FITC ap160F, Dk x Rb Cy3 (Chemicon, Temecula, CA)
cGMP (Aim 2a)	None	10% NDS	cGMP DARPP-32, ChAT, Parvalbumin or NOS	Dk anti-Gt FITC, Dk x Ms FITC ap192F, Dk x Rb FITC ap182F, Rb x Ms FITC ap160F, Dk x Rb Cy3 (Chemicon,

Table 2 Summary of reagents and dilutions used: Varying concentrations and dilutions used to assess cell phenotype and nitric oxide markers

H. RT-PCR FOR NPY mRNA

Striata were dissected from fresh frozen tissue (1 mm thickness) Tissue was homogenized and total RNA was extracted using Qiagen RNeasy mini kit (Qiagen, Valencia, CA. USA). RNA was isolated using Quick-RNA MiniPrep (Zymo Research). RNA integrity and quantification was performed using Thermo Scientific Nano Drop 1000 Spectrophotometer. The purity and integrity of the samples were determined using the ratio A_{260}/A_{280}. All samples used fell between 1.8 and 2.2. RNA was normalized to 100ng for PCR. Total RNA was reverse-transcribed to cDNA and amplified using Taqman One-Step PCR kit with TAMRA Taqman probe and custom primers: Forward – 5'GCA GAG GAC ATG GCC AGA TAC-3', Reverse-5'TGG ATC TCT TGC CAT ATC TCT GTC T-3', Probe-5'-FAM-CGC TCT GCG ACA CTA CAT CAA TCT CAT CA-TAMRA-3'. These NPY Primer and Probe for Taqman RT-PCR: GenBank Accession no. NM_023456, taken from Terroni et al. (2005). Real-time (RT)-PCR approach (Applied Biosystems Universal Thermal cycling, 7500 Real Time PCR System) measured levels of NPY mRNA in mice striata. Samples and GAPDH internal control were run in triplicate with each assay for 6 animals. $\Delta\Delta C_T$ values were calculated for paired drug and control animals.

I. QUANTIFICATION

For all histochemical assays, slides were coded and two different individuals blind to the treatment conditions performed counts to determine the number of labeled cells. For Aim 2b,2c images were taken with Hamamatsu 1394 ORCA-ERA Spinning Disk Confocal Microscope using a 60x objective lens. Data from 6 control animals and 6 animals for each drug concentration were analyzed. A 1mm area surrounding the injection site was exempted from quantification to avoid needle damage. The remaining area of the striatum was divided in to 4 quadrants for image capture. 4 images (dorsal lateral, dorsal medial, ventral lateral, ventral medial) were taken against the striatal border. Cells were quantified by quadrant and summed for each hemisphere. The aCSF hemisphere was nicked prior to acquiring slices in order to differentiate the treatments. Fluorescence intensity was measured using Volocity 5.2.0 (2008, PerkinElmer, Waltham, MA).

Background fluorescence was subtracted manually from each image. Distinct borders, morphology and significant stain throughout cell shape defined positive cells.

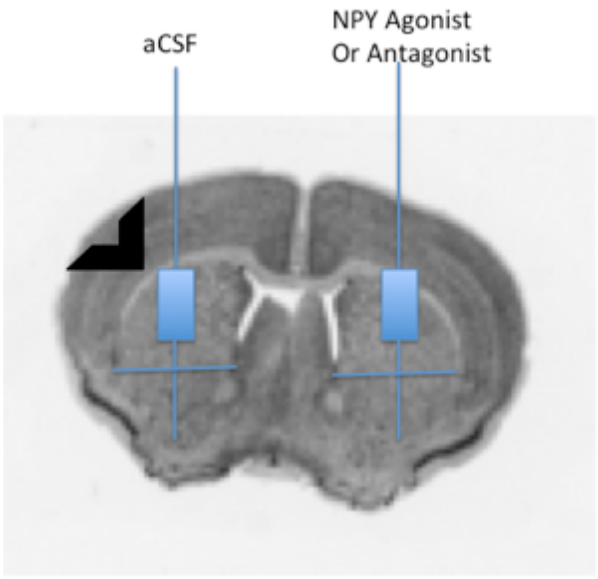


Figure 8. Quantification after Microinjection.

Fluorescent immunostaining for Aim 1a, 1b, 2a was visualized and images were taken with Leica TCS™ confocal microscope and corresponding Leica Lite LCS software system (Leica Microsystems, Heidelberg, Germany). FITC and Cy3 signals corresponded to single wavelength laser line 488 (green) and 568 (red) respectively. The striatum was divided into four regions corresponding to dorsal lateral, dorsal medial, ventral lateral, ventral medial. Z-stack images from each striatal region were taken in four-eight animals per group, six tissue sections per animal. Autofluorescence was automatically eliminated and two individuals blind to the study quantified all tissue samples. To avoid cross detection between the signals, the pinhole setting was less than 2μM and z-stacks 10μM thick were recorded sequentially between frames at 63X in a raster pattern series. Confirmation of co-label was done by reconstruction and orthogonal rotation of the images using the Leica confocal software in “view” and also “analysis” mode. Average surface area of striatum quantified per region was 1.5 mm^2. Distinct borders, morphology and significant stain throughout cell shape defined positive cells. Cells expressing NPY receptors were taken as

a percentage from total number of immunolabeled cells from each image. Cells expressing cGMP were taken as a percentage of total cells expressing cell marker.

J. STATISTICAL ANALYSIS

Statistical comparisons were performed from mean ± SEM. Differences between groups were analyzed by ANOVA followed by *post hoc* comparison using Fisher's protected least test. Differences between two groups were analyzed by Student's *t*-test. The significance criterion was set at p=0.05. Analysis was done with Graphpad Prism software (San Diego, CA).

II. Purpose

The last three decades of METH research, devoted to the study of neurotransmitter systems and DA flow, have yet to elucidate a mechanism of drug toxicity. There is a need to include neuropeptides in the dialogue surrounding this mechanism because of their known participation in neurodegenerative disorders and maintenance of other homeostatic processes. This is one of the first studies to address a role for neuropeptides in the excitotoxic process. The following studies are designed to assess the effect of NPY on acute methamphetamine toxicity (bolus dose of 30 mg/kg, sacrifice 4-16 hours after treatment). Many laboratories have described role of the mesolimbic system in METH-induced damage and the regulatory role of neuropeptides. To our knowledge, this is one of the first studies to discuss a differential role of NPY in NO modulation of specific cell populations. Our experiments take a histological and pharmacological approach to NPY receptor signaling in the striatum.

The current working model describes METH-induced cell death that is modulated through the SP-specific NK-1R. NPY is colocalized with NK-1R on SST neurons, a population of striatal interneurons that is immune to METH-induced excitotoxic damage. We propose that NPY provides neuroprotection to SST interneurons as a homeostatic response to SP-induced striatal injury. NPY also inhibits presynaptic GLU release, which counteracts the overflow of neurotransmitters during toxicity.

Our studies use a single bolus injection of METH (30 mg/kg) to study the neurotoxic effects of this drug. While this dosing is significantly lower than some scheduled doses, (15 mg/kg, 4 times, every 2 hours) it produces the same effect on DA terminal toxicity and resulting apoptosis. METH-induced hyperthermia is sustained for longer with binge rather than bolus dosing (Zhu et al., 2006). Our previous dose-dependent study shows that there is a significant toxic response to a 30 mg/kg bolus dose that is not observed at doses 20 mg/kg and lower, but that a dose of 40 mg/kg does not increase observed neurotoxicity and does increase risk for mortality (Zhu et al., 2006). Systemic binge dosing causes a greater effect than bolus on reduced Vmax and reduced DAT function (Kokoshka et al.,1998).

III. RESULTS

Specific Aim 1. To investigate the role of the striatal neuropeptide Y in the METH-induced production of nitric oxide.

1a. Test the hypothesis that NPY receptor agonists will attenuate and antagonists will potentiate the METH-induced production of striatal 3-NT

A. Neuropeptide Y Receptor (NPY-1R and NPY-2R) agonist compounds + METH

48 animals were divided into the following 8 groups based on striatal infusion of drug compound: NPY-1R agonist control (saline), 5μM, 10μM and 20μM, NPY-2R agonist control (saline), 5μM, 10μM and 20μM. The exogenous administration of both agonist compounds results in a potent dose-dependent neuroprotective effect as measured by loss of fluorescence intensity (See Methods). In saline-treated animals, very little fluorescence is visualized in the striatum. The aCSF hemisphere of saline-treated mice were used as a baseline control to calculate the percent increase of stain intensity.

Cell number and brightness of staining increased in METH-treated animals when compared to saline animals. aCSF+METH striata were averaged and normalized for all treatment groups due to the uneven sample size (METH+aCSF treated striata for each treatment group gave three times as many samples). With METH treatment, fluorescent red cells, as labeled by Cy3, can be visualized throughout the striatum (Figure 9). We chose the 4 hour time point to precede cell death observed at 12 and 16 hours (Zhu et al., 2005). METH increases the appearance of 3-NT staining by approximately 30% from animals with no drug treatment. When administered alone, both NPY-1R and NPY-2R agonists reduce fluorescence staining (5%, 3% respectively). This likely accounts for the protective effect observed by low doses of agonist treatment. Mid (NPY-1R=12%, NPY-2R=10% reduction) and High doses (NPY-1R=14%, NPY-2R=17% reduction) appear to cause a true attenuation of METH-related NO production .Data reveals a dose dependent attenuation of 3-NT fluorescent intensity with increasing NPY-1R and

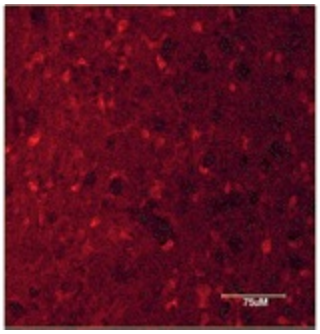
NPY-2R agonist dose when compared to the contralateral striatum injected with artificial cerebral spinal fluid (aCSF).

In this study, optimal working doses for Y1R and Y2R agonists were established. These dosages were modified from working concentrations in a previous paper (Thiriet et al., 2005). Since our experimental model applies different methods for inducing neurotoxicity, we felt it was important to perform a dosing study. The high dose of both agonist compounds (20μM) exerts a potent effect on NO reduction.

Both NPY analogs are highly potent and specific with no cross-reactivity (Gehlert et al., 1992, Grandt et al., 1992). Two-way ANOVA reveals significant effect of drug treatment on fluorescence intensity [$F=43.31$, $p<.0001$]. Post-hoc Analysis by Bonferroni's Multiple Comparison Tests shows a dose-dependent effect of each agonist alone. Y1R agonist (5μM,$p<.05$, 10μM, $p<.001$, 20μM, $p<.0001$) and Y2R agonist (10μM, $p<.01$, 20μM, $p<.0001$). Both cell number and brightness were visibly reduced by intrastriatal agonist injection. Analysis was performed from mean ±SEM. aCSF+METH, and almost all experimental conditions were significantly different from aCSF+Saline treatment (Figure 9). 20uM dose of NPY-2R agonist treatment with METH was significantly different from 5uM+METH ($p<.01$) and aCSF+METH ($p<.001$). 10uM NPY2R agonist was significantly different from aCSF+METH ($p<.05$). NPY2-R agonists demonstrated the most potent reduction in 3-NT immunofluorescence at 4 hours post-METH administration.

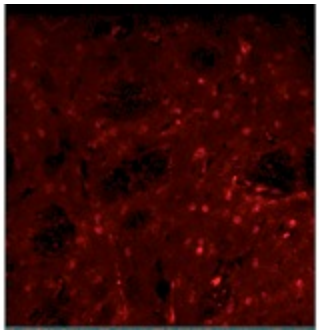
Figure 9. NPY-1R ([Leu^{31},Pro^{34}]-Neuropeptide Y) and NPY-2R (Neuropeptide Y 13-36) agonists attenuate Methamphetamine-induced 3-Nitrotyrosine production. Mice (n=6) were injected with METH (30 mg/kg, i.p.) or a matching volume of saline after intrastriatal infusions of aCSF (left striatum) and NPY Receptor agonist (right striatum). X-axis describes the agonist concentration. All animals were sacrificed at 4 hours after i.p. injection. Immunohistochemical detection was used to label an anti-3-NT antibody with Cy3. Fluorescence intensity was measured by baseline correction with Leica LCS software and measuring total fluorescence from each striatal quadrant (a-c). The percent change in fluorescence intensity of METH-treated animals was significant compared saline treated animals with aCSF infusion (d) (* p<.05 **p<.01 ***p<.001 relative to aCSF + Saline). 3-NT fluorescence intensity decreased from METH control treatment in correlation with increasing agonist dosage. High dose (20μM) of each agonist showed the strongest protective effect with NPY-2R agonist having stronger potency. Data were analyzed by one way ANOVA for each compound to determine significant change in percentage of 3-NT immunofluorescence (p<.0001). F=11.58 [Leu^{31},Pro^{34}]-Neuropeptide Y, F=55.34 Neuropeptide Y 13-36

a.



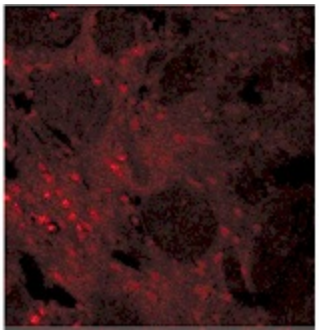
aCSF
+ METH

b.



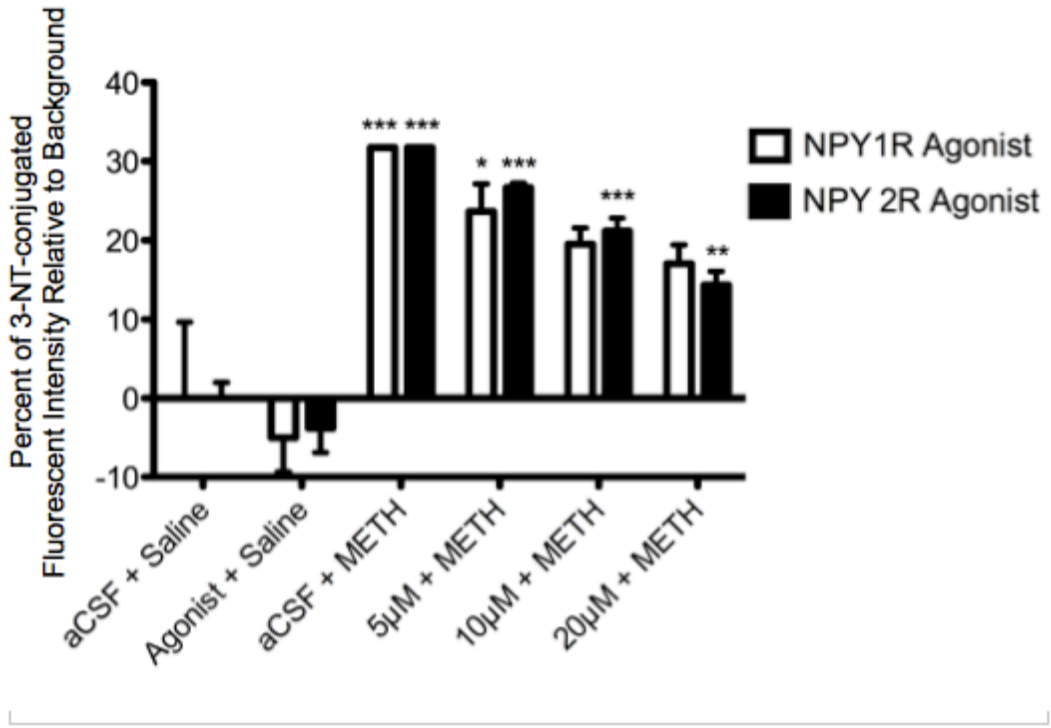
20μM NPY-1R Agonist
+ METH

c.



20μM NPY-2R Agonist
+METH

d.

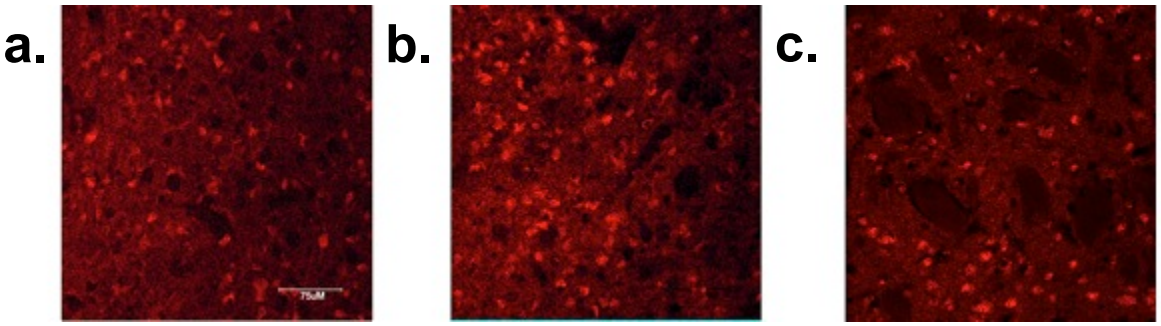


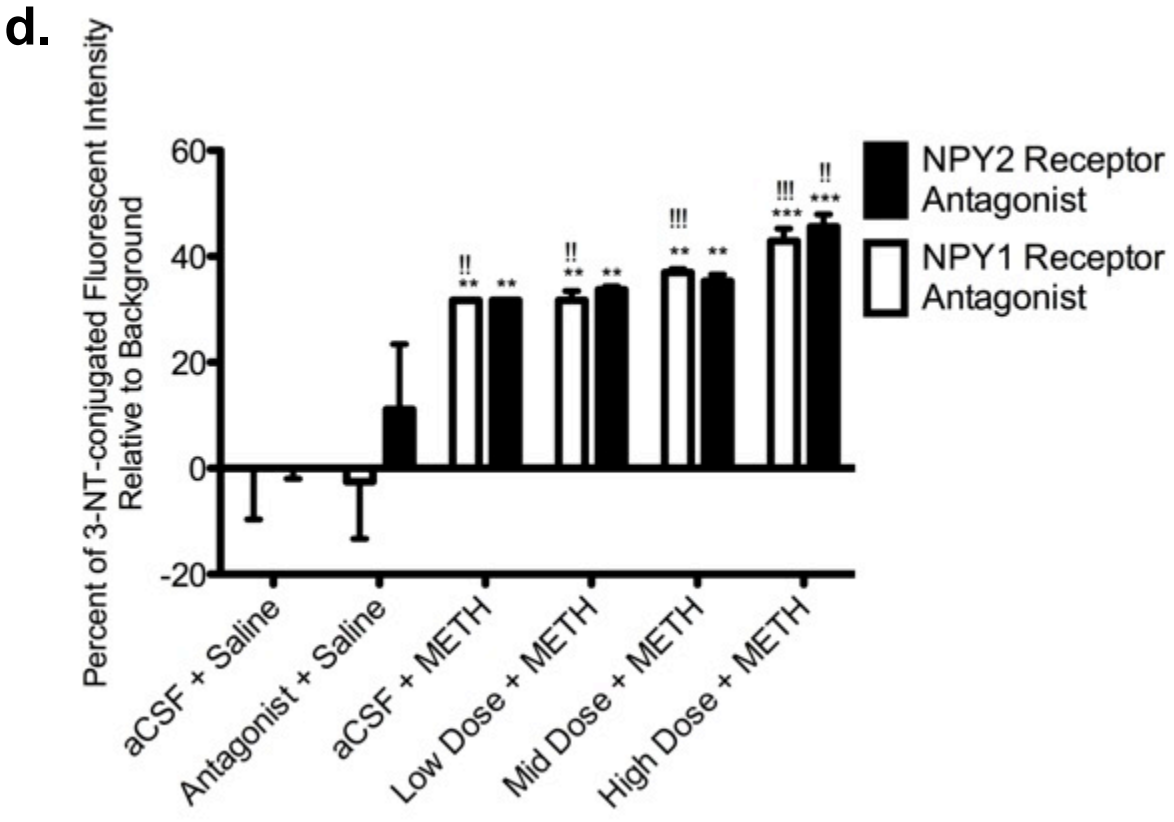
B. Neuropeptide Y Receptor (NPY-1R and NPY-2R) antagonist compounds + METH

To confirm the pharmacological validity of our observation, the techniques from above were repeated with NPY-1R/NPY-2R antagonists. aCSF + METH control striata appeared dark with approximately 100 labeled cells in each of four striatal regions whereas experimental animals had both brighter staining and a higher number of cells that expressed 3-NT. We assessed a dose-dependent response by choosing three doses based on previous literature demonstrating effectiveness of the antagonists in vivo (Chen et al. 2002, Thorsell et al. 2002).

48 animals were divided into the following 8 groups based on striatal infusion of drug compound: NPY-1R antagonist control (saline), 340 µmol, 680 µmol, 1360 µmol, NPY-2R antagonist control (saline), 1 nmol, 2 nmol, 4 nmol. The aCSF hemisphere of saline-treated mice were used to calculate the baseline percent of fluorescence (Figure 10). Cell number and brightness of staining increased in treatment (METH) animals when compared to saline animals. aCSF+METH striata were averaged and normalized for all treatment groups due to the uneven sample size. The 1360umol dose of BIBP3226 ($p=0.0192$) and the 4 nmol dose of BIIE0246 ($p=.0228$) demonstrated a significant induction in 3-NT binding at 4 hours post-METH administration. METH increases the appearance of 3-NT staining by 31.7% from animals with no drug treatment. We note that when administered alone, the NPY-2R antagonist increased fluorescent staining by 11% from control. This likely accounts for the protective effect observed by low (2.5%) and mid (6% NPY-1R, 4% NPY-2R) doses of NPY-2R antagonist treatment. High doses (12% NPY-1R and 15% NPY-2R) stimulate true induction of METH-related NO production. METH is causes significant toxicity to the brain, and thus our low percentage of potentiation is likely due to the fact that an antagonist cannot create much more toxicity than the drug treatment alone.

Figure 10. NPY-1R (BIBP 3226) and NPY-2R (BIIE 0246) receptor antagonists Potentiate Methamphetamine-induced 3-Nitrotyrosine production Both NPY-1R and NPY-2R antagonists show a dose dependent potentiation of immunofluorescence intensity as measured by conjugation to an anti-3-NT antibody at 4 hours post-METH i.p. injection. One way ANOVA for each compound was used to describe the percent fluorescence intensity from baseline control (aCSF infusion paired with saline i.p. injection) (* p<.05 **p<.01 ***p<.001 relative to aCSF + Saline). A low dose (340μmol NPY-1R or 1nmol NPY-2R) infusion of antagonist paired with saline injection did not alter fluorescence intensity significantly. A METH i.p. injection alone increased fluorescence intensity by 31.7% (a,d). This positive control was normalized across the left striata of 18 animals in the study. The percent change in fluorescent intensity of METH-treated animals continued to increase with increasing antagonist dose (d). Mid dose (680μmol Y1R or 2nmol Y2R) treatment increased fluorescence intensity by 37% and 35% respectively. High dose (1360umol dose BIBP3226, 4 nmol dose of BIIE0246) of each antagonist provided the brightest fluorescence (! P<.05 !! p<.01 !!! p<.001 relative to aCSF + Antagonist alone) including both neuron and neuropil staining throughout the stratum (b,c). Data show that administration of an NPY-1R and NPY-2R antagonist can cause significant change in percentage of 3-NT immunofluorescence (p<.0001). F=14.91 BIBP 3226 F=10.65 BIIE 0246 .

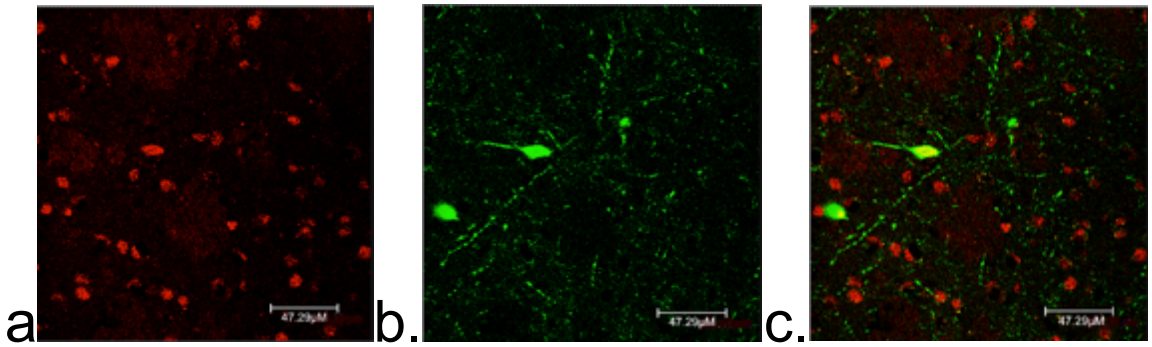

a.
b.
c.
aCSF
+ METH
1360µmol NPY-1R
Antagonist + METH
4nmol NPY-2R
Antagonist + METH

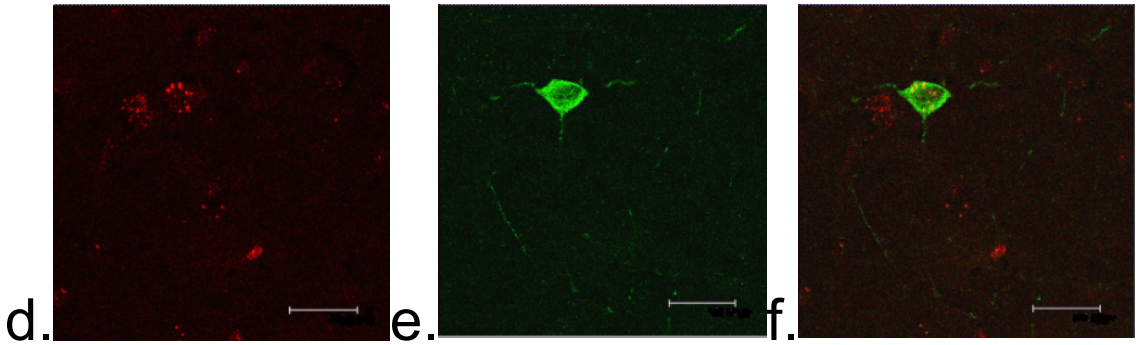

d.
Percent of 3-NT-conjugated Fluorescent Intensity
Relative to Background
60
40
20
0
-20
NPY2 Receptor
Antagonist
NPY1 Receptor
Antagonist
aCSF + Saline
Antagonist + Saline
aCSF + METH
Low Dose + METH
Mid Dose + METH
High Dose + METH

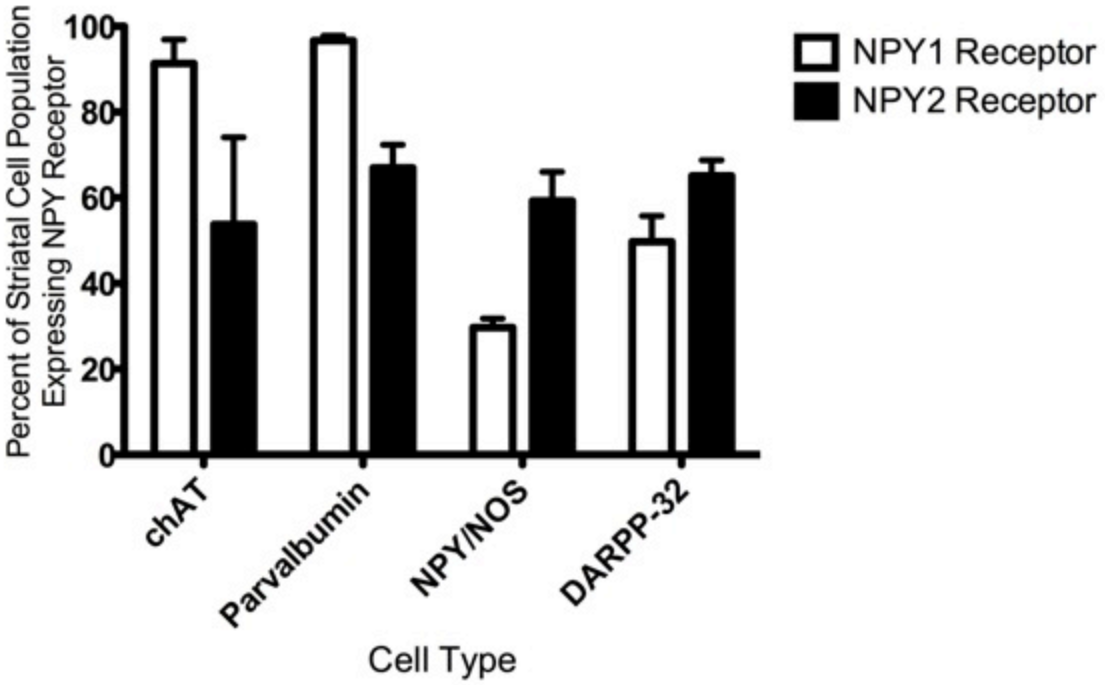
C. Neuropeptide Y receptors (NPY-1R,NPY-2R) are expressed differentially on striatal cells

Fluorescent co-label was used to quantify receptor immunoreactivity by cell type in untreated tissue (n=4). In order to characterize the receptor distribution in the striatum, phenotype markers were overlayed with markers for NPY receptors subtypes (NPY-1R, NPY-2R). NPY-ir is visualized on just 1-2% of all neurons, but data show that all cell populations express both Y1R and Y2R receptors (Silva et al., 2005). (b). For both D1R and D2R-expressing cells, an antibody against DARPP-32 was used. This population accounts for more than 95% of striatal cells, of which 50% express the NPY-1R receptor and 65% express the NPY-2R receptor. This corroborates with previous studies of that estimate about half of DA neurons express NPY-2R in the mouse hypothalamus (Fetissov et al., 2004). The same study estimated that most NOS neurons of the arcuate nucleus express NPY-2R. Almost 60% of the NPY/NOS population co-expresses NPY-2R and 30% co-fluoresce with NPY-1R. ChAT. 91% of these cells contain NPY-1R cells and 54% express NPY-2R cells. Parvalbumin-containing cells almost unanimously (97%) express NPY-1R while 67% express NPY-2Rs. NPY receptors likely exercise both autocrine and paracrine mechanisms for modulation of NO. Images show even NPY-1R and NPY-2R distribution throughout the striatum. NPY-2R appears to be expressed on smaller cells (DARPP-32, SST/NPY/NOS) whereas Y1Rs are more often expressed by larger cells (ChAT). Two way ANOVA was performed to compare effect of cell type and receptor type. This reported no significant differences in receptor distribution in the striatum.

Figure 11. NPY-1R and NPY-2R receptors are expressed on all striatal neurons with varying prevelance After anesthetization, untreated animals were sacrificed by perfusion with 4% paraformaldehyde and 0.1% PBS. Immunohistochemical label was used to determine the prevalence of NPY receptors (Y1R=e, Y2R=b) on striatal cell types (a,d). Cell phenotypes as listed on the x-axis were co-labeled with markers for NPY (NPY-1R, NPY-2R) receptors in untreated animals. The panel shown displays a red Cy3 label for NPY-2R and a green FITC label for NOS to represent the NPY/NOS population of striatal interneurons. Receptor distribution is representative of what was observed among 4 cohorts. The percent of colabeled cells was determined by dividing cells expressing both the phenotypic marker and receptor marker by the total number of cells expressing phenotypic marker for a given region. Receptor labels were distributed throughout striatum and among all cell types. NPY-1R show particular prevalence on ChAT and parvalbumin-expressing cells. Two Way ANOVA reveals significant difference between cell type but not between receptor type (**p=.0064 F=5.43).


a
b.
c.


d.
e.
f.


Percent of Striatal Cell Population Expressing NPY Receptor
100
80
60
40
20
0
chAT
Parvalbumin
NPY/NOS
DARPP-32
Cell Type
NPY1 Receptor
NPY2 Receptor

D. Neuropeptide Y mRNA production

As a hormone, NPY has some releasable pools within the neuron for release, but additional production requires time to be transcribed, modified and released. After translation, preproNPY that is produced after translation moves to the endoplasmic reticulum for further processing. Here prohormone convertases cleave the peptide to produce proNPY. Finally, truncation of proNPY produces the biologically active amidated NPY. Mature NPY can be further split in to NPY_{3-36} and NPY_{2-36} (Silva et al., 2002). We evaluated the presence of mature NPY peptide (NPY_{3-36}) after a toxic METH injection.

NPY mRNA levels in the mouse striata were measured by Taqman RT-PCR after METH or saline administration to assess utilization of the neuropeptide. Mice (n=6) were given intraperitoneal injection of METH (30 mg/kg) or a matching volume of saline. One cohort was sacrificed by cervical dislocation 4 hours after drug treatment and the second cohort was sacrificed at 16 hours after drug treatment. The two time points were chosen to capture the accumulation of NPY mRNA prior to release as well as the restoration of NPY mRNA stores after release. NPY is likely released between in response to METH 4 and 8 hours after drug treatment (see Aim 2a). Knowing this, NPY mRNA would reach a peak above control during this time. After peptide release, NPY mRNA should again accumulate to replenish lost stores. This is observed at 16 hours. All brains were immediately frozen for RT-PCR analysis. GAPDH was run as an internal control against probes for neuropeptide Y using Taqman RT-PCR. After 40 cycles, C_T values were measured and saline controls were subtracted from experimental samples. Results show that NPY mRNA is elevated at 4 hrs and 16 hours post-drug treatment. A mean fold increase of 0.36 was recorded at 4 hours and a mean fold increase of 1.65 12 hours later (Figure 12). Both saline and METH-treated samples were normalized to C_T values of GAPDH. One-way ANOVA shows significant difference between 4 and 16-hour time points [$F=226.6$, $p<.0001$]. Bonferroni's multiple comparison post-hoc test confirms statistically significant differences in treatment from control (** $p<.001$, *** $p<.0001$) as well as between cohorts (*** $p<.0001$).

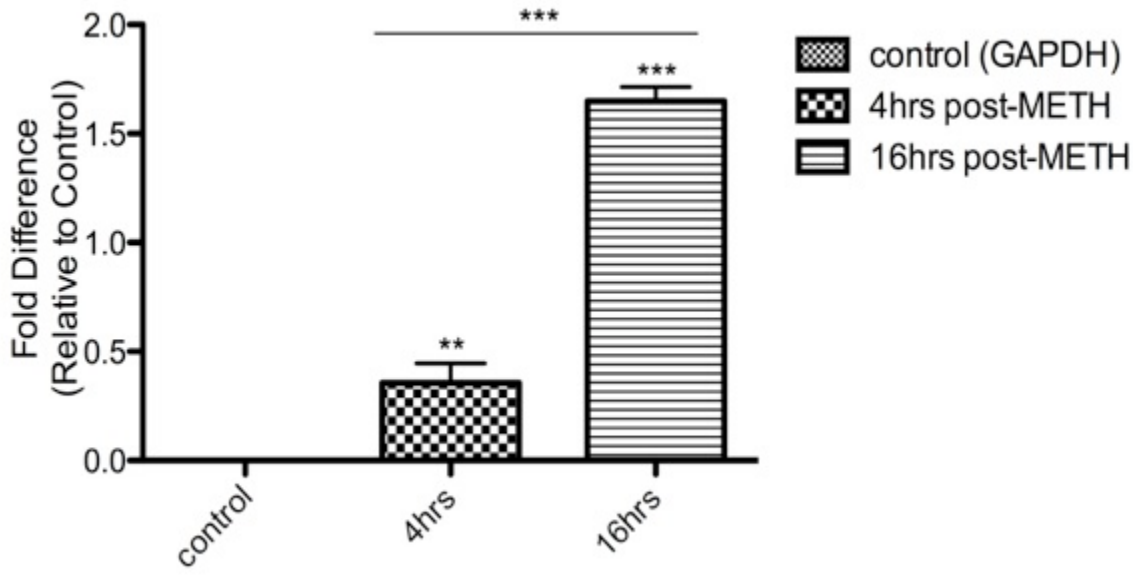


Figure 12. Neuropeptide Y mRNA is elevated during early hours after Methamphetamine treatment Mice (n=6) were given intraperitoneal injection of METH (30 mg/kg) or a matching volume of saline. Animals were sacrificed at 4 and 16 hours after drug treatment. After 40 cycles, C_T values were measured and saline controls were subtracted from experimental samples. METH treatment activates NPY utilization in the striatum both 4 and 16 hours after drug treatment. Average C_T of GAPDH=18.22 and NPY=22.34. $\Delta\Delta C_T$ values were calculated and are reported as mean fold difference from saline control. Drug treated animals show a mean fold increase of 0.36 over saline-treated animals at 4 hours and a mean fold increase of 1.65 over saline-treated animals at 16 hours. One-way ANOVA with Bonferroni's multiple comparison post-hoc test confirms statistically significant differences in treatment from control as well as between cohorts (**p<.001, ***p<.0001, F=226.6).

<u>Specific Aim 2.</u> To examine the impact of METH-induced nitric oxide production on striatal neurons.

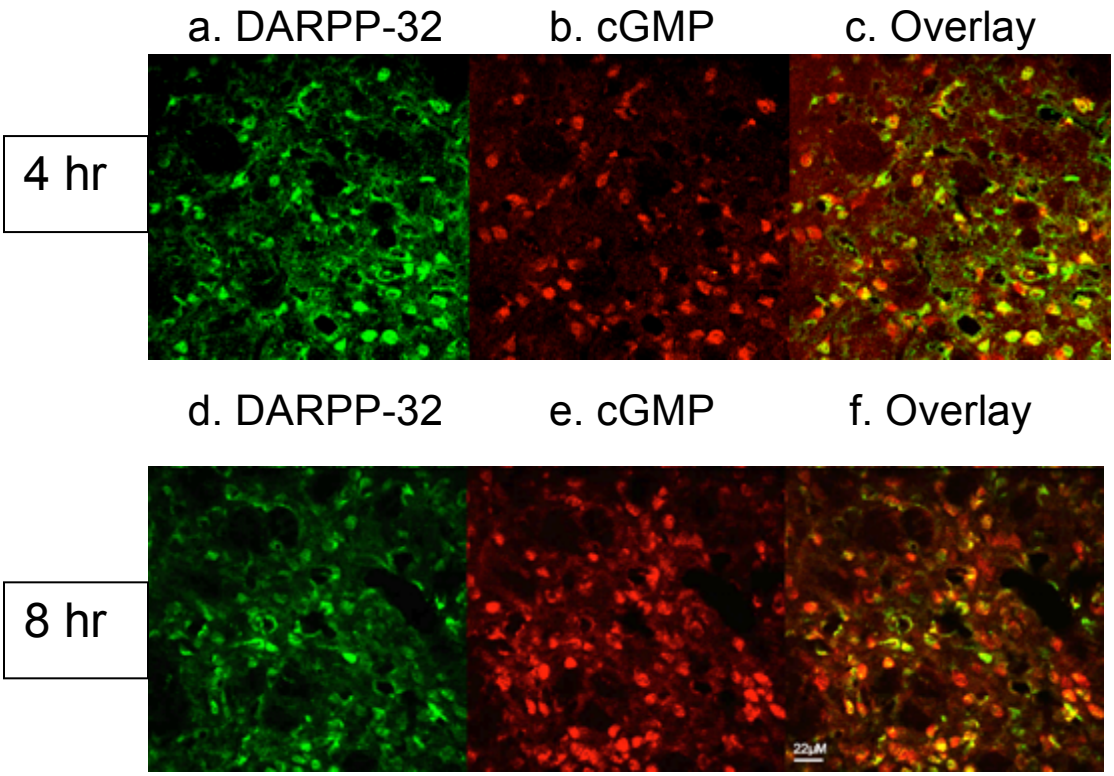
2a. Assess the neuronal targets of the METH-induced nitric oxide: Co localization of cGMP with select markers of striatal projection and interneurons

<u>A. Differential Response of Striatal Cell Populations to METH-Induced Nitric Oxide</u>

Characterization of the cellular response to METH-induced NO release was performed by fluorescent co-label. Projection neurons with dopamine and cAMP-regulated neuronal phosphoprotein-32,000 kDa (DARPP-32). Interneurons were labeled with choline acetyl transferase (ChAT), parvalbumin and somatostatin (SST), respectively. Due to the short half-life of NO, its stable byproduct cyclic guanosine monophosphate (cGMP) was labeled with cell phenotypes at 4 or 8 hours after ip injection of METH or saline (n = 8 per group). While significant fluorescence is labeled at 8 hours, no later time points were evaluated. This owes to the fact that cell death is observed at 16 and 24 hours in striatal cells (Zhu et al., 2005). While NO damage persists in the striatum, this study did not risk losing phenotypic markers during co-label. During immunohistochemical label, we encountered many of the same issues as described by previous studies including difficulty labeling projection neurons (Zhu et al., 2006). DARPP-32 was the most reliable marker assayed for immunhistochemical label as compared to commercially available D1R/D2R/SP/ENK/DYN markers.

METH-treated animals displayed increased cGMP expression in all cell types after both 4 and 8 hours by histological assessment. The results are expressed as mean percent above control levels (4 hours=DARPP-32 32.6%, ChAT 17.4%, Parvalbumin 3.6%, NPY/NOS 20.1% and 8 hours =DARPP-32 78.5%, ChAT 25.9%, Parvalbumin 25.1%, NPY/NOS 5.5%). Significance is measured by two-tailed t-test. p values are as follows (DARPP-32 p=.0439, ChAT=.0111, Parvalbumin=.0037, NPY/NOS=.2857). cGMP response persisted to 8 hours in all cell types, cGMP reactivity decreased in NOS/NPY-producing cells by 8 hours. This suggests a resistance, but not immunity, to NO-induced cell death.

Figure 13. cGMP expression in DARPP-32 neuron increases from 4 to 8 hours after METH treatment Animals were given intraperitoneal injection of either METH (30 mg/kg) or a matching volume of saline by bodyweight. One cohort (n=16) was sacrificed after 4 hours and the second cohort was sacrificed after 8 hours by perfusion. Immunofluorescence label was used to detect both DARPP-32-containing cells and cGMP expressing neurons. DARPP-32 neurons are labeled in green with FITC secondary antibody and cGMP-expressing cells are labeled in red with cGMP secondary antibody. The number of cells expressing both cGMP and DARPP-32 label was divided by the total number of DARPP-32-expressing cells for each region to determine the percent co-label relative to saline. Histochemical label of DARPP-32 neurons (a,d) reveals induction of cGMP expression (b,e) by striatal projection neurons in 32.6% of cells (c,e) at 4 hours and 78.5% of cells (a,g) at 8 hours. T-test reveals significant difference between time points (* $p<.05$, $F=1.206$) and at 8 hour treatment from saline (***$p<.001$).


a. DARPP-32
b. cGMP
c. Overlay
4 hr
d. DARPP-32
e. cGMP
f. Overlay
8 hr
22μM

g.

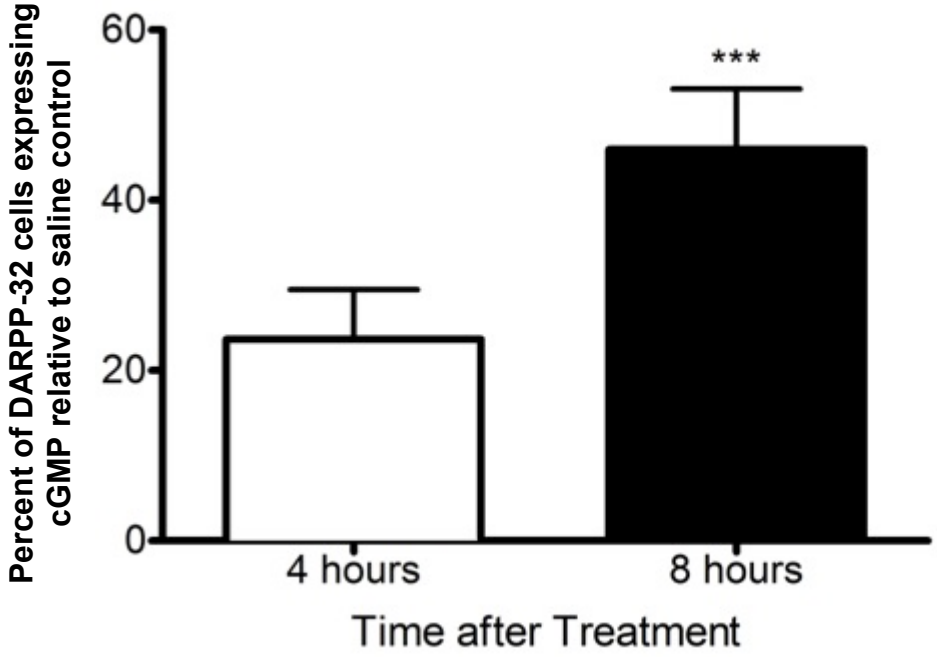
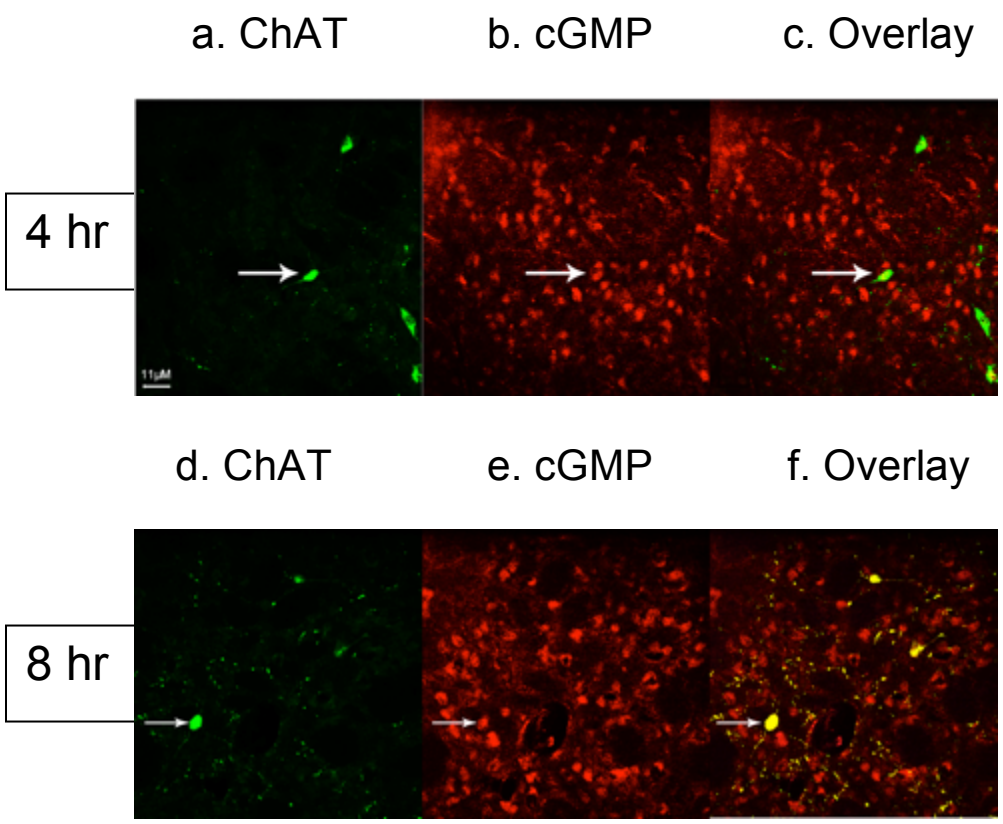

Percent of DARPP-32 cells expressing cGMP relative to saline control
60
40
20
0
***
4 hours
8 hours
Time after Treatment

Figure 14. cGMP expression in ChAT neurons increases from 4 to 8 hours after METH treatment cGMP staining is increased over time in ChAT expressing cells during the early hours after systemic methamphetamine treatment. ChAT is labeled in green by FITC (a,d) and cGMP is labeled in red by Cy3 fluorescent antibody conjugated to anti-cGMP (b,e). Animals were given intraperitoneal injection of either METH (30 mg/kg) or a matching volume of saline by bodyweight. One cohort (n=16) was sacrificed after 4 hours and the second cohort was sacrificed after 8 hours by perfusion. Immunofluorescence label was used to detect both ChAT-containing cells and cGMP-expressing neurons. The number of cells expressing both cGMP and ChAT label was divided by the total number of ChAT-expressing cells for each region to determine the percent co-label relative to saline. Histochemical label of ChAT neurons (a,d) reveals induction of cGMP expression (b,e) by striatal projection neurons in 17.4% of cells (c,e) at 4 hours and 25.9% of cells (a,g) at 8 hours. T-test reveals significant difference between time points (* $p<.05$, $F=1.046$) and in treatment from saline groups (***$p<.001$, ****$p<.0001$).


a. ChAT
b. cGMP
c. Overlay
4 hr
d. ChAT
e. cGMP
f. Overlay
8 hr

g.

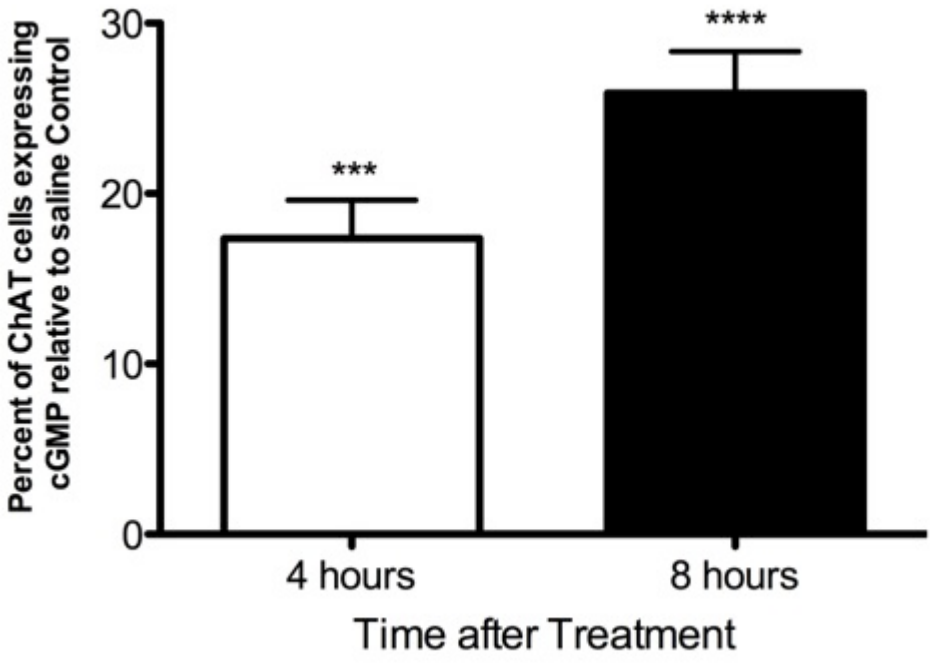
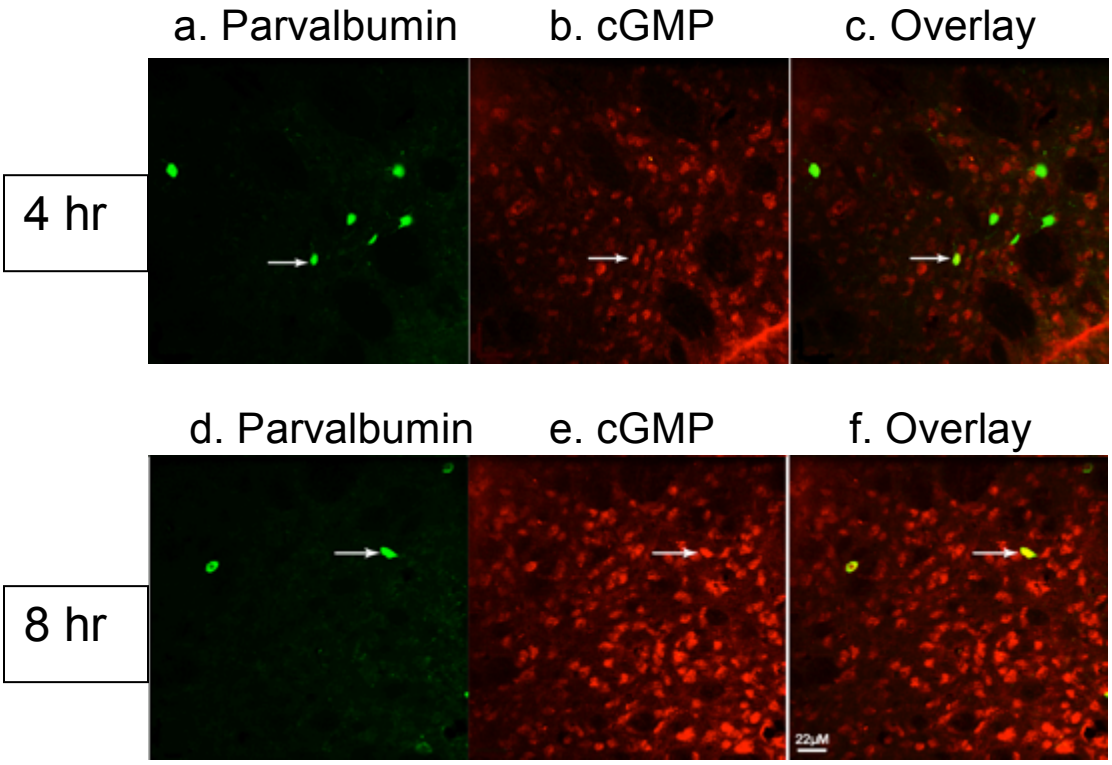

Percent of ChAT cells expressing cGMP relative to saline Control
30
20
10
0
***
****
4 hours
8 hours
Time after Treatment

Figure 15. cGMP expression in Parvalbumin neurons increases from 4 to 8 hours after METH treatment Immunohistochemical label of parvalbumin interneurons (a,d) reveals NO-related damage by cGMP expression (b,e) to striatal interneurons in 3.6% of cells at 4 hours and 25.1% of cells at 8 hours. Parvalbumin-containing cells are labeled in green by FITC (a,d) and cGMP is labeled in red by Cy3 fluorescent antibody conjugated to anti-cGMP (b,e). The x- axis denotes the time of sacrifice after methamphetamine or saline intraperitoneal injection. The number of cGMP and Parvalbumin-containing cells were divided by total number of parvalbumin interneurons for each frame to determine the percent of colabeled cells. The data represent percentage of T-test reveals significant difference between time points (* $p<.05$, $F=5.848$). The expression of cGMP in parvalbumin interneurons is significantly increased after methamphetamine injection when compared to saline treated controls at 8 hours (****$p<.0001$).


a. Parvalbumin
b. cGMP
c. Overlay
4 hr
d. Parvalbumin
e. cGMP
f. Overlay
8 hr
22μM

g.

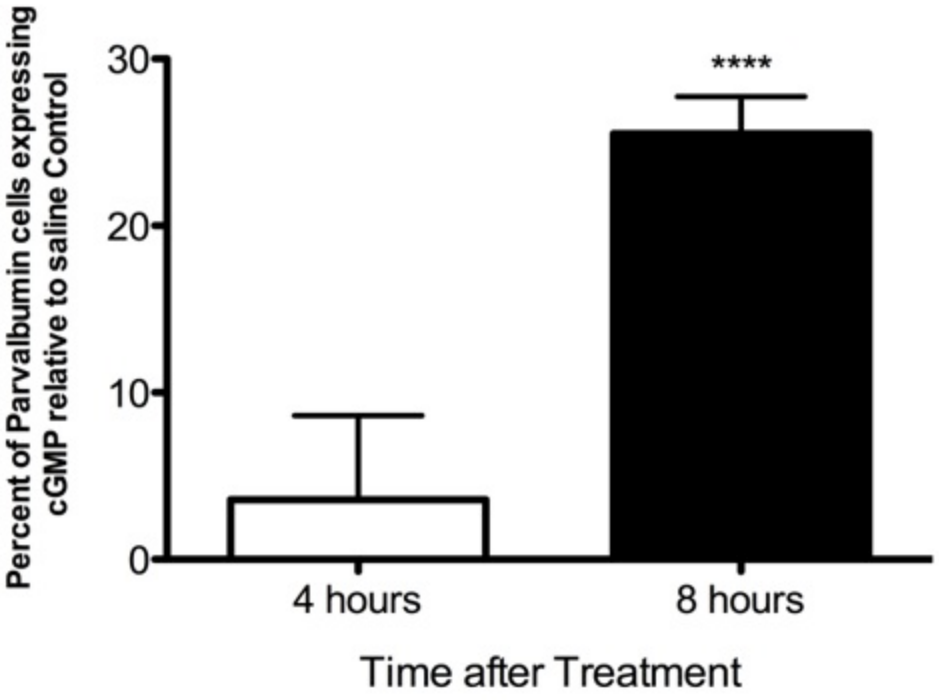
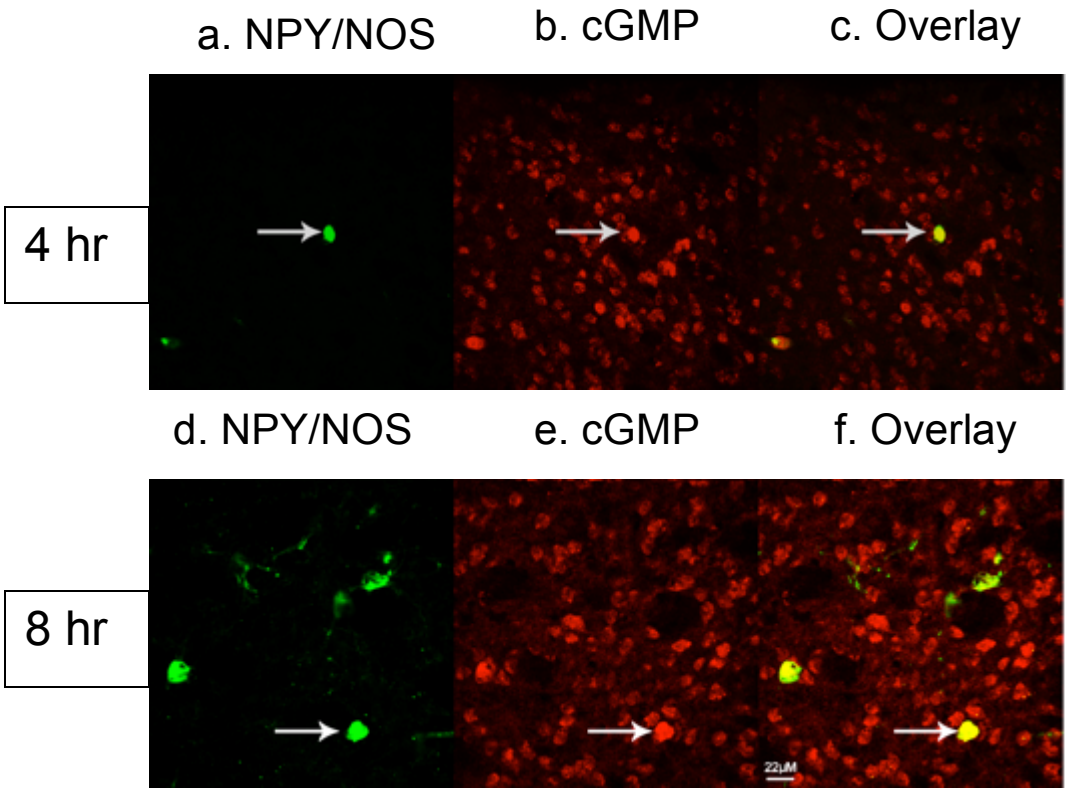
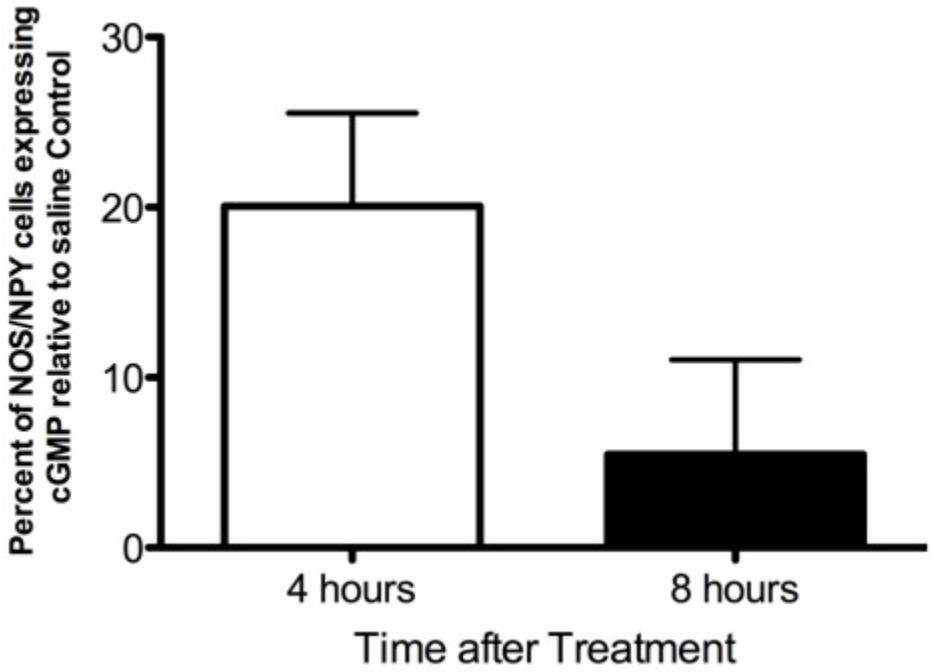

Percent of Parvalbumin cells expressing cGMP relative to saline Control
30
20
10
0
****
4 hours
8 hours
Time after Treatment

Figure 16. cGMP expression in NPY/NOS neurons increases and then decreases in the first 8 hours after systemic METH treatment Immunohistochemical label of NPY/NOS interneurons (a,d) reveals cGMP induction (b,e) is present in 20.1% of cells at 4 hours, but this number is reduced to 5.5% of cells at 8 hours. Animals were given intraperitoneal injection of either METH (30 mg/kg) or a matching volume of saline by bodyweight and sacrificed at 4 and 8 hours after drug treatment. Immunofluorescence label was used to distinguish NPY/NOS cells (FITC/green) from cGMP label (red/Cy3). The number of cells expressing both NOS and cGMP label was divided by the total number of NOS-expressing cells for each region to determine the percent co-label relative to saline. While this interneuron population shows response to nitric oxide production in the early hours following drug treatment, the level of response is not maintained. T-test reveals significant difference between time points (* $p<.05$, $F=1.046$).


a. NPY/NOS
b. cGMP
c. Overlay
4 hr
d. NPY/NOS
e. cGMP
f. Overlay
8 hr
22μM

g.


Percent of NOS/NPY cells expressing cGMP relative to saline Control
30
20
10
0
4 hours
8 hours
Time after Treatment

B. BDNF immunoreactivity is not elevated from control in the early hours following METH treatment

Aim2a results disprove our hypothesis that NPY/NOS interneurons do not respond to NO and suggest that the mechanism is more involved. At 4 hours after drug treatment, this cell population demonstrates a robust increase in cGMP corresponding to NO presence. An alternative mechanism was proposed to account for neuropeptide Y release.

Brain-derived Neurotrophic Factor (BDNF), a neurotrophic factor associated with promoting survival in the striatum, demonstrates a role in growth and neurotransmission as well as cognitive and emotional neural pathways (Ventimiglia et al., 1995). It binds to the tropomyosin receptor kinase B (TrkB) receptor and several studies have documented its role in cell death protection (Barbacid 1995). For instance, activation of extracellular signal-regulated kinase (ERK) 1/2 and phosphatidylinositol-3-kinase (PI3K)/Akt) Otherwise, METH inhibits Bcl-X_L and consequently promotes mitochondrial-dependent apoptosis (Cadet et al., 1997).

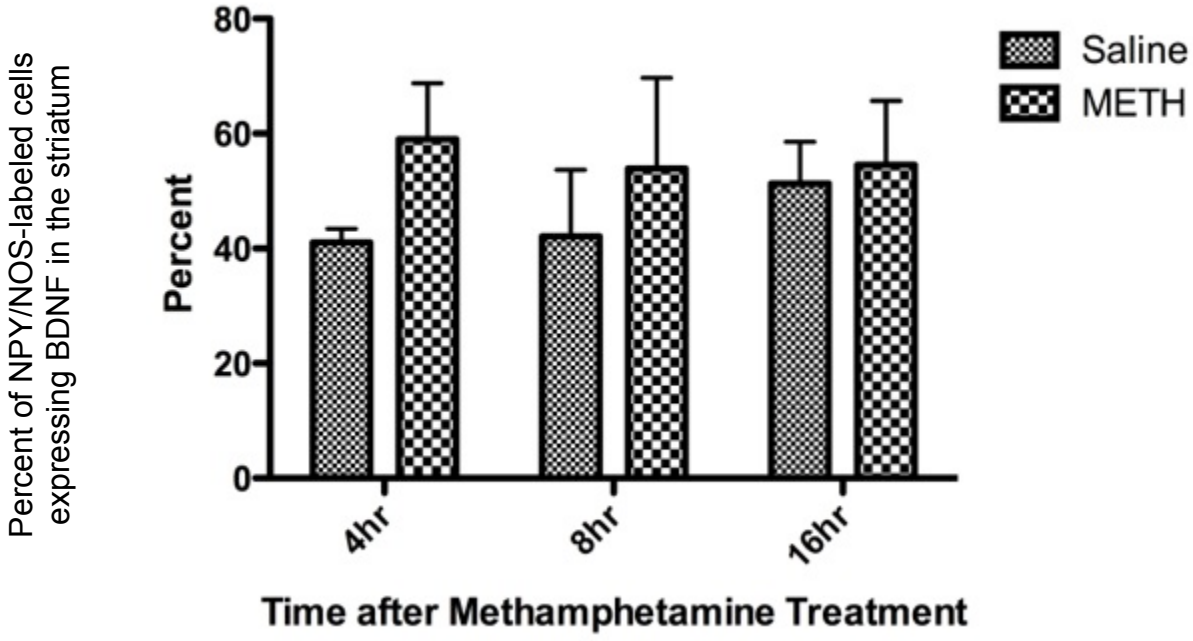
There is also an established connection between BDNF and NPY activity. BDNF induces sustained phosphorylation of extracellular-regulated kinase (ERK), and can induce the secretion of NPY via ERK-dependent and ERK-independent mechanisms (Barnea and Roberts, 2001). When a null mutation of BDNF is performed in mice, the number of immunoreactive NPY/NOS neurons is decreased. Exogenous application of BDNF also induces the appearance of striatal NPY mRNA and active peptide (Mizuno et al., 1997). Thus, it was suggested that the upregulation of NPY mRNA (aim 1c) may be responsible for the production of BDNF and downstream neuroprotective mechanisms.

Animals given an i.p. injection of METH or a matching volume of saline were sacrificed at 4, 8 and 16 hours after drug treatment. These time points were chosen to capture the downstream effect of NPY upregulation. Immunohistochemical label was used to distinguish cells expressing BDNF and the population of NPY/NOS neurons labeled with NOS1. 41% of saline treated NPY/NOS interneurons express BDNF at 4 hours and 42% at 8 hours. 51% of saline-treated NPY/NOS cells express BDNF at 16 hours after drug treatment This co-label was

elevated to 59%, 54% and 55% at 4,8, and 16 hours respectively. Two way ANOVA with Bonferroni's post hoc test show no significant differences by treatment or time point.

Figure 17. No cell specific induction of BDNF in NPY/NOS producing cells at 8,16 hours

There is no significant change of the neurotrophic factor BDNF in NPY/NOS cells during the hours following a bolus METH injection or a matching volume of saline (n=5). Histological method was used to co-label BDNF protein and NPY/NOS interneurons (NOS1). Animals were sacrificed at three time points as labeled by the x-axis. Percent co-label (y-axis) was determined by dividing the number of dual-labeled cells by the total number of NOS-labeled cells. Two way ANOVA reveals no significant difference in treatment animals from control tissue.



C. cGMP and active caspase-3 localization

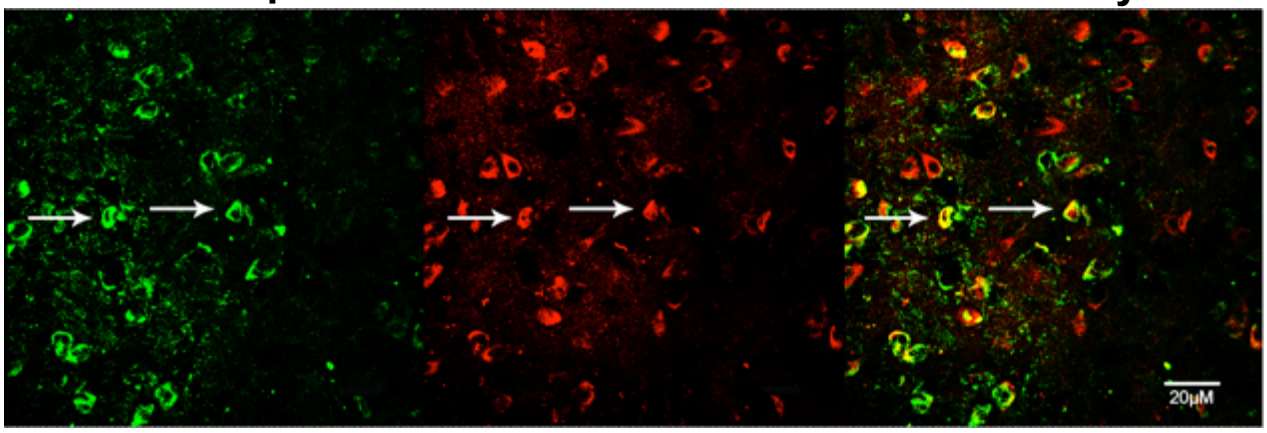
METH-induced neurotoxicity causes notable damage to cell mitochondria and the mitochondrial membrane. BAX/bcl-2 protein function is altered and causes cytochrome c release from mitochondria in cell and animal models (Jayanthi et al., 2001, Deng et al., 2002). METH can also inhibit mitochondrial complex I and IV activity (Gluck et al., 2001, Burrows.2000). We sought to determine if cells responding to NO are destined for apoptosis via this pathway since cells may become apoptotic through DA-independent and cGMP-independent mechanisms. To this end, we colabeled cGMP and active caspase-3 in animal tissue sacrificed 8 hours after saline or METH treatment.

This time point was chosen because this is when the most NO-related damage is recorded (Aim 2a). Previous study has also documented active caspase-3 release from mitochondria at 8 hours and peaking at 16 hrs (Jayanthi et al., 2004). Active caspase-3 release also begins at 8 hours in immortalized rat striatal cells (Deng et al., 2002).

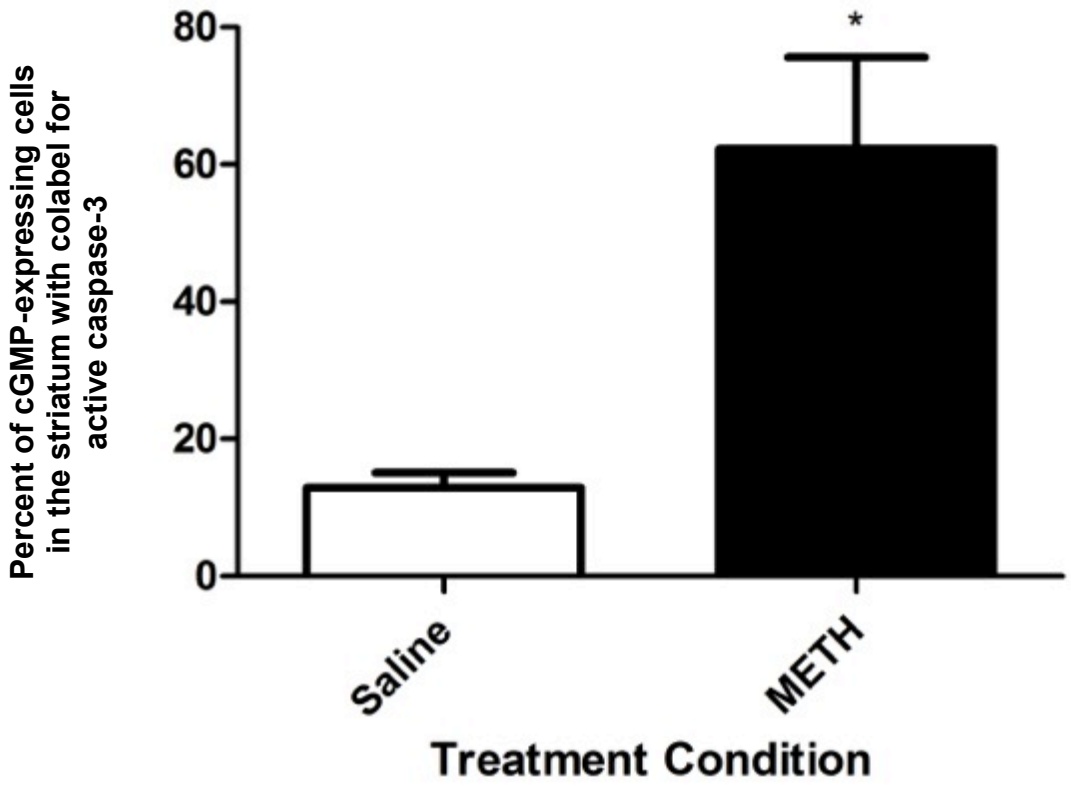
Of the striatal cells labeled with a cGMP antibody, 13% were immunoreactive with active caspase-3 after systemic saline injection (n=6 per group). METH increased this cellular colocalization to 62%, the majority of NO-responsive neurons (Figure 17). Apoptotic cells varied in shape and size, owing to the fact that both projection and interneurons are susceptible to METH-induced cell death. One tailed t test (assuming METH will have more damage markers) reveals significant difference between groups (p=.0053)

Figure 18. METH induces the appearance of apoptotic markers in cells that respond to nitric oxide overproduction Fluorescent localization of NO-responding neurons (b) reveals caspase-dependent damage (a) in a majority of striatal neurons (d) at 8 hours after METH treatment. Animals were given either METH (30/mg/kg) i.p. injection or a matching volume of saline sacrificed 8 hours later by perfusion. Immunohistochemical label was used to fluoresce an anti-active caspase-3 antibody (green, FITC) (a) and an anti-active cGMP antibody (red, Cy3) (b). 13% colabel is observed after systemic saline injection (n=6 per group). METH increased this cellular colocalization to 62%, the majority of NO-responsive neurons (a). Percent co-label (y-axis) was determined by dividing the number of dual-labeled cells (c) by the total number of cGMP-labeled cells. One tailed t test (assuming METH will have more damage markers) reveals significant difference between groups (* $p<.01$)

a. Active Caspase-3 b. cGMP c. Overlay



d.



D. NPY2R agonist attenuates appearance of apoptotic markers

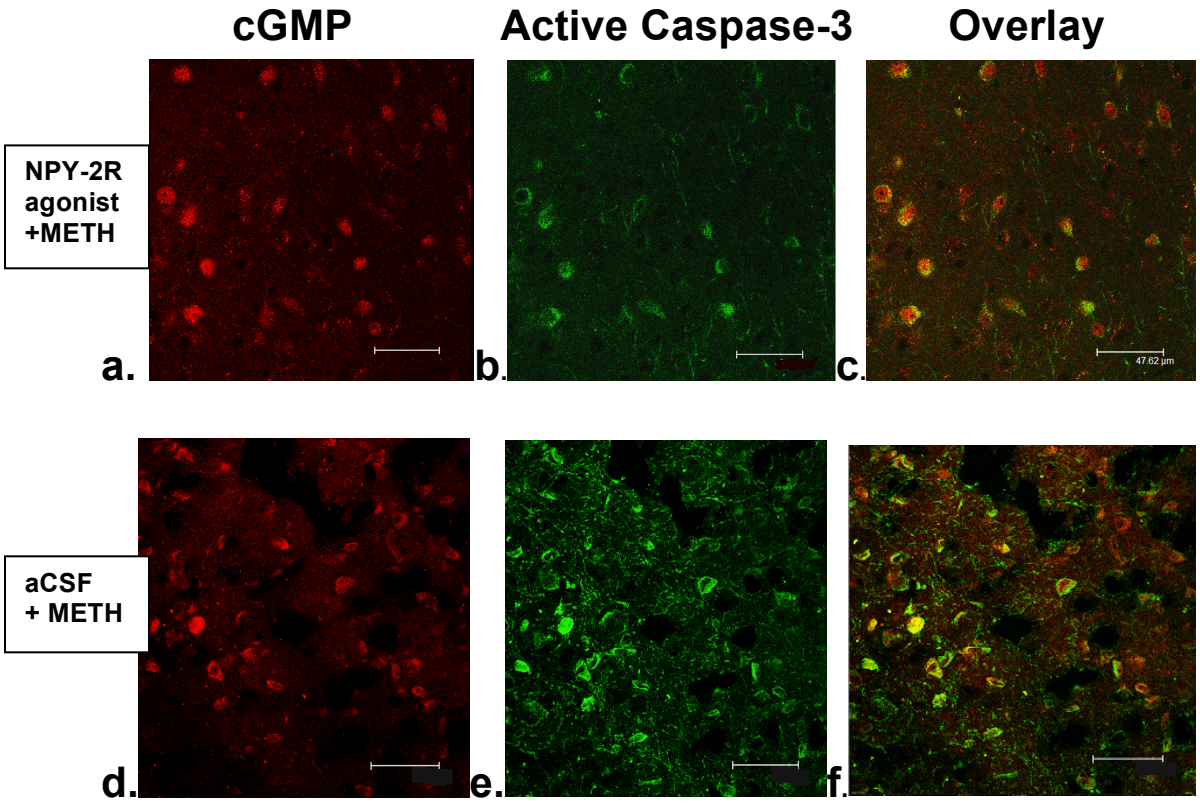
Aim 2b concluded that most cells the respond to METH-induced NO undergo caspase-dependent apoptosis. Aim 1a data show that intrastriatal infusion of NPY analogs can modulate NO production. In Aim 2a, results demonstrate that NO production peaks at 8 hours. No later time points were chosen to avoid losing phenotypic markers since TUNEL stain is observed at this time.

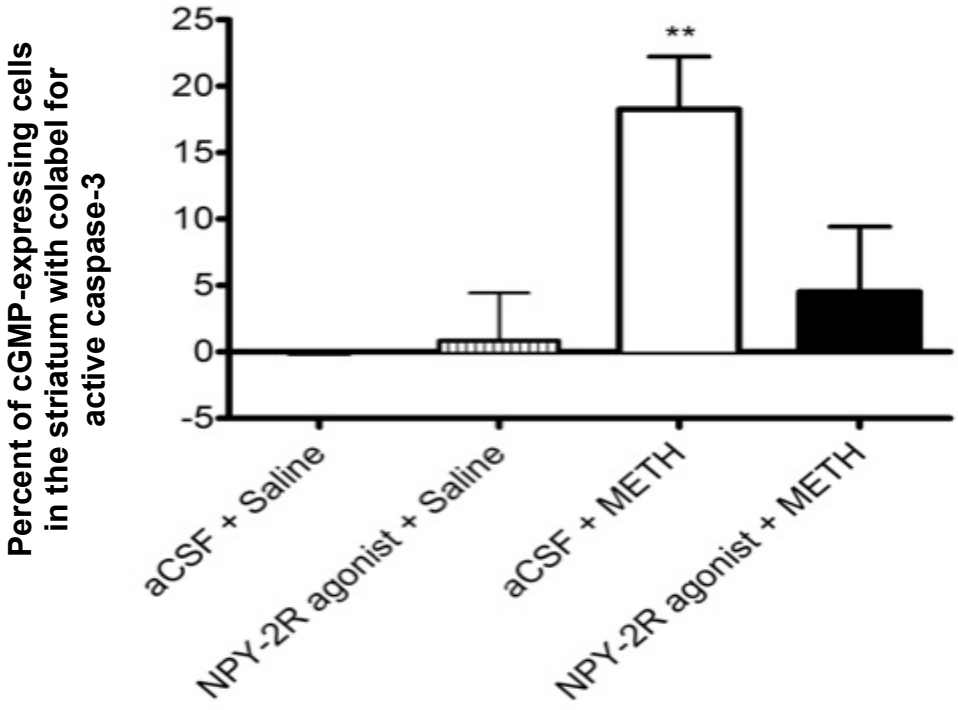
In the present aim, cGMP and active caspase-3 were again colabeled in animal tissue sacrificed 8 hours after saline or METH treatment. NPY2R agonist was infused via stereotaxic injection as described in Aim1a prior to METH injection. NPY2R agonist ($NPY_{(3-36)}$) alone had a negligible effect (<1%) on caspase-3 cleavage and METH alone (aCSF infusion) increased to 18.25%. The addition of NPY2R agonist with systemic METH reduced active caspase-3 production to 4.52% of cGMP-labeled cells.

One Way ANOVA reveals significant different between means of treatment groups [f=5.427, p=.0068]. Post-Hoc analysis with Bonferroni's Multiple Comparison Test shows METH treatment varies significantly from both saline controls (aCSF+Saline, Y2R agonist+saline). Since NPY-1R and NPY-2R agonists showed similar effect on reduction of 3-NT fluorescence (Aim 1a), we chose one agonist to test effect on caspase-3 activation.

Figure 19. Neuropeptide Y attenuates appearance METH-induced caspase-3 activation

METH induced cleaved caspase-3 (b,e) in a majority of cGMP-responding cells (a,d) and an NPY-2R agonist attenuates nitric oxide (cGMP) production (c) A high dose of NPY2R agonist was infused to assess the ability of the compound to attenuate this effect by histological method. This was not repeated with additional concentrations or agonists because the optimal dose and potent effect of our NPY-2R agonist is established. Agonist alone had a negligible effect (<1%) and METH alone increased active caspase-3 production to 18.25%. The addition of NPY2R agonist reduced this to 4.52% of cGMP-labeled cells. One way ANOVA reveals significant difference in METH group from Saline controls (** p<.01, F-5.427)


cGMP
Active Caspase-3
Overlay
NPY-2R agonist +METH
aCSF + METH
a.
b.
c.
d.
e.
f.
47.62 µm


Percent of cGMP-expressing cells in the striatum with colabel for active caspase-3
25
20
15
10
5
0
-5
**
aCSF + Saline
NPY-2R agonist + Saline
aCSF + METH
NPY-2R agonist + METH

DISCUSSION

Our lab has found that neuropeptide signaling modulates METH-induced NO production and its byproducts. For example, a NK-1R agonist (SP) exacerbates METH-induced 3-NT and L-citrulline production, whereas pharmacological blockade of the NK-1R attenuates the METH-induced production of NO (Wang and Angulo, 2011). NPY, a neuropeptide that is also strongly colocalized with NK1-Rs in the striatum, has shown a neuroprotective effect in an *in vivo* mouse model with binge METH dosing (10 mg/kg, 4 times, every 2 hours) and in *in vitro* microglial cell culture (Thiriet et al., 2005, Ferreira et al., 2010). Additionally, METH increases the number of prepro-NPY neurons in the striatum (Horner et al., 2006). NPY is implicated in other homeostatic mechanisms with SP release. For example, SP induces pain and NPY has been shown to modulate pain (Broqua *et al*., 1996, Brumovsky *et al*., 2007). We hypothesized that NPY reduces levels of NO after METH treatment, and that by doing so counteracts NO production by SP release during the neurotoxic response. **My data show that production of NPY and its precursors are upregulated during METH neurotoxicity, and that the presence of this peptide counteracts oxidative stress and excessive NO production.**

Following a bolus dose (30 mg/kg) of METH, nNOS activity and NO levels are both increased significantly in the brain (Imam et al., 2005). Imam and colleagues also documented the increase of 3-NT levels in the brain following METH injection, a product formed in the presence of NO and tyrosine (Imam et al., 2005). Both antioxidants and free radical scavengers have shown the ability to reduce or block 3-NT in models of neurotoxicity (Imam et al., 2001, Kawasaki et al., 2006). When we infused exogenous NPY-1R and NPY-2R analogs into the striatum, 3-NT levels were reduced after a single bolus dose (30 mg/kg) of METH. Antagonists increased 3-NT fluorescence in a dose dependent manner as well, thus demonstrating true pharmacological modulation of NO by NPY receptor subtypes.

Endogenous NPY mRNA is also upregulated in response to METH. Previous studies demonstrate that a toxic METH dose (10 mg/kg, 4 times, every 2 hours) increases NPY mRNA at 12 hours, 3 days and 7 days post-treatment. METH increases the number of prepro NPY-

expressing neurons in the striata of mice at 3 hours after drug treatment, and this effect is eliminated by pretreatment with SCH23390, a D1R antagonist. (Horner et al., 2006). NPY mRNA is elevated in the METH brain by a significant amount at 4 hours, and by a substantial amount at 16 hours after treatment. This variation over time suggests that NPY is produced and utilized 6-8 hours after METH treatment. NPY mRNA is likely elevated at 16 hours to replenish these lost peptide stores. NPY may exert initial action 16 hours after METH treatment, but the former hypothesis is more likely since NPY-like striatal immunoreactivity is decreased at 18 hours post-METH (Westwood and Hanson, 1999).

NPY is released from storage vesicles on the same interneurons that express SST, NK-1Rs and nNOS. NPY is likely produced by SP/NK-1R signaling, which is stimulated by the direct D1R pathway release of SP in the striatum. Electrical stimulation of cortical afferents with D1R agonist application induces nNOS activity, and striatal NO release is blocked by nNOS inhibitor, D1R antagonists or NMDA-R antagonist (West and Tseng, 2011). Since both DA and GLU excitation are needed for METH-induced striatal neurotoxicity, then the interruption of just one signaling process is enough to discontinue this feedforward transmitter release.

Once released, NPY can disrupt both DA and GLU transmission by inhibiting calcium (Ca) signaling, a necessary step for GLU and subsequent NO production (Tepper and Bolam 2004, Colmers and El Bahh 2003). NPY receptor activation inhibits adenylyl cyclase and intracellular calcium concentration can be mobilized via inositol phosphate dependent and independent pathways (Pernet et al., 1989, Motulsky et al., 1988). There is an abundance of NPY-1R and NPY-2R expression in the PFC of rats and humans, and their activation inhibits GLU release (Wang 2005, Caberlotto and Hurd 2001, Statnick et al., 1997). Study of NPY agonists in the hippocampus and dentate gyrus reveals similar action (Whittaker et al., 1999, Qian et al., 1997). NPY selectively inhibits GLU release in hippocampus via voltage-dependent Ca in presynaptic terminal (Colmers et al., 1987, Klapstein and Colmers, 1992).

DA signaling in the striatum is largely influenced by excitatory Ca-dependent GLU inputs from the PFC. *In vivo* excitation or inhibition of PFC neurons correlates with the effect of

downstream VTA neurons in the dorsolateral and dorsomedial regions of the striatum (Voorn et al., 2004, Gariano et al., 1988, Tong et al., 1996). ChAT-containing cells also regulate DA transmission by large inhibitory postsynaptic outputs and a majority of ChAT cells contain NPY receptors. (Threlfell and Cragg 2011, Bolam et al., 1991). In summary, NPY exhibits inhibitory effects on Ca-dependent GLU transmission and DA signaling, which has far-reaching effects on NO production and METH-induced feed-forward excitotoxicity. While unable to completely block cell death, NPY can slow the cycle of DA leakage and oxidative stress in the striatum.

Quinolinic acid lesions, Huntington's disease or a toxic dose of METH do not damage NPY-expressing striatal interneurons (Koh et al., 1986, Ferrante et al., 1985, Thiriet et al., 2005). This population of cells is also immune to METH toxicity when observed 3 days post-METH treatment (Thiriet et al. 2005). It has been suggested that NPY-2R signaling acts in an autocrine fashion to reduce susceptibility of this cell to NO-related apoptosis. Our data show that NPY-2R signaling is not exclusively autocrine and that NPY/NOS cells must rely on another mechanism for immunity from oxidative stress and toxicity. Characterization of receptor expression in the hypothalamus, hippocampus and amygdala reveals a presynaptic role for NPY-2Rs, which are mostly localized to nerve endings (Stanic et al., 2011). Both receptors are co-expressed with NPY in the dorsal root ganglion after injury (Landry et al., 2000) and are expressed in non-NPY-expressing neurons as well (Wettstein et al., 1995, Caberlotto et al., 2000), though Y2R receptors are more abundant than Y1R in the mouse brain. Since NPY-1R and NPY-2R receptors are widely expressed throughout the striatum, we cannot conclude that signaling through either or both of these receptors alone is enough to rescue the NPY/NOS neuron from oxidative damage.

The project also investigated the possibility that cell populations of the striatum respond differently to NO production, and perhaps SST/NPY/NOS cells are immune to METH-related cell death for this reason. In Aim2a, we used immunohistochemical label for cGMP since it is produced in the presence of NO and guanylyl cyclase (GC), and is a more stable molecule than NO itself. Agonists of the direct D1R pathway and antagonists of the indirect D2R pathway enhance tissue levels of cGMP. The opposite is seen with D1R blockade or D2R agonist

administration (West and Tseng, 2011). This effect is eliminated in nNOS knockout mice, confirming the effect of DA transmission on NO production (Siuciak et al., 2006).

This study considers cGMP modulation to correlate with NO production, but acknowledges that there are other means by which cGMP is produced. NO can interact with soluble GC cGMP (Chinkers and Garbers, 1991, Miki et al., 1977). cGMP can also be produced by the action of natriuretic peptides (type C) on guanylyl cyclase B (GC-B) (Potter et al., 2006). Our data show that NPY/NOS cells respond to NO release in a similar manner to other striatal neuron populations, but that they are later rescued.

Further study is needed to determine the mechanism of cGMP reduction in these neurons and to describe NPY action in this process. cGMP exerts an effect downstream by the activation of cGMP-dependent protein kinases (PKG) of which PKG-I is expressed in striatal MSNs (Ariano 1983, Lohmann et al., 1981). PKGs are associated with modulation of neurotransmitter release and long-term potentiation, both associated with substance abuse physiology (Zhuo et al., 1994, Guevara-Guzman et al., 1994, Lin et al., 1995). Phosphodiesterase 10a (PDE10a) is specifically expressed in striatal tissue in both projection and interneurons and regulates cGMP/PKG and cAMP/PKA signaling by hydrolyzing these cyclic nucleotides (Kehler and Nielsen, 2011, Seeger et al., 2003). The PDE10a knockout mouse shows decreased exploratory behavior and delayed conditioned avoidance behavior, but no difference in AMPH-induced locomotion (Siuciak et al., 2006). Interestingly, PDE mRNA, protein, and activity are elevated in DA-depleted rats (West and Tseng 2011, Sancesario et al., 2004; Giorgi et al., 2008). PDE10a is of interest for its therapeutic potential in PD and schizophrenia models. PDE10a deserves investigation in our model of METH toxicity due to our observations of differential cGMP production in striatal cells.

BDNF or other neurotrophic factors may be intermediaries in the control of NPY release and reduction of toxic markers. BDNF has shown protective effects in neurodegenerative disease such as AD and Multiple Sclerosis (Nagahara et al., 2009, Linker et al., 2010) and dysfunction in the BDNF gene Val66Met may be predictive of Alzheimer's (Voineskos et al., 2011). Low serum

levels of BDNF are a hallmark of depression and the protein can be used for supplemental treatment (Siuciak et al., 1997). Most significantly, BDNF shows a protective effect in the METH brain (Matsuzaki et al., 2004) and control over mesolimbic dopamine release (Narita et al., 2003). Polymorphisms of the BDNF gene and elevated serum levels show correlation with substance abusers (Cheng et al., 2005, Kim et al., 2005)

To the contrary, we found no specific effect of BDNF on NPY/NOS cells. Immunohistochemical study was used to investigate the METH-induced effect of BDNF protein levels in NPY/NOS cells after an acute METH injection. A third time point was added at 4 hours to account for the idea that the study was not measuring the early induction of BDNF after METH treatment, but this also yielded no significant change BDNF levels in this cell population.

This supports data collected from a similar paradigm. Intrastriatal pretreatment with BDNF (1 day prior to METH) provides no protection from METH-induced loss of DA as measured by HPLC 7 days after treatment. BDNF levels can also be depleted by amphetamine treatment (Angelucci et al., 2007). In human studies, serum NPY and BDNF are both elevated in stress-induced responses of abstinent addicts, but show no correlation with one another (Meng et al., 2011). While BDNF is widely expressed in the striatum and displays protective and therapeutic effects, we could not capture this in the NPY/NOS cell population. The effect of BDNF on NPY production is established in cell culture, but may not show a disproportionate effect in the METH brain (Williams et al., 1998, Barnea et al., 2004). BDNF may show drastic changes only after binge or chronic METH treatment. It may also exhibit protective effect in larger cell population that was not measured.

While unlikely, it is also possible that BDNF gene changes are not transcribed to show a protein effect. In a second interpretation, BDNF levels may be elevated as an immediate response to METH toxicity, and this in turn regulates transcription activation of the NPY gene (Reibel et al., 2000, Scharfman et al., 2002, Barnea et al., 2004, Wirth et al., 2005). This mechanism explains the appearance of NPY-mediated action at 8 hours after drug treatment

since the peptide precursor is modified and released prior to this time. BDNF is not responsible for the mechanism by which NPY exhibits protective effect in the brain during METH toxicity.

Striatal apoptosis may occur through a cGMP-independent mechanism, and thus we co-labeled active caspase-3 with cGMP in striatal tissue to measure the pro-apoptotic cells that respond to NO (Aim 2b). In this pathway, METH increases the autoxidation of DA and triggers oxidative stress in the endoplasmic reticulum and on mitochondrial respiration. This leads to inhibition of mitochondrial ATP production. (Acanthi, et al. 2004) Calpain protease activation in the cytoplasm and caspase-12 cleavage occurs almost immediately following ip methamphetamine administration. Active caspase-3 and cytochrome c release are also seen as soon as 30 minutes after methamphetamine injection (Jayanthi et al., 2004). Additionally, activate caspase-3 (fractin) is substantially increased in a 6-OHDA-infused (6-hydroxydopamine) striatum with 50% striatal DA depletion (Ariano et al., 2005).

Active caspase-3 is expressed in over 60% of striatal cells with cGMP immunoreactivity. These data confirm a strong correlation between oxidative stress (cGMP independent) and cGMP-dependent cell death. It is assumed that an even larger number of NO-responsive cells are destined for apoptosis since peak apoptosis is observed at 16 hours post-treatment (Zhu et al., 2005). When we administered a potent dose of NPY-2R agonist (20uM) in the presence of METH, NO was drastically reduced. The same dose was used to measure active caspase-3 expression, which returned to near-control levels in the presence of NPY-2R analog. METH increases mitochondrial membrane permeability, allowing proteins to enter the cytoplasm. It can then be assumed that NO signaling occurs in step with mitochondrial disruption, leading to cytochrome c release and caspase cleavage, which results in apoptosis. These data suggest that NPY counteracts some deleterious effects of NO, and attenuates further apoptotic signaling.

CONCLUSIONS

The current study makes a contribution to our understanding of NPY function during METH excitotoxicity. Data demonstrate that an acute dose of METH induces significant appearance of damage markers in the early hours (4-8 hr) following treatment in an *in vivo* mouse model, but that NPY administration can attenuate this response. Our measures of 3-NT, active caspase-3 and cGMP demonstrate that METH induces marked changes in cell signaling pathways that produce NO, and lead to subsequent striatal cell death. We show that all striatal cells respond to NO in the first 8 hours after METH administration, and that both cGMP-dependent and ROS mechanisms are engaged to induce cell death. These markers precede apoptosis in all striatal neuron populations except for the NPY/NOS neuron. This cell population shows some recovery from the oxidative stress as soon as 8 hours after METH treatment.

We were unable to explain the mechanism of NPY/NOS cell immunity to METH toxicity, but suggest that it is likely through the inhibition of calcium-dependent GLU transmission from the cortex. Further research is needed to explore the actions of NPY on Ca signaling and GLU transmission in the METH brain. This may involve microglial activation or activation of neurotrophic factors. While BDNF does not appear to contribute to resistance of NPY/NOS cells to METH-induced toxicity, GDNF and other neurotrophic factors may show a more potent effect.

NPY is clearly involved in countering inflammatory signaling. AD and PD are both characterized by the appearance of NO, oxidative damage, ER and mitochondrial stress, as well as microglial activation. Our data and others show that NPY decreases caspase-dependent cell death and lowers levels of NO during the striatal stress response. There is therapeutic potential for NPY in inflammatory disease treatment. The next stage of research will determine if NO can be modulated when NPY mRNA is knocked down. This is necessary to more fully describe the interaction between neuropeptides and NO.

NPY is elevated in response to stress in human and animal models, and demonstrated significant protection when administered after METH treatment (Hirsch and Zukowska 2012, Zhou et al., 2008, Heilig and Thorsell 2002). In clinical studies, variants in NPY expression also show differential responses to stress and alcoholism (Xu et al., 2011, Lappalainen et al., 2002).

Patients with variants of the NPY1R gene show correlation with METH dependence and psychosis (Okahisa et al., 2009). Taken together, NPY expression and receptor function are important to maintenance of homeostasis in the hypothalamus and striatum. Altered expression may predispose patients to substance abuse.

Our study of neuropeptides in a mouse model of drug toxicity is important to research of disease states. As a ubiquitous and endogenous peptide, the therapeutic potential of NPY is limited by its inability to cross the blood brain barrier and its ubiquitous presence in the brain, which lends itself to side effects. These challenges should be addressed to bring us closer to the clinical relevance of NPY treatment.

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