

City University of New York (CUNY)

CUNY Academic Works

Dissertations, Theses, and Capstone Projects

CUNY Graduate Center

5-2015

Attenuation Of Methamphetamine And Nmda-Induced Toxicity By Leptin In Murine Striatum

Nawshin Hoque Kutub

Graduate Center, City University of New York

[How does access to this work benefit you? Let us know!](#)

More information about this work at: https://academicworks.cuny.edu/gc_etds/1014

Discover additional works at: <https://academicworks.cuny.edu>

This work is made publicly available by the City University of New York (CUNY).

Contact: AcademicWorks@cuny.edu

**ATTENUATION OF METHAMPHETAMINE AND NMDA-INDUCED TOXICITY BY LEPTIN IN
MURINE STRIATUM**

by

NAWSHIN HOQUE KUTUB

A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the
requirements for the degree of Doctor of Philosophy
The City University of New York

2015

© 2015

NAWSHIN HOQUE KUTUB

All Rights Reserved

This manuscript has been read and accepted for the
Graduate Faculty in Psychology in satisfaction of the
dissertation requirement for the degree of Doctor of Philosophy.

_____ Dr. Jesus A. Angulo _____
Date

Chair of Examining Committee

_____ Dr. Joshua C. Brumberg _____
Date

Interim Executive Officer

Dr. Peter Serrano _____

Dr. Michael Lewis _____

Dr. Syed F. Ali _____

Dr. Shirzad Jenab _____
Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

ABSTRACT

ATTENUATION OF METHAMPHETAMINE AND NMDA-INDUCED TOXICITY BY LEPTIN IN MURINE STRIATUM

by

NAWSHIN HOQUE KUTUB

ADVISER: DR. JESUS A. ANGULO

Methamphetamine (METH) is an addictive illicit psychostimulant that is neurotoxic and causes permanent brain injury. METH-induced neurological damage affects areas of the brain that mediates emotions, motivation, cognition and critical thinking. In the striatum METH neurotoxicity intertwines several factors such as dopamine (DA) overflow, glutamate signaling, and free radicals formation causing oxidative stress. In addition, excessive dopaminergic innervation leads to severe reduction in DA terminals, DA transporters (DAT), and vesicular monoamine transporters (VMAT)-2. METH use causes permanent damage which cannot be recovered even after three years of abstinence. Understanding the mechanism of METH-induced neurodegeneration will provide an avenue towards identifying effective therapeutic targets for treatments of METH abuse. Leptin is an important peripheral hormone produced mainly by adipose tissue in proportion to fat stores, which circulates in the plasma, and found ubiquitously in the central nervous system (CNS). Though leptin is primarily known for its regulation of energy homeostasis mediated by its receptors, ObRb, it was shown to serve

other functions as ObRbs are expressed in hypothalamic and extra hypothalamic areas, specifically in the ventral tegmental area (VTA), substantia nigra (SN), and nucleus accumbens (NAc), striatum, cortex, cerebellum and hippocampus. The precise molecular pathway underlying the direct effects of leptin in these regions is mostly unknown. But studies report that leptin administration decreased the firing rate of DA neurons in the VTA. The exact cellular mechanism for this reduced excitability by leptin remains to be determined. We show evidence that leptin signaling is neuroprotective in striatal neurons upon METH-induced injury. We hypothesized leptin would attenuate METH-induced striatal neural injury. Our data suggests that leptin produced a dose dependent attenuation of apoptosis upon METH administration. METH caused about 25% of the striatal neurons to undergo apoptosis. However, leptin treatment attenuated apoptosis by 18% suggesting that it protects striatal neurons from METH toxicity. Leptin did not prevent METH-induced hyperthermia or weight loss, one reason may be that it is an anorexigenic peptide and causes animals to increase activity and energy expenditure. In support of our hypothesis leptin treatment attenuated the over activation of the astrocytes and microglia caused by METH toxicity. It also dampened oxidative stress. Furthermore, we demonstrated here that leptin mediates striatal neuroprotection by modulating glutamate transmission. *N*-Methyl-D-aspartate (NMDA) -mediated apoptosis was attenuated by leptin treatment. It also reduced the NMDA-induced formation of nitric oxide (NO). However, leptin failed reduce NMDA-induced striatal over activation of astrocytes and microglia. A plethora of evidence demonstrates that METH induces neural damage in the striatum and other parts of the brain. Our contribution to this area of research is the finding that peripheral hormone, leptin, can protect degeneration caused by METH in the striatum.

ACKNOWLEDGEMENTS

There are a host of people that supported me throughout the years in my pursuit of this Ph.D. First, I want express my deep appreciation and gratitude to my adviser Dr. Jesus Angulo for the patient guidance and mentorship he provided to me since I joined his lab. I would not have been able to work on such an interesting and intellectually stimulating project and learn the practice of science if I had not worked under his direction. Dr. Angulo allowed me the freedom to make decisions that would shape my research and also allowed me to learn from my mistakes. His intellectual heft is matched only by his genuine kind nature and humility. Without exaggeration, I am truly fortunate to have the privilege to be a graduate student in his laboratory and if I'm ever half as good a scientist as Dr. Angulo, I'll be satisfied.

I would also like to humbly thank my doctoral committee members for the friendly guidance, brilliant comments and thought provoking suggestions over the years. Each member is an expert and experienced scientist and their advice and support has been a big part of the learning process and instrumental in the completion of my thesis. I want to specially thank Dr. Peter Serrano for encouraging me through various formal and informal conversations that stimulated a great deal of query in my research especially at the onset of my thesis process and during the committee meetings. I also want to recognize Dr. Syed Ali for always taking the time to discuss my project and provide advice each time I saw him at neuroscience and drug abuse conferences over the last couple of years, his kindness and helpful feedback were something positive I always brought back with me after each meeting. I want to thank Dr. Michael Lewis for his advice and recommendation to rotate in Dr. Angulo's lab which was the genesis of my thesis project and for his and Peter's suggestions during the pre-approval

committee meeting. Finally, I want to thank Dr. Shirzad Janeb for his time and feedback on my writing. I want to acknowledge Dr. Nieves Angulo for her advice and encouragement on both research and career planning. In a similar vein, I want to thank Drs. Rae Silver and Joseph LeSauter for the contributions each made to my intellectual growth and curiosity during my years of study at Barnard College. Working in Rae's lab provoked me to pursue a Ph.D.

I want to thank my husband, Sal Kutub, his love, support, and encouragement through this academic endeavor was a key backbone in reaching the end. He was understanding of my unorthodox work hours for these past few years. He often picked me up from lab in bad weather, late nights and weekends and also was my weekend companion in the lab while I conducted experiments. I'd be remiss if I didn't acknowledge my parents. There aren't enough words to adequately express my gratitude to them, my mother Samsun Neher and father Mominul Hoque supported me and my education over twenty years. It is hard to imagine that the past few years in the lab were just the last bit of a journey that started when I was just a kid and my parents were there every step of the way. I want to express my utmost gratitude for their innumerable sacrifices in shouldering far more than their parenting duties and burdens while I pursued college and this final degree. Also, I want to thank my siblings Tasnuba and Adnan Hoque for their undeniable love, friendship and advice that has sustained me thus far, and reassurance to complete this long journey, I'm truly grateful to them. I want to thank my father and mother-in laws Mohammed Kutubuddin and Selina Kutub for their love, kindness and support during this research process. Also, I want to thank my twin nieces and nephew Lana, Lia and Amaan. In the short one year they have been in this world so far, their tiny smiles and laughter were breaks that helped me during data analysis and the writing process. Finally, I want to thank my sisters-in-laws Farzana, who always remembered to find good espresso for me which I enjoyed during the writing process, and Farzana as well as my brother in-law Mohammed for their friendship and kindness during the past several years.

I would also like to thank my fellow co-workers, graduate students and friends who started the Ph.D. journey together. I value their friendship, scientific expertise, technical advice and support in weathering this arduous journey. I want to thank and acknowledge the many research assistants/ honor students I had the chance to train and work with the last several years: Vartan Pahalyants, Jing Liang, Jenny Mamtora, Isabelle Ament, Liza Yelin and Nick Burney. Each person dedicated tireless hours and days to collecting data, helping with analysis and editing manuscripts. Their unfailing presence, dedication and passion for science helped me to complete this project efficiently. They not only afforded their technical assistance but their friendship which I value greatly, thank you.

I appreciate the backing of The Graduate Center and Hunter College for extending support and teaching experience in various lectures and labs. These experiences are invaluable. The efforts of Ellen Breheny at the program office were also essential and helpful in navigating through the paperwork these past years. Ellen has always promptly and efficiently helped with transitioning from first year to the final. I could ask her almost any questions or come to her with a problem and she would get back promptly. I also want to thank Dr. Patricia Glennon and Barbara Wolin of Animal Facility who have allowed for easy communication and help with scheduling experiments. Finally, I want to thank the bio-imaging facility for providing technical support when using the facility in gathering my data.

TABLE OF CONTENTS

ABSTRACT		iv
LIST OF FIGURES		xii
LIST OF ABBREVIATIONS		xiv
CHAPTER 1	INTRODUCTION	
1.1	History and Use of Methamphetamine	1
1.2	Pharmacology and Addictive Properties of Methamphetamine	3
1.3	Mechanism of Action and Neural Toxicity of Methamphetamine	4
1.4	Basal Ganglia System and Striatal Neurochemistry	7
1.5	Dopamine, Glutamate and Oxidative Stress in Methamphetamine Toxicity	9
CHAPTER 2	ROLE OF LEPTIN IN NEUROPROTECTION	
2.1	History and Pharmacology of Leptin and Leptin Receptor Activity	19
2.2	Role of Leptin in Dopaminergic Pathway	22
2.3	Leptin Receptor Signaling	22

CHAPTER 3	RESEARCH DESIGN	
3.1	Specific Aim 1 and Relevance	28
3.2	Specific Aim 2 and Relevance	29
CHAPTER 4	METHODS AND MATERIALS	
4.1	Animal and Systemic Drug Administration	30
4.2	Intrastriatal Infusion of NMDA and Leptin Injection	32
4.3	TUNEL Immunofluorescence	32
4.4	NeuN, GFAP, Iba-1, NOS1, cGMP, Active Caspase-3, 3-Nitrotyrosine Immunohistochemistry	31
4.5	Tyrosine Hydroxylase and Leptin Receptor Western Blot	34
4.6	Quantification and Statistical Analysis	35
CHAPTER 5	ROLE OF LEPTIN IN METHAMPHETAMINE- INDUCED NEURAL INJURY	
5.1	Attenuation of Apoptosis by Leptin	37
5.2	Role of Leptin in Striatal Overactivation of Astrocytes and Microglia	46
5.3	Role of Leptin in Methamphetamine-Induced Striatal Nitric Oxide and Caspase-3 Activation	49
5.4	Discussion	55

CHAPTER 6	LEPTIN MEDIATED STRIATAL NEUROPROTECTION BY MODULATING GLUTAMATE TRANSMISSION	
6.1	Contribution of Leptin in NMDA-Induced Striatal Apoptosis and Dopamine Terminal Toxicity	57
6.2	Role of Leptin in NMDA-Induced Formation of Nitric Oxide	61
6.3	Role of Leptin in Striatal Expression of Astrocytes and Microglia Upon NMDA	63
6.4	Discussion	67
CHAPTER 7	CONCLUSIONS	67
REFERENCES		72

LIST OF FIGURES

Figure 1 – 1:	Widespread distribution of methamphetamine in the USA	15
Figure 1 – 2:	Molecular events during METH-induced DA terminal damage and apoptosis in the striatum	16
Figure 1 – 3:	Glutamate transmission affected by DA overflow in METH toxicity	17
Figure 1 – 4:	Nitric oxide signaling through cGMP	18
Figure 2 – 1:	Leptin mouse and feedback loop	26
Figure 2 – 1:	Leptin receptor signaling pathways	27
Figure 5 – 1:	Leptin alone does not cause apoptosis	40
Figure 5 – 2:	Dose dependent attenuation of METH-induced apoptosis in the striatum by leptin	41
Figure 5 – 3:	Leptin does not protect dopamine terminals from METH toxicity	42
Figure 5 – 4:	Leptin protects against METH-induced cell death without preventing hyperthermia	43
Figure 5 – 5:	Leptin does not protect against METH-induced weight loss	44
Figure 5 – 6:	Leptin receptor expression in mouse striata	45
Figure 5 – 7:	Activation of astrocytes measured using glial fibrillary acidic protein (GFAP)	47
Figure 5 – 8:	Activation of microglial cells in the mice striatum by METH is attenuated by leptin	48
Figure 5 – 9:	Photomicrographs of nNOS stained striatal sections	52
Figure 5 – 10:	Cyclic guanosine monophosphate (cGMP) expression in all cell types in the mouse striata	53
Figure 5 – 11:	Photomicrographs of activated caspase-3 stained striatal sections	54

Figure 6 – 1:	Leptin attenuation of NMDA-mediated apoptosis in striatum	59
Figure 6 – 2:	Leptin does not protect dopamine terminals from NMDA toxicity	60
Figure 6 – 3:	NMDA-mediated striatal NO attenuated by leptin	62
Figure 6 – 4:	NMDA-induced activation of astrocytes measured using glial fibrillary acidic protein (GFAP)	64
Figure 6 – 5:	Activation of microglial cells in the mice striatum by NMDA-infusion is not attenuated by leptin	65

LIST OF ABBREVIATIONS

3-NT:	3-nitrotyrosine
5-HT:	serotonin
ACh:	acetylcholine
aCSF:	artificial cerebrospinal fluid
AgRP:	agouti-related peptide
AMPA:	α -amino-5-hydroxy-3-methyl-4-isoxazde propionic acid
ATP:	adenosine triphosphate
Ca ²⁺ :	calcium ion
CART:	cocaine and amphetamine regulated transcript
cGMP:	cyclic guanosine monophosphate
CNS :	central nervous system
CuZn-SOD:	cooper/zinc-superoxide dismutase
Cy3:	cyanine 3
D1:	dopamine 1 receptor
D2:	dopamine 2 receptor
DA:	dopamine

DAPI:	4',6-diamidino-2-phenylindole
DAT:	dopamine transporters
<i>db</i>	diabetic gene
DEA:	Drug Enforcement Agency
DOPAC	dihydroxyphenylacetic acid
Enk:	enkephalin
eNOS:	endothelial nitric oxide synthase
ERK:	extracellular signal-regulated kinase
ETC:	electron transport chain
FDA:	Food and Drug Administration
Fe ²⁺	iron II
GABA:	gamma-aminobutyric acid
GAD:	glutamic acid decarboxylase
GC:	guanylyl cyclase
GFAP:	glial fibrillary acid protein
GluR:	glutamate receptor
GP:	globus pallidus
GPCRs:	G-protein coupled receptors
GTP	Guanosine-5'-triphosphate
Iba1:	ionized calcium-binding adapter molecule 1

ICV:	intracerebroventricular
IHC:	immunohistochemistry
iNOS:	inducible nitric oxide synthase
IP:	Intraperitoneal
JAK:	Janus Kinase
K ⁺ :	Potassium ion
KO:	knockout
LHA:	lateral hypothalamic area
MAPK:	mitogen-activated protein kinase
MDMA:	3,4-methylenedioxymethamphetamine
METH:	methamphetamine
mRNA	messenger RNA
MnSOD:	manganese superoxide dismutase
mTOR	mechanistic target of rapamycin
NADPH:	nicotinamide adenine dinucleotide phosphate
NAc:	nucleus accumbens
NDIC:	National Drug Intelligence Center
NK-1R:	neurokinin 1 receptor
NMDA:	N-methyl-D-aspartate
nNOS:	neuronal nitric oxide synthase

NO:	nitric oxide
NOS:	nitric oxide synthase
NPY:	neuropeptide Y
NueN:	neuron-specific nuclear protein
ObRb:	leptin receptor long form
ONDCP:	Office of National Drug Control Policy
PBS:	phosphate buffer saline
PD:	Parkinson's disease
PV:	parvalbumin
PFC:	prefrontal cortex
PI3K:	phosphoinositide kinase-3
PKA:	protein kinase A
POMC:	pro-opiomelanocortin
RNS:	reactive nitrogen species
ROS:	reactive oxygen species
SAMSHA:	Substance Abuse and Mental Health Services Administration
SERT:	serotonin transporter
SHP2:	Src homology phosphatase 2
SNc and SNr:	substantia nigra par compacta and reticulata
SOCS3:	suppressor of cytokine signaling 3

SP:	substance P
SST:	somatostatin
STAT:	signal transducers and activators of transcription
STN:	subthalamic nucleus
SOD:	superoxide dismutase (SOD)
TUNEL:	terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
TPH:	tryptophan hydroxylase
TH:	tyrosine hydroxylase
Tyr:	tyrosine residues
UNODC:	United Nations Office on Drugs and Crime
VTA:	ventral tegmental area
vGLUT:	vesicular glutamate transporters
VMAT:	vesicular monoamine transporters

CHAPTER 1

INTRODUCTION

1.1 History and Use of Methamphetamine

METH, an amphetamine analog, is a mind-altering drug behaving as a nervous system stimulant. It was first discovered from ephedrine around the early 1900s in Japan (Grinspoon and Hedblom, 1975). Ephedrine and pseudoephedrine are the most valuable precursor commodities for METH production in the illicit market. Because of its synthetic nature and potency many variations are produced. Some common terms used to describe METH and the combination of METH and other drugs are: crank, crystal, ice, speed, and poor man's coke (UNDOC, 2014). These names are not representative of the quality, but rather used for the purposes of street marketing and illicit use. METH is a white, odorless, bitter-tasting crystalline powder that is highly soluble in water or alcohol (UNDOC, 2014). METH can be ingested orally, smoked, snorted, or injected. Even though it has a 10 –12 hour half-life in humans (Cook et al, 1992; Krasnova and Cadet, 2009) compared to 60 – 70 min in mice and rats (Brien et al., 1978; Melga et al., 1995), tolerance for METH occurs instantaneously upon use, and the “high” lasts for hours. The typical mode of usage is a “binge and crash” the drug is eliminated long after the diminished pleasurable effects (Schepers et al., 2003; Krasnova and Cadet, 2009). Also, unlike other psychostimulants like cocaine, METH is disseminated in the human and rodent brain (Fowler et al., 2008; O'Neil et al., 2006; Segal et al., 2005).

Upon its discovery METH, was heavily used to increase alertness and concentration during World War II among British, Japanese and American soldiers. The grave popularity of legal use during

the war fueled the development of the illegal market and soon METH trafficking became popular (Grinspoon and Hedblom, 1975). Also, it was commonly prescribed in America and abroad in the late 1940s and 1950s to treat opiate addiction, cerebral palsy, seasickness, narcolepsy and radiation sickness, alcoholism and obesity (Miller and Hughes, 1994). It wasn't until the 1960s that the Food and Drug Administration (FDA) began to scrutinize amphetamine production causing pharmaceutical companies to stop its manufacture. But with so much of the population addicted, the illicit production rose to meet the demand (Brecher, 1972). The Controlled Substances Act passed in 1970 by the U.S. government grouped METH, barbiturates, opiates and narcotics into five schedules according to the rate of abuse and addiction levels. METH is classified as a Schedule II drug (DEA, 2015).

The influx of METH into the local market followed the California Outlaw Motorcycle Gangs (OMGs), as they started conducting transactions with common users and formed strategic networks. Not only did the OMGs controlled 90% of METH trade in California they started working with Mexican drug traffickers to sell Mexican METH in America. METH's popularity and synthetic nature fueled a shift from the clandestine super-labs to a rise in small local laboratories that could produce limited quantity but high quality products. From the early to mid-1990s, the number of people hospitalized for METH abuse (as their primary drug) increased from 1,400 to 42,000 (SAMSHA, 2011). In juxtaposition, the number of METH labs increased from about 7,000 to 8,500 (NDIC, 2011). By early 2000, although motorcycle gangs and Mexican cartels were still responsible for controlling METH trafficking in the U.S., local production increased tremendously. Legal ingredients like pseudoephedrine fueled local manufacturing. Only in 2004, laws were passed to restrict the purchase of pseudoephedrine and currently, every state has some type of limitation on purchasing this product (ONDCP, 2006). From 2005 – 2007, upon implementing the pseudoephedrine legislation seizures of METH and the number of people seeking treatment decreased, for the first time in many years (SAMSHA, 2011). However, the decline in METH abuse was ephemeral as currently 70 – 90% of the METH in the U.S. is imported from Mexico (NDIC, 2011). In addition, local labs began to use cheaper ingredients to make METH.

Mexico efficiently smuggles METH in pill form or its precursor chemicals like ephedrine and pseudoephedrine from Southeast Asian and East Asian countries, to produce METH at very low cost and with high purity (UNODC, 2011). METH seizure is used by the governmental agencies as an indicator of METH supply in the country. One of the largest seizures of small METH labs took place in 2012 due to recent increase in trafficking and high demand. At the same time, the largest amount of finished METH was seized in Mexico. In the U.S., the laws passed since 1970 to present, on regulating the different precursor chemicals for METH production has not significantly reduced the overall quantities of METH seized (UNODC, 2011). In the U.S., METH is the second most prevalent illicit drug used, other than cannabis, with an estimated economic cost of use to be \$23.4 billion in 2005 with 1.2 American users (Nicosia et al., 2009; UNODC, 2011). A recent survey showed that four percent of the American population have tried METH at least once due to very low cost and an intense euphoric feeling (UNODC, 2011). The METH market and illicit manufacturing is difficult to study because the U.S. and global market formed large and small networks that adapt to control efforts. In the U.S. METH use is one of the most poorly understood and most serious illicit drug problems that affects our society today (NDIC, 2011). See Figure 1 – 1.

As defined by the American Psychiatric Association, drug addiction is paralleled to substance dependence (American Psychiatric Association, 2013). According to Koob and LeMoal (1997) addiction is deemed as a nonstop process of hedonic homeostatic dysregulation, governed by brain neurotransmitters, hormones and reinforcements. Thus, the brain fails to properly maintain regulation, ultimately leading to addiction and neural damage.

1.2 Pharmacology and Addictive Properties of Methamphetamine

Amphetamines and METH show no differences in terms of the neural injury caused by these drugs, according to human discrimination studies (Melega et al., 1995; Lamb, 1994; Shoblock, 2003). METH is chemically similar to the endogenous neurotransmitter dopamine, DA, which enables its

vehement actions in the brain. The immediate effects upon METH use are feeling of euphoria, increased sexuality, productivity and energy, and decreased anxiety and appetite. The negative consequences are tachycardia, paranoia, hallucinations, aggression, convulsions, and hyperthermia (sometimes to lethal level) (Ellinwood, 1971; Homer et al., 2008; Krasnova and Cadet, 2009). Chronic METH users suffer from anxiety, depression, aggressiveness, mood disturbances and other psychological dysfunctions (Daker et al., 2008; Homer et al., 2008; Scott et al., 2007). These users show deficits in attention, decision making and working memory, that persist even after abstinence (Gonzalez et al. 2004; Woods et al., 2005; Salo et al., 2002 Krasnova and Cadet, 2009). Some of the withdrawal effects of METH are irritability, fatigue, impaired social functioning, and intense craving (Brecht et al., 2004; Homer et al., 2008).

Compared to other psychostimulants, METH remains unchanged in the body for a longer duration causing a prolonged stimulatory effect. Over the years, progressive increase in METH abuse and the neural deficits among users attracted attention from researchers and the government. It wasn't until the 1970s that several studies began to research and report the neural degeneration caused by METH. Because the chemical structure of METH resembles the structure of neurotransmitter DA, scientists looked at the DA innervations in the brain and found that prolonged exposure to METH in cats elicit neural damage (Escalante and Ellinwood, 1970). More specifically, scientists found that about 80% of the DA in the striatum of monkeys upon METH administration is depleted (Seiden, 1975). Similar reductions were found in humans in cortical and limbic brain areas (McCann et al., 1998; Sekine et al., 2003; Volkow et al., 2001).

1.3 Mechanism of Action and Neural Toxicity of Methamphetamine

Immediately upon taking METH, excess DA is released from the vesicles into the cytoplasm (Sulzer et al., 2005). The euphoria experienced is associated with the high DA levels at the synapses caused by this excessive release (Volkow et al., 1999; Liechti and Vollenweider, 2000; Winslow et al.,

2007). With repeated exposure to METH, the monoaminergic system is compromised. It causes damage to the dopaminergic innervations which results in a reduction in the levels of DA, DAT (Wagner et al., 1980; Eisch et al., 1992; Nakayama et al., 1993; Cass and Manning, 1999) TH and tryptophan hydroxylase (TPH) (Hotchkiss et al., 1979; Ricaurte et al., 1982; Seiden et al., 1988; Johnson, et al., 1989) and depletion in the activity and quantity of VMAT-2 (Frey et al., 1997; Villemagne et al., 1998; Hogan et al., 2000; McCann and Ricaurte, 2004). Typically, DATs remove excess DA from the synapse and VMAT-2 transports cytoplasmic DA into vesicles for storage, release and protection. METH enters the DA neurons via the DATs on the cell membrane. Once inside the neuron, METH enters the vesicles through the VMAT-2 initiating the excessive release, thus causing depletion. METH also compromises the passive diffusion of DA and changes the internal pH balance (Cubells et al., 1994; Sulzer et al., 2005). The function of VMAT is to maintain high concentration of neurotransmitters in storage vesicles. They recognize serotonin (5-HT), DA, noradrenaline, adrenaline, and histamine. VMAT-1 is present in the peripheral organs and VMAT-2 is found mainly in monoaminergic neurons in the CNS (Kavanaugh 1998). DAT knockout (KO) mice lack the gene for DAT, have high levels of extracellular DA, are hyperactive and fail to show further stimulatory activity in response to psychostimulants (Giros et al., 1996). However, these animals will continue to self-administer cocaine, indicating that DAT functionality is not the only culprit in the rewarding properties of psychostimulants (Rocha, et al., 1998). Human neuroimaging studies show that after one year of abstinence, DAT reduction was similar to patients with Parkinson's disease (PD) (Volkow, et al., 2001). Among the users, density of DAT reduction corresponds to the amount and duration of METH used and the amount of METH used negatively correlates with DAT recovery (Sekine, et al., 2001; Volkow et al., 2001). Interestingly, after abstinence, DAT recovered but cognitive deficits persisted. Even with acute METH administration synaptic DA levels greatly increased (Stephans and Yamamoto, 1995). See Figure 1 – 2.

A neurotropic role of glutamate signaling in METH-induced toxicity is its excessive release in the striatum (slightly reduced in the nucleus accumbens (NAc) (Stephans and Yamamoto, 1994; Nash and Yamamoto, 1992; Stephans et al., 1998). METH inhibits the binding of glutamate to its receptors in the striatum. Striatal neurons receiving dopaminergic innervations contain glutamate receptors α -amino-5-hydroxy-3-methyl-4-isoxazole propionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) (Betarbet and Greenamyre, 1999). Studies show that when an NMDA receptor antagonist, MK-801, is administered upon METH treatment, hyperthermia is prevented, and blocks the decreases in DA content and TH activity (Sonsalla et al., 1989, 1996) and thus contributes to neuroprotection (Bowyer et al., 2001; Albers et al., 1995; Ali, 1994). However, there is some inconsistency as another study demonstrated that attenuation of METH-induced deficits with NMDA antagonists is independent of body temperature regulation (Fuller et al., 1992). Another group of researchers used a different antagonist, mGluR5, to prevent METH-toxicity by preventing the release of glutamate (Rodrigues et al., 2007). Increase in glutamate transmission (acting via the NMDA receptors) produces reactive oxygen species, ROS, through the activation of nitric oxide synthases (NOS) and the generation of nitric oxide (NO) via neuronal nitric oxide synthase (nNOS) (Dawson et al., 1996; Yamamoto and Zhu, 1998). See Figure 1 – 3.

In the CNS, NO is a signaling molecule, involved in synaptic plasticity to maintain neurosecretion, appetite, and temperature regulation. NOS catalyzes the conversion of amino acid, L-arginine to L-citrulline and NO in the presence of oxygen. There are three types of NOS: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS); with nNOS being present in the CNS (Xie and Nathan, 1994). Among the limbic structures, nNOS is ubiquitously present in the striatum, NAc, amygdala, hippocampus, and olfactory bulb. nNOS is activated by the formation of Ca^{2+} /calmodulin complex is found in astrocytes and involved in regular cellular functions such as neurotransmission, regulation of blood vessels, immune response. In addition, it uses nicotinamide-adenine dinucleotide phosphate (NADPH) as cofactors for catalytic activity. NO undergoes chemical

changes to form toxic compounds like reactive nitrogen species (RNS) or reactive oxygen species (ROS) causing cellular damage (Dawson and Dawson, 1996; Cadet and Brannock, 1998). Overproduction of ROS and RNS leads to structure damage by the formation of superoxide anions and peroxynitrite/ nitrogen (III) oxide respectively (Itzhak and Ali, 1996). More specifically, peroxynitrite is formed by glutamate-induced oxygen species damages axon terminals (Ali and Itzhak, 1998; Pacher et al., 2007). In the brain, NO behaves as a neuromodulator. Its signaling cascade initiates when it interacts with guanylyl cyclase (GC) to stimulate intracellular activity and increases cyclic guanosine monophosphate (cGMP) which stimulates neurotransmission and in excess can damage axon terminals (Imam et al., 2001).

1.4 Basal Ganglia System and Striatal Neurochemistry

Neuroimaging studies in human have shown various deficits and neural degeneration in the structure and chemistry mainly in the striatum and other brain areas of METH users (Chang et al., 2007). In the 1980s, researchers looked at long-lasting influences of METH outside of the traditional monoaminergic pathways by investigating their effects on cortical neurons. They saw METH induced cortical neuron degeneration by using the silver stain method (Ricaurte, et al., 1982; Ricaurte, et al., 1984). With the same methods they saw METH-induced degeneration of nigrostriatal dopaminergic fibers innervating the striatum (Ellison and Switzer, 1993; Bowyer et al., 1994). The striatum is the key area in the basal ganglia system that receives glutamatergic cortical inputs and is affected by METH. Typically the basal ganglia system is comprised of: cortex, striatum (which is comprised of caudate- putamen and NAc), internal and external segments of globus pallidus (GPi and GPe), subthalamic nucleus (STN), substantia nigra par compacta and reticulata (SNc and SNr). Overall, the dorsal basal ganglia are mainly involved in motor associative functions, while ventral basal ganglia are linked to limbic emotional functions. The major difference between the basal ganglia of mammals (mainly primates) and rodents is in the striatum. The rodent striatum lacks the unique

separation seen in the primate striatum, which has a distinct caudate nucleus (receive innervations from the prefrontal cortex (PFC) and putamen areas (innervations from motor and somatosensory areas) (Heinz and Tseng, 2010).

About 95% of the neurons in the striatum are medium spiny gamma-aminobutyric acid (GABA) neurons that receive major inputs and send outputs to GPi and SNr. Also, these neurons synthesize the neuropeptides substance P, dynorphin, and enkephalin (ENK) (Kawaguchi, 1997). The remaining striatal neurons are interneurons. There are two types of interneurons; one type is the large aspiny acetylcholine (ACh) synthesizing neurons, which consists about 1-3% of the remaining cells. The ACh neurons receives inputs from the cortex, thalamus and axon collaterals of striatal projection neurons. They also contain most of the striatal dopamine 2 receptors (D2R), ACh muscarinic receptors and, to a lesser extent, dopamine receptors (D1R), NMDA receptors, the neurokinin receptors (NK-1R) (for the neuropeptide substance P), and glutamate receptors 1 and 2 (GluR1 and GluR2) (Kawaguchi et al., 1995). These interneurons are responsible for mediating interactions between both direct and indirect pathways between the thalamus and striatum. The second type of interneurons is the medium aspiny GABA-ergic neurons (Kita, 1993). These neurons contain the calcium binding protein parvalbumin (PV). They receive inputs from cortex and contain most of the striatal glutamate receptor (GluR2, GluR3, GluR4), NK-1 and some D1 and D2 receptors. These GABA-ergic interneurons are also involved in NO synthesis and produce somatostatin (SST) and neuropeptide Y (NPY) (Kawaguchi, et al., 1995).

With respect to the stimulatory effects upon drug use, the striatal neurons are intricately affected since they receives mostly excitatory inputs on dendritic spines from cortical and limbic structures. These excitatory inputs communicate via DA, which increases the excitatory effect of the direct pathway and reduces the inhibitory effect of the indirect pathway. METH use exacerbates these pathways and causes damage. Mainly, the direct pathway works as follows: glutamatergic cortical projections send excitatory message via axon collaterals. Striatal GABA-ergic projection neurons

receive the excitatory message and send inhibitory message to the GPi and SNr complex, which then provides less inhibition of the thalamus (composed of mainly glutamatergic neurons), thus sending excitatory projections to the cortex. The indirect pathway works in the following ways: it also starts from cortical projections to the striatum, which sends axon collaterals to GPe (also contains GABA neurons). It receives more inhibition from the striatum and there is a net reduction of inhibition. This enables the STN to release glutamate to the SNr – GPi complex, which is stimulated to send more GABAergic inhibition to the thalamus and as a result prevents glutamate from being released into the cortex and prevents the motivated action. Also, most innervations to the GPe contain neuropeptide Enk and projections to the GPi-SN complex contain SP and dynorphin.

1.5 Dopamine, Glutamate and Oxidative Stress in METH toxicity

METH toxicity alters the normal DA and glutamate signaling among other crucial neurotransmitters, by tipping the balance between release and reuptake. All drugs of abuse increases DA release in various brain regions. There are four different DA systems: nigrostriatal, mesolimbic, mesocortical and tuberoinfundibular. METH use affects the nigrostriatal (originating in the SN, it projects to the caudate – putamen of the dorsal striatum) and the mesolimbic DA system (originating in the VTA and projects to the NAc (ventral striatum), bed nucleus, septum, amygdala, and hippocampus (Kandel et al., 2000). METH use leads to abnormal functioning (attenuation or over expression of DA) that is similar to neurodegenerative diseases such as PD, schizophrenia, and depression. The intertwining relationship between glutamate and DA plays a role in the dendritic spines of neurons in the striatum. DA modulates postsynaptic events that influence glutamatergic synaptic events. The effects of METH. DA release in the striatum increases GABA release in the SN, which reduces nigro-thalamic flow of GABA, thus causing disinhibition of thalamo-cortical afferents, which allows excessive release of GLU in the cortico-striatal pathway. See Figure 1 – 4.

METH-induced DA trafficking increases extracellular and cytosolic DA. DA auto-oxidizes form quinones and semiquinones which produce ROS that causes neural damage (LaVoie and Hastings, 1999; Larsen et al., 2002; Krasnova et al., 2001). Excess DA is also mediated by an increase in NO production. METH toxicity overwhelms the antioxidant free radical scavenging systems. Toxic METH breaks down DA which increases hydroxyl radical production, via superoxides and hydrogen peroxide and cause oxidative stress, mitochondrial dysfunctions and lipid peroxidation (Giovanni et al., 1995; Cadet and Brannock, 1998; Yamamoto and Zhu, 1998). Typically NO scavengers act as a feedback system to inhibit release of harmful molecules. METH-induced damage as a result of NO can be attenuated by blocking its synthesis (Cadet and Brannock, 1998). One possible way of reversing the long-term effects of METH-induced decrease of striatal DA content is by antioxidants (Yamamoto and Zhu 1998). METH-induced toxicity is attenuated in the superoxide dismutase (SOD) transgenic mice. They overexpress the antioxidant enzyme, copper-zinc-superoxide dismutase (CuZn-SOD) which neutralizes the NO-induced accumulation of superoxide radicals (Cadet et al., 1994; Cadet and Brannock, 1998). In addition, pre-treating monkeys with powerful antioxidants like n-acetyl-L-cysteine before METH administration prevents terminal damage (Hashimoto et al., 2004). Oxidative stress caused by METH in DA and glutamate signaling can cause mitochondrial damage (Jayanthi et al., 2004). Mitochondria are the site of enzymatic reactions that make adenosine triphosphate (ATP) used by neurons to carry out general carry out general functions. METH inhibits these reactions, which then increases reactive oxygen species resulting in DA toxicity (Burrows et al., 2000; Brown et al., 2005). In some cases, it has been shown that METH-induced DA toxicity can be attenuated when inhibition of enzymatic reactions is counteracted (Stephans and Yamamoto, 1994).

METH can induce apoptosis through multiple pathways by inhibiting electron transport chain activity in mitochondria, decreasing anti-apoptotic Bcl-2 related protein, increasing pro-apoptotic protein (Bax, Bad, Bid) or activating caspase death pathway. In attempt to find a relationship between

NO-induced cGMP accumulation and apoptosis we plan to co-label cGMP with activated caspase-3, which is an early marker of apoptosis (Jayanthi et al., 2004).

D1 receptors on striatal medium spiny neurons co-localize with striatal interneurons that express NPY/SST/NOS. Pretreating rats with D1 receptor antagonist, SCH23390, prevents DA deficits upon METH administration (O'Dell, 1992; Sonsalla et al., 1989). Also, this antagonist prevents decrease of DA uptake upon acute METH-treatment (Gerfen et al., 1990). Since nNOS contributes to the DA deficiency caused by METH, D1 receptor agonist, SKF-82958, further exacerbates this system. However, pretreatment with nNOS inhibitor prevents this type of deficiency (Di Monte et al., 1996; Itzhak and Ali, 1996). Further, in nNOS deficient mice, METH doesn't induce this deficiency (Imam et al., 2001). Furthermore, D1 receptors form complexes with NMDA receptors (Fiorentini et al., 2003) and when D1 receptors are activated, they enable NMDA transmission (Cepeda et al., 1993) which is evidence of DA and glutamate signaling working together to accentuate toxicity.

D2 receptor activation also plays a role in METH-induced deficits of DA. Administration of D2 antagonists, prior to METH delivery, prevents the decrease in vesicular DA uptake and prevents the continuous damage that is independent of preventing hyperthermia (Xu, 2005; Broening et al., 2005). Peripherally, intense hyperthermia is an immediate reaction to METH. Some have shown that METH-induced neurotoxicity caused by excess DA can be attenuated by preventing hyperthermia (Albers and Sonsalla, 1995; Broening, 2005; Bowyer et al., 1992). Within the last 15 years, it has become clear that when studying METH toxicity, researchers must take into account increased body temperature, as drugs used in most studies, D1, D2, agonists, antagonists alike show a reduction of METH-induced hyperthermia (Albers, 1995). For the purposes of this proposal, we will also measure body temperature.

METH-induced toxicity increases levels of some but not all striatal neuropeptides. Our lab has identified that 25% of striatal neurons undergo apoptosis 24-hours post METH administration (Zhu et al., 2005). Relevant to this proposal, METH-induced apoptosis of striatal cells, shown by TUNEL

staining, a late marker for apoptosis, co-localized with neuronal marker neuron-specific neuronal nuclear protein (NueN) (Zhu et al., 2005). Some of the neurons that die upon METH toxicity are GABA-parvalbumin interneurons, which mediate inhibitory signaling and receive innervations from glutamatergic cortical neurons (Zhu et al., 2006). The other type of interneurons affected by METH are the cholinergic interneurons, which affects the output of the projection neurons and mediate excitatory signaling (Zhu et al., 2006). Thus, the cholinergic transmission increases DA release and sensitivity of projection neurons to glutamate (Steiner and Tseng, 2010). Both the GABA and cholinergic interneurons are affected by METH, the inhibitory and excitatory signaling systems by these interneurons do not cancel each other out upon METH-induced toxicity. Rather, transmission is more favorable for SST/NPY/nNOS interneurons because they are spared from METH-induced toxicity (Zhu et al., 2006).

Substance P, SP, is an excitatory neuropeptide with pro-toxic capacity upon METH. Neurons that release SP sends projections outside of the striatum. SP signaling via the NK-1R increases NMDA receptor activity and causes METH-induced overproduction of NO (Wu et al., 1994; Wang et al., 2008). NK-1R is co-localized with the cholinergic and SST/NPY/nNOS synthesizing interneurons (Kawaguchi et al., 1995). METH administration increases NK-1R signaling in these interneurons (Wang and Angulo, 2010). Therefore, overexpression is indicative of SP binding and signaling connected to NO formation (Wang et al., 2008; Wang and Angulo, 2010). Work in our laboratory demonstrated that, when animals are pretreated with NK-1 receptor antagonist (WIN-51,708), NO production was attenuated significantly (Wang et al., 2008; Zhu et al., 2009). NO production was quantified by measuring 3-nitrotyrosine (3-NT) levels, an indirect measure of NO production. 3-NT is formed when NO reacts with superoxide anion to produce peroxynitrite, which causes nitration of tyrosine, forming 3-NT (Wang et al., 2008). Therefore, we can state that SP modulates or amplifies its response via its NK-1R, which can further exacerbate the toxic effects of METH.

NPY and SST both have inhibitory influences on striatal glutamatergic release and transmission. They modulate intracellular calcium influx which is how they protect from striatal excitotoxicity (Thiriet et al., 2005). The corticostriatal neurons release abundant amount of glutamate to the SST/NPY/nNOS interneurons (Kawaguchi et al., 1995) under METH. NPY can inhibit glutamate release and stimulate SST release (Silva et al., 2005). In the hippocampal cultures, NPY reduces AMPA degeneration (Silva et al., 2003). METH administration increases NPY mRNA expression in the striatum (Thiriet et al., 2005). Work in our laboratory have shown that NPY1 and 2 receptor agonist attenuate METH-induced NO accumulation, in a dose dependent manner (Yarosh and Angulo, 2012). ICV administration of NPY reduces METH-induced apoptosis in the striatum (Thiriet et al., 2005). SST, expressed in the striatal projection neurons which regulates the release of GABA, glutamate and DA (Thermos et al., 2006). SST can cause presynaptic inhibition of glutamate release. Therefore, it can be deemed to have neuroprotective capacity upon glutamate toxicity (Cervia et al., 2008). When NMDA receptors are activated in cortical and striatal cultures, SST is released (Forloni et al., 1997). Furthermore, NMDA mediated toxicity elevates SST mRNA expression. It has the ability to decrease NMDA function which could lower NO formation (Kumar, 2008). SST plays a tenet role in survival of SST, NPY, nNOS neurons during excitotoxicity, since ablation of SST enhances cell death (Kumar, 2008).

Further relevance to the current research, apoptosis peaks at 24 hours post METH and DA terminal marker for degeneration peaks at 3 days post METH (Zhu et al., 2005). This may indicate that SP is mediating its connection to DA neurons via an indirect pathway (perhaps by coupling with the corticostriatal pathway) even though cell loss in the striatum is mediated by localized NK-1R. Another way METH can induce NO production is via the microglia and astrocytes which both express NK-1R (Rasley et al., 2002). Perhaps, there are other neuromodulators that can provide neuroprotection to striatal neurons upon METH toxicity. Recently, peripheral hormones have been

considered to have neuroprotective capacity. Understanding the molecular mechanism of how these peripheral hormones provide neuroprotection can help researchers find therapeutic targets.

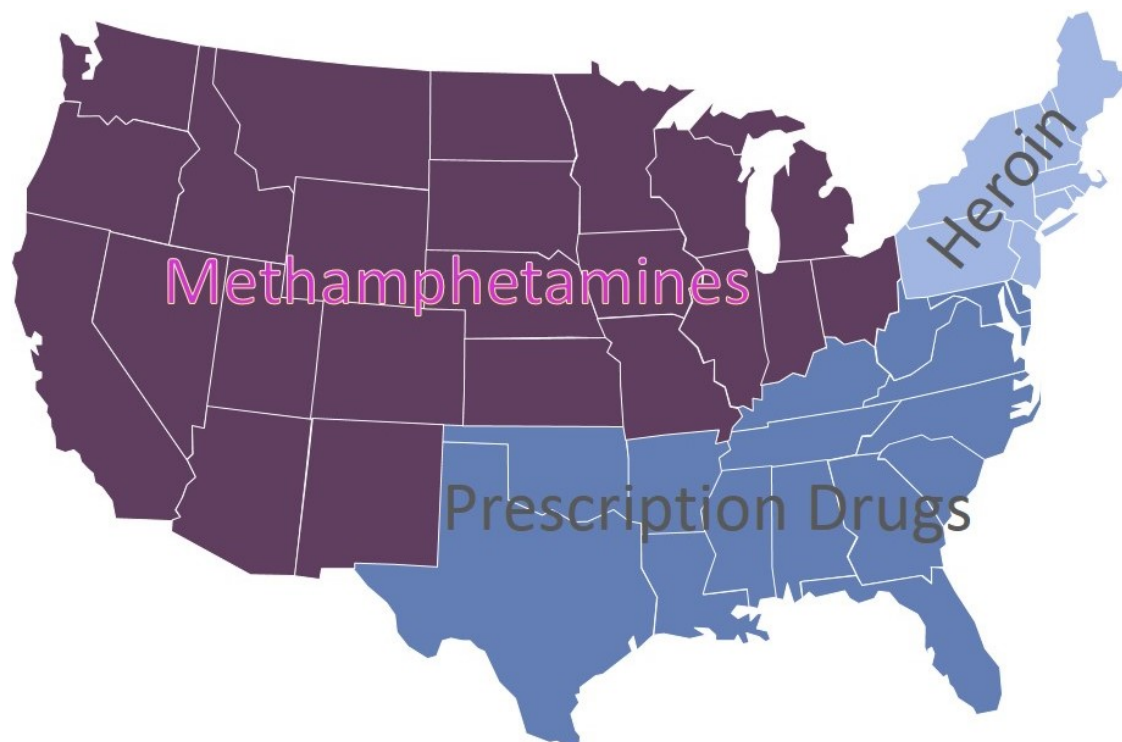


Figure 1 – 1: Widespread distribution of Methamphetamine in the USA. Over 1.2 million Americans reported using METH (US Dept. of Health and Human Services, 2012). Methamphetamine use occurs across the country in both urban and rural areas. Distribution of methamphetamine is widespread in Pacific, Southwest and West. Currently, trafficking has spread across central regions, Great Lakes, Southeast regions and limited but rising spread in the Northeast and Mid-Atlantic regions. It is expanding eastward, with most of the METH, 70-90%, in the U.S. imported from Mexico (NDIC, 2011)

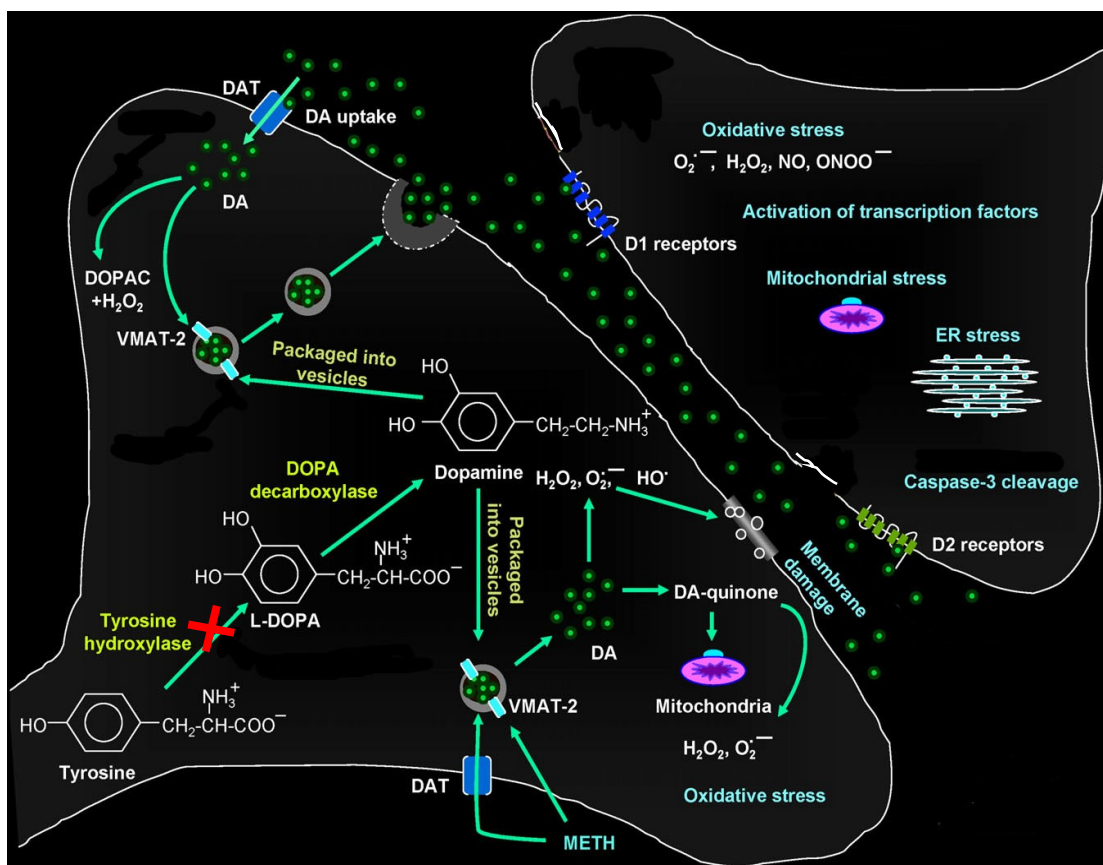


Figure 1 – 2: Molecular events during METH-induced DA terminal damage and apoptosis in the striatum (adapted from Krasnova and Cadet, 2009 with modification): The figure summarizes the role of DA, oxidative stress, and other mechanisms in METH toxicity. Within the neuron, METH enters via the terminal DAT and passive diffusion. It then enters into the vesicles through VMAT-2 and causes excessive DA release. In the cytoplasm, DA auto-oxidizes to form DA quinones, the generating of superoxide radicals and hydrogen peroxides. Subsequent formation of hydroxyl radicals through interactions of superoxides and hydrogenperoxide leads to oxidative stress, mitochondrial dysfunctions and peroxidative damage to pre-synaptic membranes. Also, with continuous excessive release, TH (the rate limiting enzyme of DA production) is exhausted leading to a significant reduction of TH and DA content. Finally, the toxic effects of released DA might occur through activation of DA receptors because interactions of DA with D1 receptors on post-synaptic membrane cause activation of transcription factors and upregulation of death cascades (active-caspase 3) in post-synaptic neurons resulting in apoptosis.

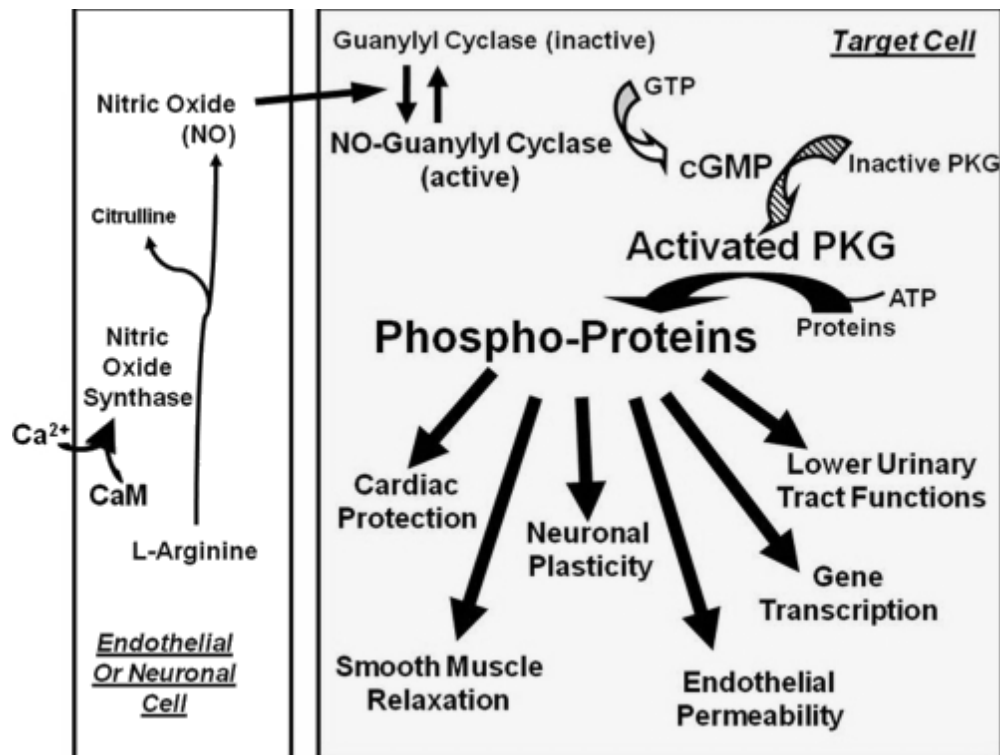


Figure 1 – 3: Nitric oxide signaling through cGMP (Adapted from Francis and Corbin, 2005) NO is synthesized from L-arginine by NO synthases located in neuronal. Calcium that enters the cell form complexes with calmodulin and activates NOS. The NO produced then diffuses through the intercellular space and crosses the cell membrane of a nearby target cell. Then NO binds to and activates guanylyl cyclase, which increases synthesis of cGMP from GTP and results in activation of PKG phosphotransferase activity. These processes initiate a cascade of reactions that are amplified at each step as shown by the arrows. In the current study, we measured the increase of NOS and cGMP after METH administration followed by leptin treatment.

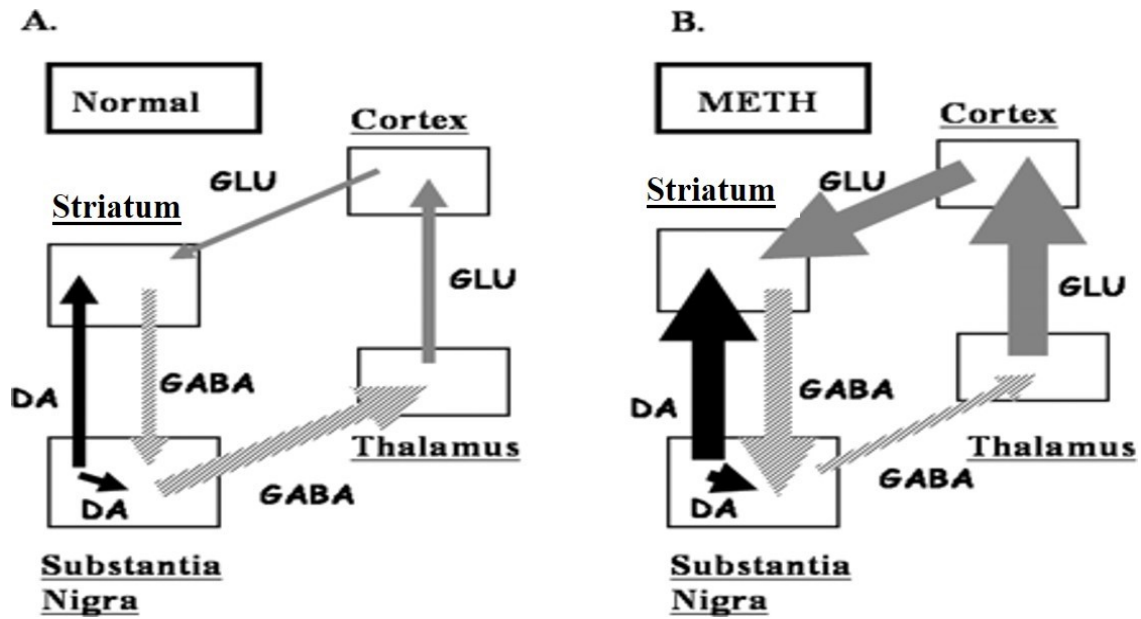


Figure 1 – 4: Glutamate transmission affected by DA overflow in METH toxicity (Adapted from Mark et al., 2004): (A) under normal condition: basal activity of SN regulates both da and glutamate release in striatum. (B) Under METH: DA release in striatum increases GABA release in SN, which decrease nigrothalamic activity leading to disinhibition thalamocorticol activity and subsequent increased release of corticostriatal glutamate. Arrow thickness indicates degree of activity.

CHAPTER 2

ROLE OF LEPTIN IN NEUROPROTECTION

2.1 History and Pharmacology of Leptin and Leptin Receptor Activity

Under the idea of identifying neuroprotective agents that can inhibit degeneration, leptin may be a candidate. Leptin is a 16 kDa endogenous protein hormone that is peripherally synthesized mainly by white adipose tissue and secreted into the bloodstream (Maffei et al., 1995; Zhang et al., 1994). In the brain, it is mediated by the leptin receptors, which helps it to cross the blood-brain barrier. Leptin was discovered when scientists saw a random recessive homozygous mutation in the mouse obese (*ob*) gene (Coleman, 2010). The mutation produced the *ob/ob* mice that weighed 300% more than normal mice, and a similar mutation is present in morbidly obese humans (Coleman, 1973; 2010). Researchers encoded the *ob* gene and found a hormone they named leptin (from the Greek word “leptos” meaning “thin”). The mutations caused these mice to be obese, completely deficient of the *ob* gene product, leptin, and these mice do not synthesize leptin (Zhang et al., 1994). But when leptin was administered to *ob/ob* mice they lost weight significantly, which indicates that leptin receptors are encoded by a different gene. The diabetic (*db*) gene was also identified around the same time and mice deficient of this gene (*db/db*) also do not synthesize leptin. But when leptin was administered peripherally it had no effect in these mice. Therefore, researchers concluded that *db* gene must encode for leptin receptors and the *db/db* mice lack functional leptin receptors which are required for leptin to produce its effects (Zhang et al., 1994; Gajiwala et al., 1995; Lee et al., 1996). See Figure 2 – 1.

Since all fat cells are able to secrete leptin, its circulation is proportional to the amount of fat mass (Maffei, et al., 1995) (See Figure 2 – 1). Therefore, obese individuals have higher levels of leptin than lean individuals and levels decrease with weight loss (Maffei et al., 1995). Leptin-induced weight loss is a result of fat mass reduction without changes in lean body mass. It is important to note that a single meal cannot alter leptin levels and administering leptin will not simply inhibit meal consumption (Maffei et al., 1995). Leptin behaves as a modulator of energy expenditure by maintaining a set-point for satiety rather than participating in the short-term regulation of food intake (Spiegelman and Flier, 1996; Myers et al., 2009; Berthoud, 2007). Prolonged fasting can decrease blood leptin levels (Boden et al., 1996). The hormone is released into the bloodstream rhythmically, entrained by meal patterns, but its levels are typically high in the morning and low in the afternoon (Licinio et al., 1997). There are slight sex differences in leptin concentration, as women tend to have greater leptin concentration than men (Mantzoros and Moschos, 1998).

As briefly described above, leptin causes its effect via its receptors, ObR, coded by the *db* gene (Tartaglia et al., 1997; Lee et al., 1996). The general structure of the leptin receptor is similar to cytokine receptors (Tartaglia et al., 1997). So far, six leptin receptors have been found: ObRa, ObRb, ObRc, ObRd, ObRe, and ObRf. All of these receptors have homologous extracellular domains and unique cytoplasmic domains varying in sequence and length (Lee et al., 1996; Tartaglia et al., 1997). ObRa and ObRc are short form, involved in transporting leptin across the blood-brain barrier (Bjorbaek, et al., 1998; Hileman, et al., 2002). The long form, ObRb, is ubiquitously expressed in the CNS and is mainly involved in leptin signaling in the brain (Elmqvist et al., 1998; Fei et al., 1997; Gao et al., 2008). The presence of leptin in the CNS causes it to play a critical role in controlling its actions peripherally. Studies found a strong association between plasma leptin and cerebrospinal fluid DA levels (Hagan et al., 1999). Glial cells, astrocytes and microglia, express the long and short form of the leptin receptors (Hosoi et al., 2000). In mice and rats, ObR mRNA has been detected in the cortex,

hypothalamus, hippocampus, brain stem, cerebellum, amygdala, and substantia nigra (Hommel et al., 2006; Figlewicz et al., 2006; Fulton et al., 2006; Grill et al., 2006; Leshan et al., 2009).

Though leptin receives most of its attention from studies on its neuroendocrine targets in the satiety centers of the hypothalamus, as it is very important for energy homeostasis, it also regulates the incentive value of food and rewards, suppresses depression and anxiety-like behaviors (Fulton et al., 2000; Lu et al., 2006; Figlewicz et al., 2006). Recently, several studies looked at leptin's wider coverage in the CNS. When leptin was administered peripherally, it activated neurons in various brain areas. ObRb-projecting neurons are expressed in other brain regions including the ventral striatum, amygdala, cerebellum, hippocampus, brain stem, and substantia nigra (Harvey, 2007; Figlewicz et al., 2003). However, the precise molecular pathway underlying the direct effects of leptin in these regions is mostly unknown.

In the hypothalamus, ObRb colocalizes with NPY and Pro-opiomelanocortin (POMC) and STAT3 (see description below). When leptin levels are decreased, expression of orexigenic neuropeptides agouti-related peptide (AgRP) and neuropeptide Y (NPY) is increased, while the expression of anorexigenic neuropeptides POMC and cocaine-and amphetamine-regulated transcript (CART) is decreased (Elmquist et al., 2005; Morton et al., 2006; Berthoud, 2007; Gao and Horvath, 2007). As discussed previously, VTA neurons send projections to limbic areas (NAc, and amygdala). Leptin was shown to modulate the DA-dependent food and drug rewards. In addition, VTA neurons express ObRb and regulate lateral hypothalamic area (LHA) neurons expressing ObRb (Fulton et al., 2000; Figlewicz et al., 2006; Hommel et al., 2006; Leininger et al., 2009). It remains to be understood how leptin can influence the mesolimbic DA system. So far, there is a lack of information about the direct projections from ObRb neurons into and within the mesolimbic DA system.

2.2 Role of Leptin in Dopaminergic Pathways

Addictive behaviors such as drug or alcohol abuse are driven by brain circuits that results in neuroadaptation generated by learned habits and powerful rewarding reinforcements. Among the four major dopamine pathways in the brain, mesolimbic and nigrostriatal pathways are of interest as they play a critical role in the reinforcing and rewarding properties of substance abuse. Mesolimbic dopaminergic neuron are found in the VTA and project to the NAc, whereas nigrostriatal neurons arise in the SN and project to the striatum. Leptin may control the mesolimbic DA system by its ObRb expressing neurons and their projections to the NAc. ObRb containing cells are present in the VTA as shown by leptin-induced STAT3 phosphorylation, pSTAT3 (Fulton et al., 2006; Hommel et al., 2006). A majority of the dopaminergic neurons in the VTA and SN are also ObRb expressing neurons (Elmqvist et al., 1998). We and other labs have also found leptin receptors to be expressed in the striatum. However, the signaling pathway critical for its role in leptin-mediated neuroprotection in the striatum still remain to be elucidated. Finally, leptin crosses the blood-brain barrier, which further demonstrates that it acts in several pathways outside of the hypothalamus (Banks et al., 1996).

2.3 Leptin Receptor Signaling

In general, leptin resembles the Janus kinase (JAK) family of tyrosine kinase and its receptors are similar to the class-1 cytokine receptors (Madej, et al., 1995). The signal transduction pathway consists of leptin binding to its receptor, which contains an intracellular domain and autophosphorylates the JAK-binding site. The kinase then phosphorylates and activates the signal transducer and activator of transcription (STAT)-binding site. In rodents and humans, there are four JAK family members. JAK1, JAK2, JAK3 and TYK2. JAK2 is the main player in survival and mediate its signals through a variety of cytokine receptors pathways.

Downstream leptin signaling requires leptin to bind to ObRb receptor, activating tyrosine kinase JAK-2 (activated by autophosphorylation), which phosphorylates the three tyrosine residues,

Tyr985, Tyr1077, Tyr1138, on the intracellular domain of the receptor. The phosphorylated tyrosine residues promote the recruitment of signaling proteins with their own specialized phosphotyrosine-binding domains, meaning each tyrosine phosphorylation site has specific downstream signaling proteins according to their amino acid chain. Tyr985 recruits two different proteins: Src Homology-domain containing phosphatase-2 (SHP2) and suppressor of cytokine signaling-3 (SOCS-3). SHP-2 binds to phosphorylated Tyr985 and mediates the activation of ERK (extracellular signal-regulated kinases). SOCS-3 attenuates cytokine signaling and is known to mediate feedback inhibition of ObRb signaling. Tyr985 signaling is important for the attenuation of leptin action via ObRb signaling. Phosphorylation of Tyr1077 activates STAT5. Though, Tyr1077 mediates most of the ObRb dependent STAT5, Tyr1138 also mediate STAT5, but, the exact mechanisms of how STAT5 regulates gene expression is not clearly understood. Phosphorylated Tyr1138 also activates STAT3. STAT3 activation leads to translocation of STAT3 to the nucleus where it regulates transcription of SOCS3, which acts as a feedback inhibitor and negatively regulate ObRb signaling (Schindler and Darnell, 1995) (See Figure 2 – 2). JAK-STAT3 signaling leads to an upregulation of antioxidant enzyme, manganese superoxide dismutase (MnSOD) and anti-apoptotic protein, B-cell lymphoma-extra-large (Bcl-xL). MnSOD functions is to protect mitochondrial damage from superoxide, it is the cell's primary defense against free radical mediated damage. Bcl-xL is located at the outer mitochondrial membrane and regulates mitochondrial membrane channel.

Leptin can also activate PI3 kinase-Akt pathway and mitogen-activated protein (MAP) kinase pathways (Harvey et al., 2000; Najib et al., 2002). MAP-kinase pathways promote neuronal survival but little is known about leptin treatment's effect on the level of phosphorylated MAP kinases (ERK1/2) in cultured hippocampal neurons. The STAT3, STAT5, ERK activation is mediated by the phosphorylation sites of ObRb, but there are other downstream signaling events that also take place. The activation of phosphoinositide-3 kinase (PI3-K), the regulation of mechanistic target of rapamycin

(mTOR) and the inhibition of the AMP-activated kinase (AMPK) in leptin signaling are all minimally understood.

Neurotropic factors that activate JAK/STAT or PI3 kinase/Akt pathways can promote survival (Cheng et al., 2003). Neurotropic conditions in hippocampal cultures were shown by inducing oxidative stress with exposure to Fe^{2+} which induces hydroxyl radical production and membrane lipid peroxidation. Also, overactivation of glutamate receptors in the hippocampus leads to excessive Ca^{2+} influx through the glutamate receptors and voltage-dependent Ca^{2+} channels (Mattson, 2003). However, when cultures were pretreated with leptin the neurons survived under both neurotropic conditions (Guo et al., 2007). Oxidative stress and excitotoxicity can cause mitochondrial alterations leading to apoptosis. Therefore, researchers checked if leptin can attenuate mitochondrial insults by measuring mitochondrial ROS levels. They found that leptin treatment stabilized mitochondrial function and protected from apoptotic death in hippocampal slices (Guo et al., 2007). Also, when hippocampal neurons were treated with a specific JAK2 inhibitor, AG490, STAT3 phosphorylation was attenuated. AG490 can block the neuroprotective effects of leptin when neurons are exposed to toxic levels of glutamate. Therefore, JAK2 and STAT3 activation is necessary for leptin-mediated cell survival signaling of hippocampal neurons (Guo et al., 2007). Next, several studies demonstrated that STAT3 induces expression of Bcl-xL and MnSOD (Terui et al., 2004). Treating tissue cultures with JAK2 or STAT3 blocker also blocked the ability of leptin to increase the levels of Mn-SOD and Bcl-xL (Guo et al., 2007). Thus, we know that leptin induces the expression of Bcl-xL and Mn-SOD. Neurons treated with PI3 kinase inhibitor (LY294002) blocks leptin's ability to prevent glutamate-induced mitochondrial damage and ROS production. Therefore, PI3K-Akt pathway is likely to be involved in mitochondrial stabilization by leptin. Leptin showed neuroprotective ability when delivered (by intraventricular cannula in mice) to the hippocampal CA1 and CA3 regions prior to inducing seizures. Leptin modulates the excitability of hippocampal neurons via ObRb by activating K^+ channels (Shanley et al., 2002). It inhibits hippocampal neurons via PI3-Kinase-driven activation of big K^+ (BK channels) which are

calcium-activated potassium channels. At rest, leptin inhibit hippocampal neurons via BK channel activation (Shanley et al., 2001). BK channels do not normally contribute to the resting excitability of neurons but act as modulators if Ca^{2+} rises. Leptin receptor activation brings BK voltage to a physiological normal range without the increase of Ca^{2+} (Shanley et al., 2002). The neuroprotective mechanism of leptin may include $\text{JAK2} \rightarrow \text{ANTI-APOPTOSIS}$; $\text{TYR985} \Rightarrow \text{SHP2} \rightarrow \text{ANTI-APOPTOSIS}$; $\text{TYR985} \Rightarrow \text{SOCS3} \rightarrow \text{SIGNAL ATTENUATION}$; and $\text{TYR1077} \Rightarrow \text{STAT5} \rightarrow \text{ANTI-APOPTOSIS}$. See Figure 2 – 2.

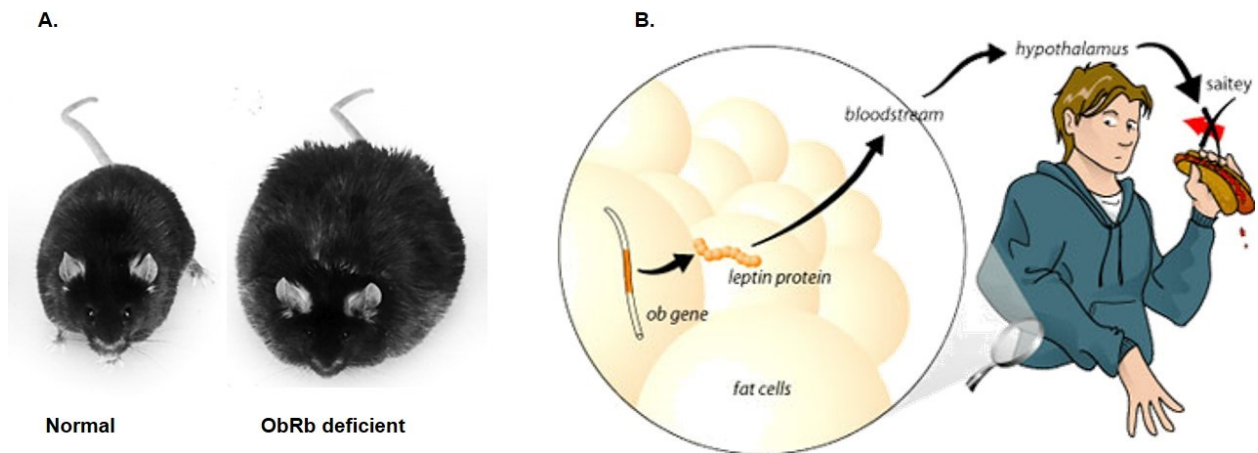


Figure 2 – 1: Leptin mouse and feedback loop. (A) A comparison of a mouse unable to produce leptin, resulting in obesity (right), and a normal mouse (left). (B) The ob gene in fat cells encodes the leptin protein, which triggers the hypothalamus to suppress appetite (adapted from Liu, 2004).

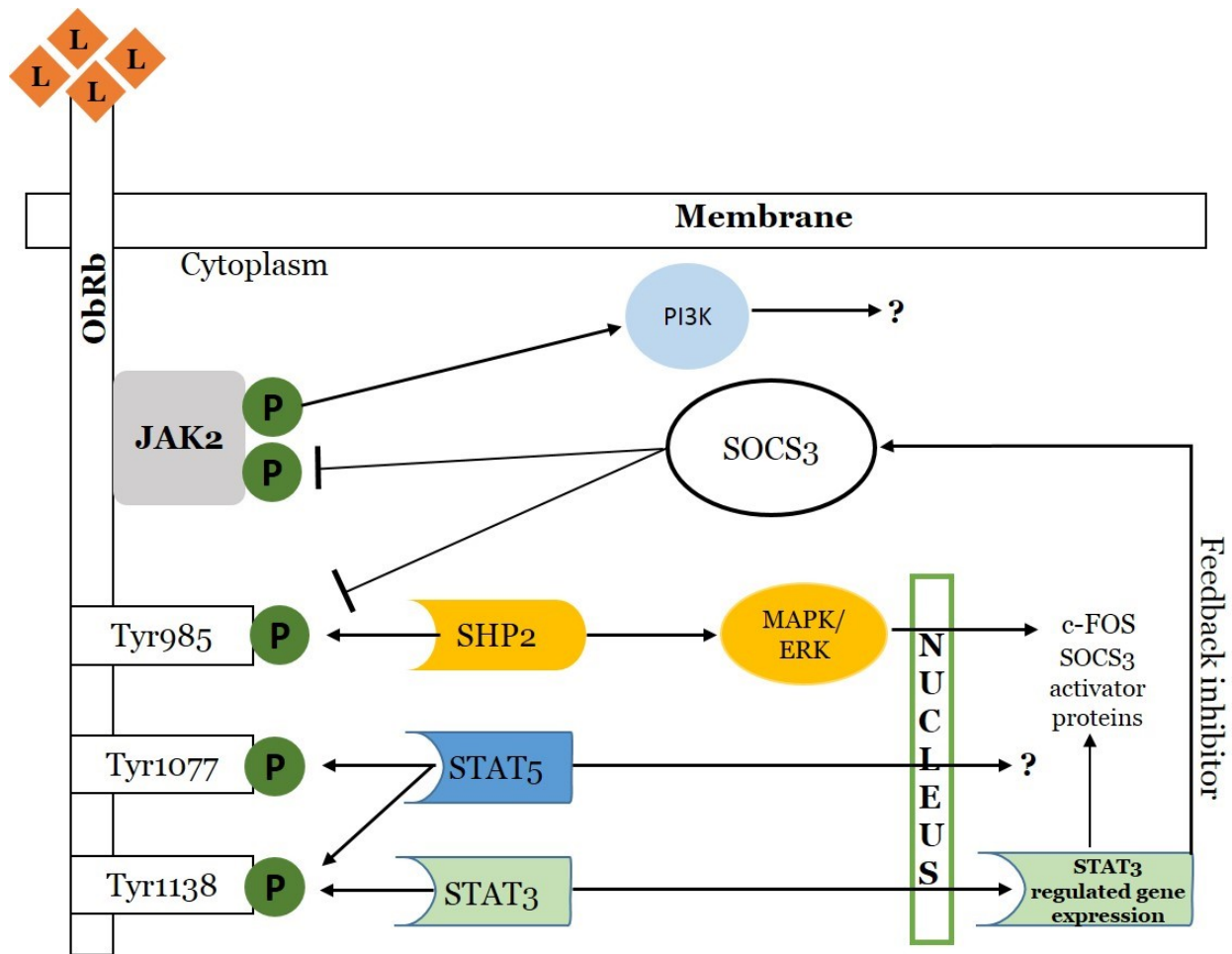


Figure 2 – 2 Leptin receptor signaling pathways: leptin binds to ObRb and activates JAK2 resulting in autophosphorylation. JAK2 phosphorylates ObRb on Tyr 985/1077/1138, which provides docking sites for signaling proteins. Phospho Tyr985 allows binding of SHP2 and mediates activation of MAPK pathway. It also allows binding of SOCS3 and inhibit leptin signaling in a negative feedback manner. Phospho Tyr1077/1138 recruits STAT5, but little is known about its impact on gene expression. Phospho Tyr1138 recruit STAT3. STAT3 proteins translocate to the nucleus to induce gene expression such as c-Fos, activator proteins, SOCS3. SOCS3 negatively regulate signal transduction by binding to phosphorylated tyrosines where they inhibit the binding of STAT proteins and SHP-2. Autophosphorylated Jak2 can activate PI3K pathway. Leptin (L), Leptin receptor (ObRb), tyrosine (Tyr), Janus kinase (JAK2), Src homology phosphatase 2 (SHP2), signal transducers and activators of transcription (STAT), suppressor of cytokine signaling (SOCS3), Phosphoinositide Kinase-3 (PI3K), extracellular signal regulated kinase (ERK).

JAK2 → ANTI-APOPTOSIS; TYR985 ⇒ SHP2 → ANTI-APOPTOSIS;

TYR985 ⇒ SOCS3 → SIGNAL ATTENUATION; TYR1077 ⇒ STAT5 → ANTI-APOPTOSIS AND

TYR1138 ⇒ STAT3 → HYPERPROLIFERATION, CYTOKINES AND SOCS3

CHAPTER 3

RESEARCH DESIGN

METH-induced neurological damage colocalizes with degeneration that occurs in various neurodegenerative diseases such as Parkinson's disease and Huntington's disease. Therefore, understanding the mechanism of METH-induced neurodegeneration will provide an avenue towards identifying effective therapeutic targets for treatments of METH abuse and neurological disorders. Several labs including ours have identified the neuroprotective capacity of endogenous peptides, such as somatostatin (SST) and neuropeptide Y (NPY), upon METH-induced toxicity in striatal neurons. Similarly, leptin is an important peripheral hormone produced mainly by adipose tissue and is produced in proportion to fat stores, which circulates in the plasma, and is found ubiquitously in the CNS. Plasma leptin communicates energy stores in the periphery to the CNS. Most of the effects of leptin are attributed to the effects in the CNS. Though leptin is primarily known for its regulation of food intake and energy homeostasis governed by its receptors on hypothalamic neurons, it has been shown to serve other functions that deviate from its traditional role. We know that METH-induced striatal injury is a multimodal function from DA overflow, glutamate signaling, free radicals formation and oxidative stress and published work from our lab and others provide some of the basis of the relationship between METH, DA, glutamate and oxidative stress. These identified pathways of how METH affects the striatum helped us to demonstrate the ways leptin can play a role. We hypothesized leptin will attenuate the METH-induced striatal neural injury.

The specific aims outlined here are intended to measure and test the overall hypothesis that METH-induced striatal neural injury is attenuated by leptin. We know that METH-induced striatal injury is a multimodal function from DA overflow, glutamate signaling, free radicals formation and oxidative stress and published work from our and other labs provides some of the basis of the relationship between METH, DA, and oxidative stress. These identified pathways of how METH affects the striatum will help us to demonstrate the ways leptin can play a role. We will demonstrate how leptin attenuates METH-induced striatal injury and mediates striatal neuroprotection. The specific aims are:

3.1 Specific Aim 1 and Relevance: Does Leptin Attenuate the METH- induced Striatal Neural Injury?

To test the hypothesis that:

- a) Leptin will attenuate the apoptosis and dopamine terminal toxicity induced by METH in the striatum.
- b) Leptin treatment prevents the METH-induced striatal overactivation of astrocytes and microglia.
- c) Leptin treatment prevents METH-induced hyperthermia and weight loss.
- d) Leptin protects by inhibiting the METH-induced production of striatal nitric oxide: role of neuronal nitric oxide synthases.
- e) Leptin will prevent the overproduction of cGMP induced by METH in the striatum.

3.2 Specific Aim 2 and Relevance: Does Leptin Mediate Striatal Neuroprotection by Modulating Glutamate Transmission?

To test the hypothesis that:

- a) Leptin will attenuate the NMDA-induced striatal apoptosis and dopamine terminal toxicity.
- b) Leptin treatment attenuates NMDA-induced formation of nitric oxide.
- c) NMDA-induced overactivation of astrocytes and microglia will be attenuated by leptin.

CHAPTER 4

METHODS AND MATERIALS

4.1 Animal and Systemic Drug Administration

All experimental animal use procedures were in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*, Hunter College of the City University of New York's Institutional Animal Care and Use Committee and Animal Welfare regulations. Experimental Male of imprinting control regions (ICR) mice were purchased from Taconic laboratories (Germantown, NY, USA) at 10 weeks of age. We used this mouse strain due to previous published work from the lab established baseline and significant results when measuring the various markers. Mice were housed individually in translucent propylene cages (29 x 19 x 12.5 cm) and habituated to a 12 hour light – dark (LD) cycle (light, 300 lux) for 2 weeks prior to commencement of intraperitoneal (IP) drug administration. The animal rooms were maintained at $21 \pm 1^{\circ}\text{C}$ and were provided with food and water *ad libitum*. For animal used in Western blot studies, received a change of bedding and wet food every 24 hours for 3 days post-drug administration.

(+)-Methamphetamine hydrochloride (Sigma, St. Louis, MO) was dissolved in 10mM phosphate-buffered saline (PBS); pH 7.4. All animals received one IP injection of saline or one bolus METH (30 mg/kg of body weight) in a volume of 200 μl . In addition to drug or saline treatment, leptin from mouse (Sigma, St. Louis, MO) (0.25, 0.5, 1, 2, or 3 mg/kg of body weight) was injected to animals of different groups to determine optimal dose. Afterwards, all leptin injections were at a dose of 1 mg/kg. Control animals receive equivalent volumes of saline. Animals were sacrificed after 8, 24, or

72 hours post drug administration. For intrastriatal infusion study, NMDA (20 nM) was also dissolved in aCSF and injected into the striatum. Animals also received a saline or leptin injection 30 minutes prior to infusion. Animals for Western blot analysis of TH levels were sacrificed 72 h after treatment by decapitation. Brains were dissected, frozen on dry ice, and stored in -80 °C until use. Animals for cGMP and activate caspase-3 were sacrificed 8 hours post-treatment. For 3-NT, NOS1, NeuN, immunofluorescence or TUNEL, animals were sacrificed 24 hour post-treatment and animals for GFAP and Iba1 immunofluorescence were sacrificed 72 hours post-treatment via intracardiac perfusion.

Perfusion

Each animal received IP injection to be deeply anesthetized for sacrifice and tissue collection (8, 24 or 72 hours after METH injection), with 1:3 mixture of ketamine/acepromazine (100 mg/kg of body weight). Animals were perfused intracardially with 30 mL of phosphate buffer saline (PBS) followed by 30 mL of 4% paraformaldehyde in in PBS. Brains were dissected out and immediately post-fixed for 24 hours in 4% paraformaldehyde at 4°C and to enhance the penetration of the immunoreagents, the brains were equilibrated in a cryoprotectant solution (20% sucrose in PBS solution) in at 4°C for 48 hours. The brains were frozen at -80°C until ready to be used. Coronal sections 25 µm in thickness were collected using a cryostat at -20°C and stored in antifreezing solution (30% glycerin solution in ethylene glycol) at -20°C until used in immunohistochemistry assays.

Immunohistochemistry

For all immunohistochemical studies perfused brains were sliced using a cryostat at 25 µm serial coronal brain sections were collected between bregma 0.38 mm ± 0.1 mm. Sections were used either free-floating or mounted on glass slides prior to staining methods. For each immunohistochemical assay, we used one entire well (6 – 7 sections) per animal.

4.2 Intrastriatal Infusion of NMDA and Leptin Injections

NMDA infusion protocol was adapted from Afanador et al., 2013. Thirty minutes prior to intrastriatal infusions, mice received one IP injection of leptin (1 mg/kg; Sigma, St. Louis, MO) or saline. The animals were anesthetized with inhaled isoflurane (2.5% for induction, 2.0% for maintenance). Their heads were immobilized in a stereotaxic frame (Model 5000; David Kopf Instruments, Tujunga, CA) and a burr hole was drilled into the skull at the following coordinates: +0.5 mm rostral-caudal; +/- 2.0 mm medial-lateral from bregma; -2.5 mm dorsal-ventral from dura (Franklin and Paxinos, 1997). A 2 μ L microinjection needle (25 gauge, Hamilton, Reno, NV) was lowered into the striatum and allowed to remain in position for 5 minutes. NMDA 1.0 μ L (20 nM, Sigma, St. Louis, MO), or aCSF were injected into the striatum using the quintessential stereotaxic injector (Stoelting, Wheat Lane, IL) at a rate of 0.1 μ L/minute and the needle remained in place for an additional 5 minutes before its removal. NMDA was dissolved in artificial cerebrospinal fluid (aCSF). The wound was closed with VetBond (n-butyl cyanoacrylate, 3M) tissue adhesive and the animal was allowed to recover.

4.3 TUNEL immunohistofluorescence

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) histochemistry methods adapted from (Zhu et al., 2005) with minor modifications. Pre-mounted brain sections were washed in phosphate-buffered saline, (PBS) at pH 7.4 and then immersed in 0.4% Triton X-100 in PBS for 30 minutes at 70 °C. Sections were then washed in PBS and incubated in TUNEL reactions mix (Roche Applied Science, Indianapolis, IN) according to protocol in the manual, in a humidified chamber (37 °C) for 2 hours. Washed in PBS and coverslip with Vectashield + DAPI mounting medium (Vector Laboratories, Burlingame, CA).

4.4 NeuN, GFAP, Iba-1, NOS1, cyclicGMP, active caspase-3, and 3-Nitrotyrosine (3-NT)

Immunohistochemistry

Free-floating sections were washed in PBS with 0.3% Triton X-100 (PBS-TX) and blocked for non-specific binding using 10 % Normal Donkey Serum (NDS) in PBS-TX for antibodies: cGMP, active caspase-3 or 5% Normal Goat Serum (NGS) in PBS-TX for antibodies: NeuN, Iba-1 or Mouse- on-Mouse IgG (BMK-2202, Vector Laboratories, Burlingame, CA) for antibodies: 3-NT, NOS1, NOS2, GFAP at room temperature (RT) for 1 hour. Sections were then incubated in primary antibody in 5% NDS or NGS in 0.2% PBS-TX or M.O.M diluents buffer (80 μ L/mL in PBS-TX for 15min). All primary antibody incubations were at 4°C overnight. Sections incubated with: mouse anti-NeuN (1:50; Chemicon, Temecula, CA) a monoclonal anti-mouse antibody against 3-NT (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); mouse anti-NOS1 (1:250; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); rabbit anti-cGMP (1:500; Millipore Billerica, CA); goat anti-active caspase-3 (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); rabbit anti-Iba-1 (1:1000; Wako Pure Chemical Industries, Japan) or Cy3-conjugated mouse anti-GFAP (1:30; Sigma, St. Louis, MO). Sections were then rinsed in PBS three times (10 minutes each) and incubated in secondary antibody at RT for 1 hour; for NeuN; Cy3-conjugated goat anti-mouse (1:100; Chemicon, Temecula, CA); for 3-NT, NOS1: donkey anti-mouse conjugated to Cy3 (Chemicon, Temecula, CA); for cGMP: donkey anti-rabbit cy3 (1:500; Chemicon, Temecula, CA); for active caspase-3: donkey anti-goat FITC (1:500; Chemicon, Temecula, CA) and for Iba-1: Alexa Fluoro® 488 Donkey anti-rabbit (1:1000; Invitrogen, Carlsbad, CA). All immunohistochemical sections were washed three times with PBS (5 min each) and mounted onto super frost glass slides, sealed and coverslipped with Vectashield hard set mounting medium for fluorescence with DAPI (Vector Laboratories, Burlingame CA).

4.5 Tyrosine Hydroxylase and Leptin Receptor Western Blot

Using a brain blocker on ice, a 2 mm thick coronal section of the striatum was removed. The samples were homogenized in approximately 150 μ L of lysis buffer (40 mM Tris-HCL, 274 mM NaCL, 2.0 mM EGTA, 20% glycerol, 1 mM Na₃VO₄, 1 mM PMSF, 1 mM β -glycerophosphate, 2.5 Na₄P₂O₇, 50 mM NaF, 1% NP40, and protease inhibitor cocktail: 1.0 mM AEBSF, 0.8 μ M aprotinin, 0.02 mM leupeptin, 0.04 mM bestatin, 0.015 mM pepstatin A, and 0.014 mM E-64) with a QSonica Sonicator 3000 cup horn at 7 cycles of 30 seconds of sonication and 60 seconds of cooling. The mixture was first centrifuged at 4°C at 3000 rpm for 5 minutes and then the supernatant was centrifuged at 5000 rpm for 5 minutes. The supernatant was removed once more and centrifuged for one final cycle of 6000 rpm for 10 minutes. The protein content was assayed by the Bradford method (Bio-Rad, Hercules, CA). Ten μ g of protein were loaded on a 10% Tris-HCL (Invitrogen, Carlsbad, CA) SDS-PAGE and transferred to an iBlot stack membrane (Invitrogen, Carlsbad, CA). After blocking nonspecific binding using Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1 hour at room temperature, membranes were probed overnight with polyclonal rabbit anti-TH (1:5000, Millipore, Temecula, CA) antibody and monoclonal mouse anti- β -actin antibody (1:20,000, Sigma, St. Louis, MO) in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) with 0.2% Tween 20 at 4°C. The next day the membranes were rinsed with 0.1% Tween 20 PBS followed by 3 washes at 5 minutes each. They were then incubated in a mixture of Odyssey's IRDye® secondary antibodies donkey anti-rabbit 800CW (1:15,000) and donkey anti-mouse 680LT (1:30,000) within Odyssey's blocking buffer for 1 hour at room temperature. After an additional 3 washes at 5 minutes each with 0.1% Tween 20 PBS as well as a final 15-minute wash with PBS alone, the proteins bands were then detected via the Odyssey infrared imager. Bands were quantified using the Odyssey Imager analysis software and normalized against β -actin.

4.6 Quantification and Statistical Analysis

All images were taken with either Nikon Eclipse TE 200 inverted epifluorescence microscope and Molecular Devices imaging software Metafluor with FITC/TRITC filter used to capture fluorescent images with varying laser intensities (0.5 to 5 seconds) Or with a Leica SP2 confocal microscope and the corresponding Leica Lite LCS software system (Leica Microsystems, Heidelberg, Germany) using a 20x or 63x objective lens. FITC and Cy3 signals corresponds to single wavelength laser line 488 (green) and 588 (red) respectively. The striatum was divided into two regions and z-stack images from each region were taken in 6 to 8 animals for each primary antibody. The pinhole setting was less than 2 μ m and z-stacks of 10 μ m thick were recorded sequentially between frames in series. Images were analyzed using NIH ImageJ (Schneider and Rasband, 2012) analysis program. All tissues selected, from the NMDA infusion studies stained with the different antibodies, must have a visible needle tract to ensure that the effect observed is due to the injected solution. The region chosen is adjusted to each side of the needle tip but avoiding the visible needle damage. They were scanned once by individuals that were blind to the experimental conditions. Analysis of the NMDA infusion studies were done using the Leica confocal microscope (described above).

Statistical analysis of the data was conducted using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) software. The differences between groups means were determined utilizing one way ANOVA (mean \pm SEM) to detect statistically significant differences at the 95% confidence level when experimental groups were compared to controls. The analysis was followed by planned pairwise comparisons or *Post hoc* analysis using Tukey's post-hoc test to determine significance between experimental groups. All statistical analysis were conducted with a significance criterion value set at $p < 0.05$.

CHAPTER 5

ROLE OF LEPTIN IN METHAMPHETAMINE-INDUCED NEURAL INJURY

It is well established that METH is a highly addictive illicit psychostimulant that is neurotoxic and causes permanent brain damage. METH-induced neurological damage colocalizes with degeneration that occurs in various neurodegenerative diseases. Understanding the mechanisms of METH-induced neurodegeneration will provide an avenue towards identifying effective therapeutic targets for treatments of neurological disorders. Several labs including ours previously identified the neuroprotective capacity of endogenous peptides, such as SST and NPY (Yarosh and Angulo, 2012; Afanador et al., 2013). Similarly, leptin is a peripheral hormone that circulates in the plasma and found ubiquitously in the CNS. Under this idea of identifying neuroprotective agents that can inhibit degeneration, leptin may be a candidate as it mainly acts via its receptor, ObRb, found in the mesolimbic DA pathway, specifically in the VTA, SN, and NAc of the ventral striatum. The precise molecular pathway underlying the direct effects of leptin in these regions is mostly unknown. But studies report that leptin administration decreases the firing rate of dopaminergic neurons causing decreased DA release in the VTA. We have demonstrated that leptin attenuates striatal apoptosis in a dose dependent manner. Therefore, understanding the mechanism by which leptin can prevent or reverse toxicity of DA-terminals or apoptosis in the striatum will shed new light into the role of this endogenous peptide.

5.1 Attenuation of Striatal Apoptosis by Leptin

METH-induced apoptotic cells in the striatum were measured by TUNEL immunohistofluorescence, which is a late marker of apoptosis. We tested various doses of leptin (0.25, 0.5, 1, 2, and 3 mg/kg) alone and with METH (30 mg/kg) of body weight on the induction of TUNEL-positive nuclei in the striatum. We tested the hypothesis that leptin alone will not show leptin-induced apoptosis but leptin + METH will have a dose dependent attenuation of METH-induced apoptosis. Our lab has previously shown that a single high dose of METH (30 mg/kg) causes peak striatal apoptosis at 24 hours post-METH (Zhu et al., 2006). Therefore, the groups tested are: leptin alone of varying doses (0.25, 0.5, 1, 2, and 3 mg/kg), METH alone (30 mg/kg), leptin + METH, and Saline. Brain tissue was collected by perfusion and processed with TUNEL immunohistofluorescence. Variables that was assessed is the number of striatal neurons positive for this marker. Statistical analysis done using One-way ANOVA (95% confidence level) with planned comparisons to saline and METH treated animals. To determine the percentage of neurons undergoing METH-induced apoptosis, a reliable baseline of the total number of neurons in the entire striatum following Zhu et al., (2005) was established. Neuron-specific marker (NeuN) IHC staining was done on six serial sections of six animals and images were taken by Nikon Eclipse TE 200 inverted epifluorescence microscope. Neurons were counted manually. The average number of NeuN positive neurons for all sections were quantified and used to determine the percentage of TUNEL-positive neurons for the dose dependent study.

Treatment of leptin alone did not show any apoptotic cell death when labelled with TUNEL (Figure 5-1 (a, b)). Next we assessed the optimal dose of leptin necessary to attenuate METH-induced apoptosis. Induction of TUNEL-positive nuclei in the striatum was measured. Compared to METH alone treated animals that shows 25% apoptotic cell death in the striatum, when animals received co-administration of leptin + METH, the dose of 1 and 2 mg/kg leptin of body weight demonstrated a statistically significant attenuation of METH-induced apoptosis (Figure 5-2 (a,b)). Apoptotic death

reduced to 5 – 6% in the striatum. There was partial attenuation with lower dosage of leptin (0.25 and 0.5 mg/kg) but the lowest amount of leptin administration that caused the highest significant attenuation of METH-induced cell death was 1 mg/kg leptin. Therefore, this dose was used in future experiments.

Attenuation of METH-induced dopamine terminal toxicity by leptin was measured via TH levels assessed by Western blots. Since TH is the rate-limiting enzyme in DA biosynthesis (Fibiger and McGeer, 1971), the amount of TH protein in the striatum is a reliable measure of DA terminal viability, which was determined 72 hours post treatment following decapitation and dissections. Our lab has shown that TH depletion peaks at 72 hours post METH administration (Zhu et al., 2005). Leptin dose of 1 mg/kg showed significant reduction in apoptotic cell death as established from the previous experiment (Figure 5-2b). Henceforth this dose was used in all other experiments unless noted otherwise. Treatment with leptin prior to METH did not demonstrate protection of dopamine terminals. TH levels for the leptin + METH group remained almost equivalent to levels in the METH alone group. TH levels for the leptin alone group were comparable to baseline levels in saline group (Figure 5-3 (a, b)).

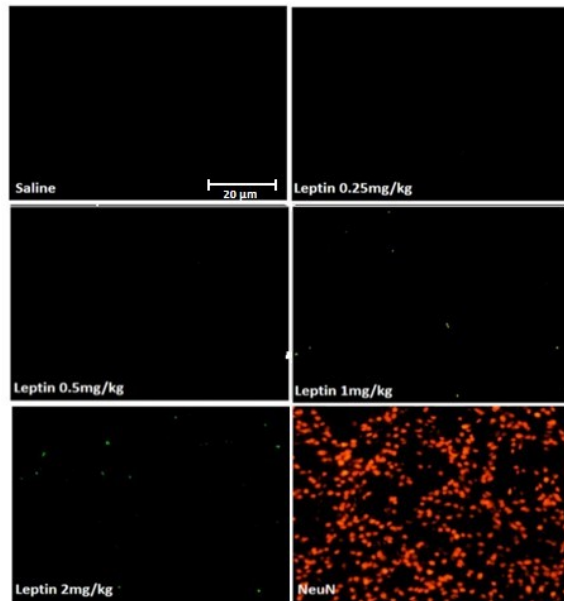
It has been well established that temperature plays a pivotal role in METH-induced neurotoxicity (Ali et al., 1994, Krasnova and Cadet, 2005). To evaluate the severity of apoptotic cell death that is independent of METH-induced hyperthermia, we assessed core body temperature post treatment for saline, leptin alone, leptin + METH and METH alone groups. To determine if the neuroprotective effects of leptin were independent of METH-induced hyperthermia, rectal body temperature was measured with a BAT-12 thermometer coupled to a RET-3 mouse rectal probe (Physitemp Instruments, Clifton, NJ) every 2 hours for up to 8 hours post treatment. Room temperature was maintained at 20 – 22°C. We found that leptin protects against METH-induced cell death without preventing hyperthermia as both the leptin + METH and METH group showed similar 2.9°C increase

from the average core body temperature of 36.6°C (Figure 5-4). Also, by the eight hour, temperature returned to normal baseline for all four groups.

METH use is well known to change appetite and induce weight loss but the interaction between hormones that regulate energy balance and satiety signals (leptin and ghrelin) and the use of club drugs is a poorly understood phenomenon. More than half of all drug addicts show significantly lower body weight. In both acute and chronic use, METH leads to the development of pharmacologically induced extreme weight loss similar to anorexia, which changes the body mass index (BMI) and eating habits (Salisbury and Wolgin, 1985; Cho et al., 2001). When rats were given IP injection of METH (2 mg/kg) there was a significant reduction of food intake (Ginawi et al., 2005). Also, researchers have attributed drug-induced anorexia with decreased blood-leptin levels (Santolaria et al., 2003). Leptin production in the body is proportionate to the amount of adipose tissue in the body. However weight loss as a result of METH use may be mediated by a different pathway that is involved in satiety signals rather than blood hormone levels. In a study done by Kobeissy et al., (2007) found no significant difference between serum leptin and ghrelin levels when rats were administered different doses of METH that ranged from 5 mg/kg to 40 mg/kg in the various time points tested 6, 12, 24 and 48 hours. In the current study, similar results were observed when mice were in the four groups were administered either METH (30 mg/kg) or leptin (1 mg/kg) or leptin + METH or saline, the leptin and saline group displayed no change in body weight over a four-day period. However, the leptin + METH and METH groups displayed slight weight loss which were not significant when compared to their weights before injection (Figure 5 – 5).

Several studies have shown that leptin mediates its activity via its receptors (Russo et al., 2004). Leptin receptors are expressed in many mouse brain areas (Zhang et al., 2007). Here, like other, labs we show both short and long isoforms of the leptin receptor were expressed in the striatum. Additionally, show that both leptin receptors are expressed in saline, leptin alone, leptin + METH and METH alone groups as assessed 24 hours post treatment (Figure 5 – 6).

(A)



(B)

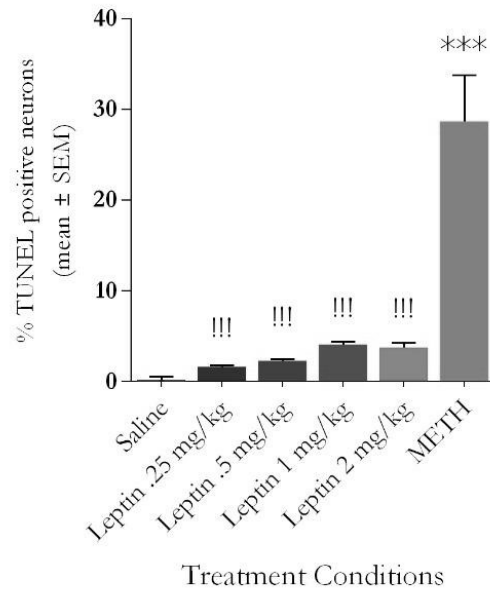
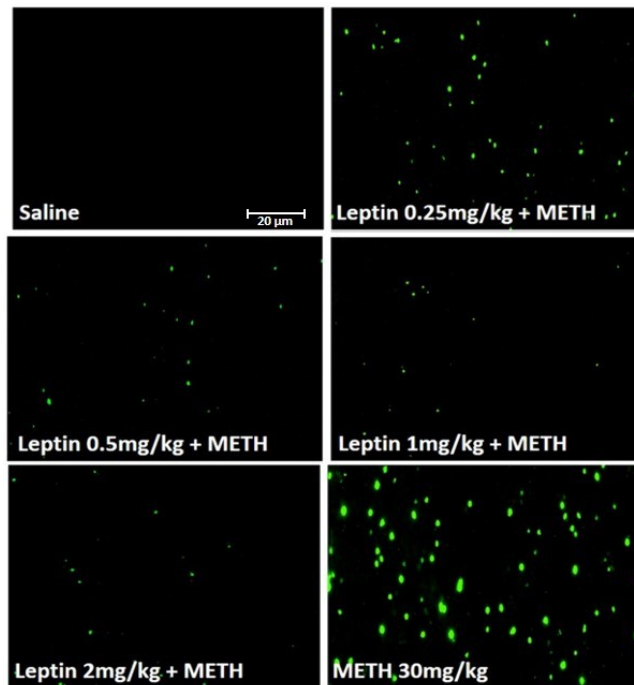


Figure 5 – 1: Leptin alone does not cause apoptosis. (A) Treatment with leptin alone (0.25, 0.5, 1, 2 mg/kg) of body weight does not induce apoptosis measured by TUNEL-staining in the mouse striata in a dose dependent manner. **(B)** Graph show mean ± SEM percentage of TUNEL-positive staining relative to total neuronal cell counts with NeuN. Differences between groups were analyzed by One-way ANOVA, followed by Tukey's post-hoc comparisons. Significance was set at $p < 0.05$ compared with saline and METH alone. ($***p < 0.001$ compared to saline, $!!!p < 0.001$ compared to METH).

(A)



(B)

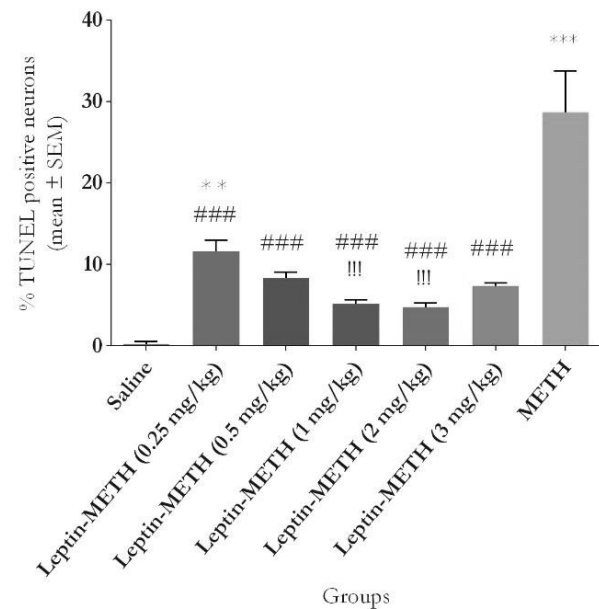


Figure 5 – 2: Dose dependent attenuation of METH-induced apoptosis in the striatum by leptin.

Mice were injected once with leptin (IP) at various doses and once with METH (30 mg/kg) (10 per group). Animals sacrificed 24 hours post treatment. Striatal tissue was processed for TUNEL immunohistofluorescence. **(A)** Micrographs of striatal tissue stained with TUNEL with FITC-conjugated dUTPs in saline, leptin (0.25, 0.5, 1, 2 mg/kg) of body weight and METH (30 mg/kg). **(B)** Counts of TUNEL positive neurons computed by percent TUNEL positive nuclei with respect to NeuN signals quantified using ImageJ for dorsal and ventral striatal region (mean \pm S.E.M.). The lowest optimal dose that showed peak attenuation is 1mg/kg with 18% reduction in apoptosis. (* p <0.01, *** p <0.001 compare to saline; ### p <0.001 compare to METH; !!! p < 0.001 compare to leptin 0.25 mg/kg).

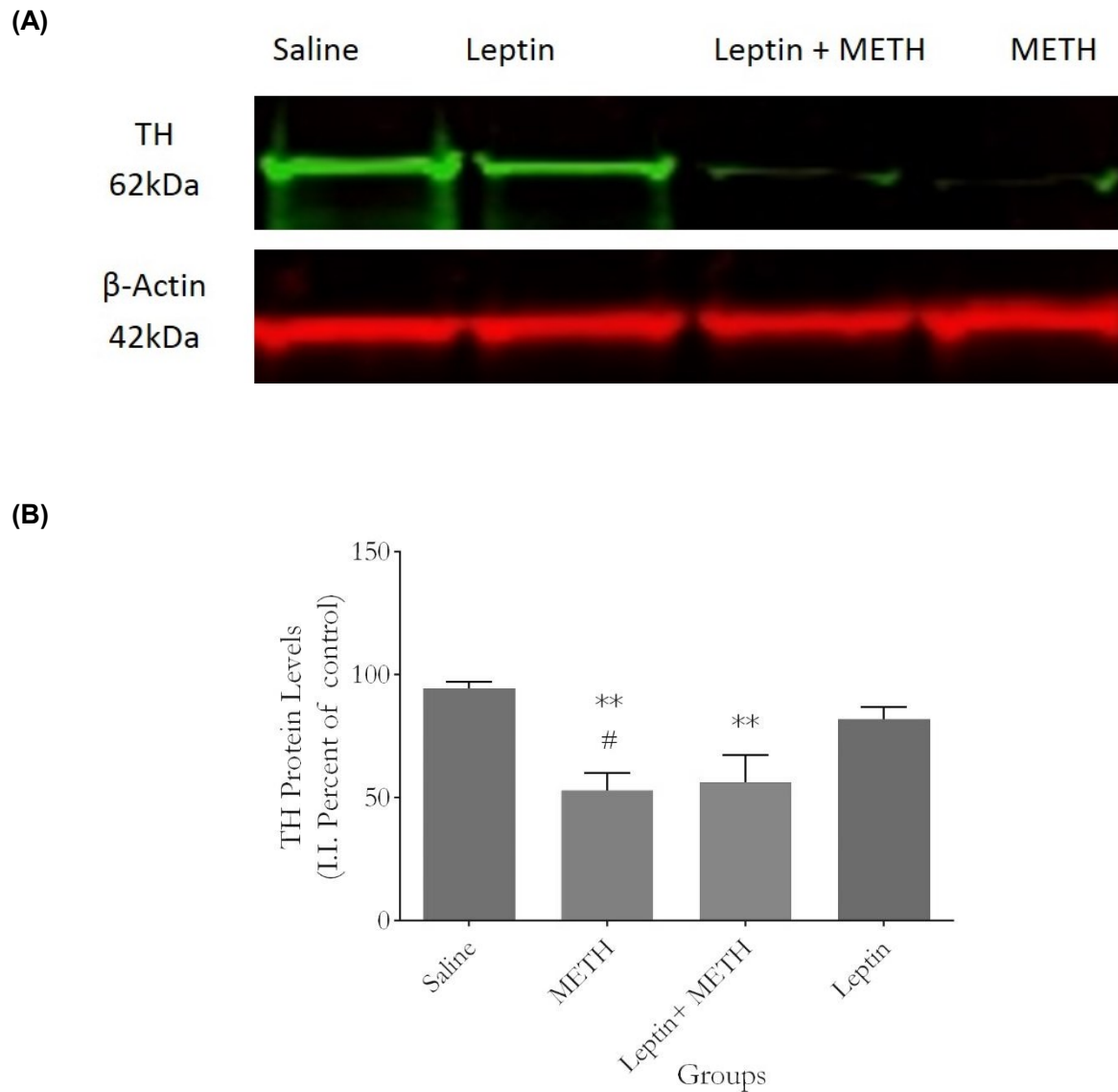


Figure 5 – 3: Leptin does not protect dopamine terminals from METH toxicity. (A) Western blot analysis was used to measure striatal tyrosine hydroxylase (TH) protein levels and thus determine dopamine terminal viability. (B) Pretreatment with leptin failed to protect from METH-induced dopamine terminal degeneration. Four groups of mice (n=6 per group) received IP of saline, leptin (1 mg/kg) or leptin + METH (30 mg/kg) or METH alone. Animals were sacrificed 72 hours after treatment. (** $p < 0.01$ as compared to saline group, # $p < 0.05$ as compared to leptin group).

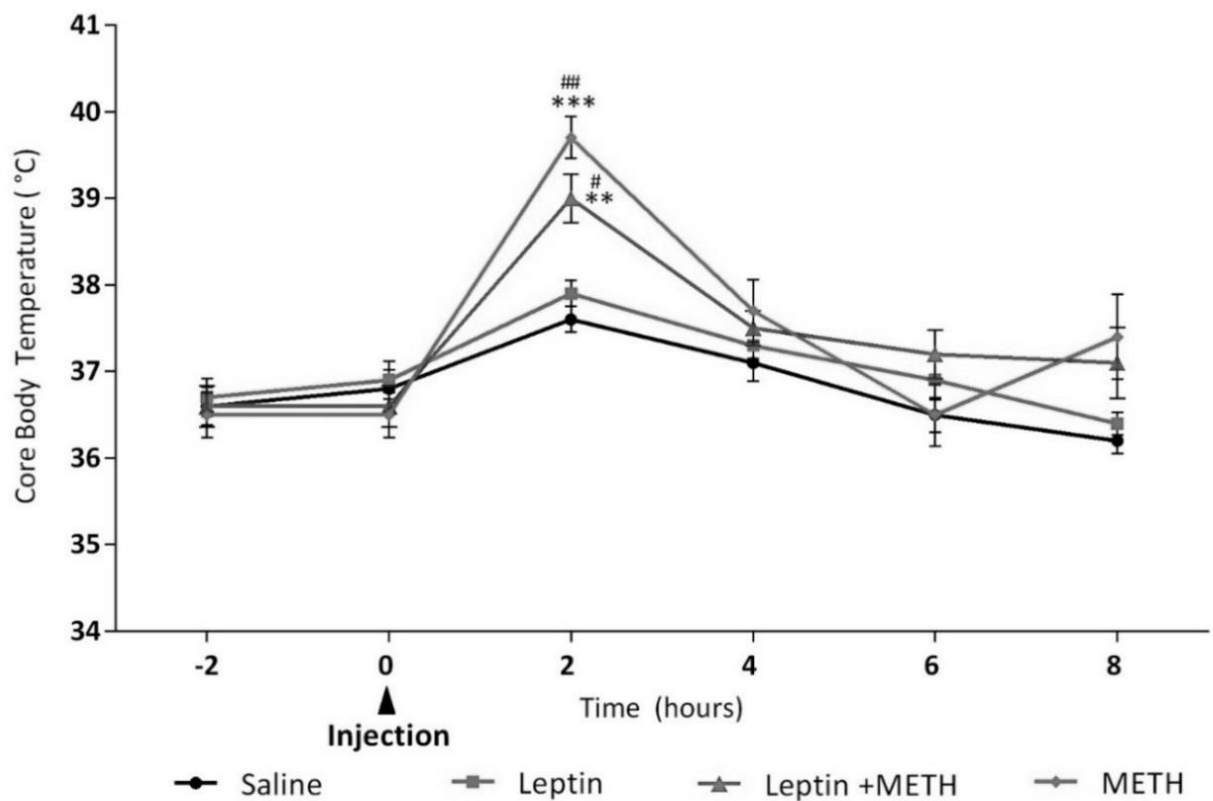


Figure 5 – 4: Leptin protects against METH-induced cell death without preventing hyperthermia. Rectal body temperature was recorded every two hours starting from the onset of METH treatment for up to eight hours post treatment. Results were from mean \pm SEM of 6 to 10 animals per experimental group. Ambient room temperature was maintained at 20 – 22°C. (** p < 0.01, *** p < 0.001 compared to saline. # p < 0.05, ## p < 0.001 compared to leptin alone).

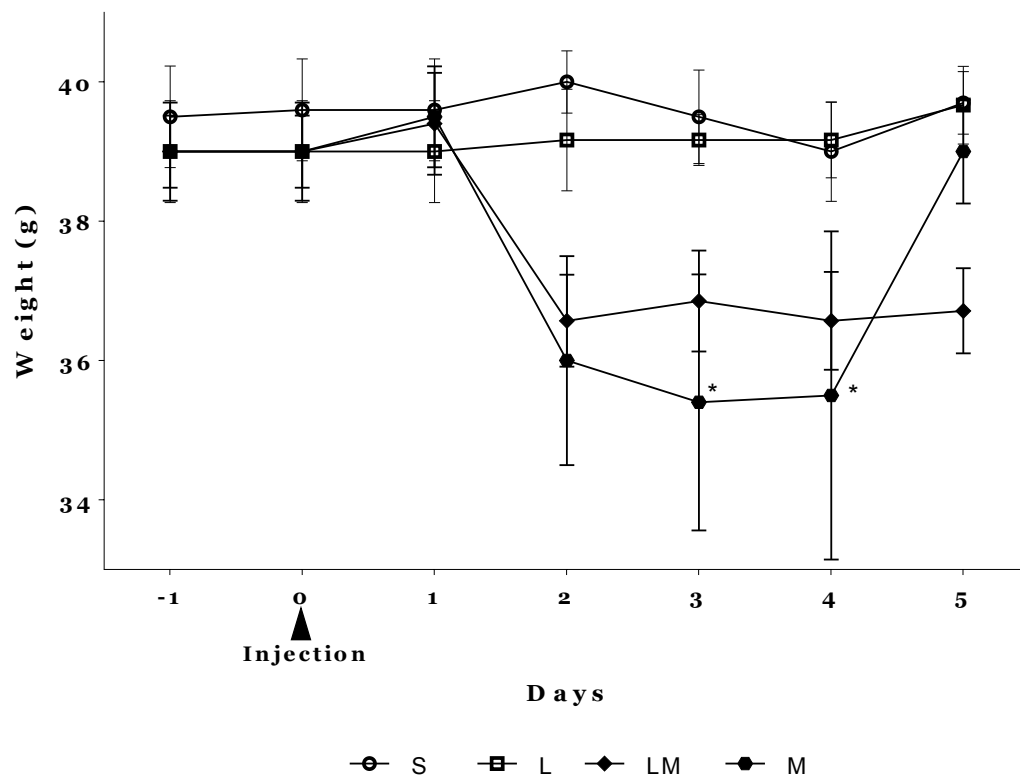
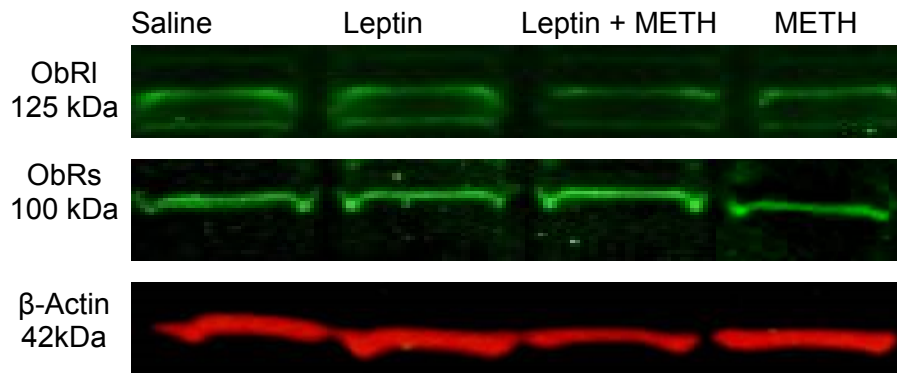


Figure 5 – 5: Leptin does not protect against METH-induced weight loss. Body weight was recorded one days prior to injections and for five consecutive days after injection. Saline and Leptin alone groups did not show any change in weight post injection. Leptin + METH and METH alone groups showed slight decrease in weight which is not significant. Ambient room temperature was maintained at 20 – 22°C. (* $p < 0.05$ compared to day 1 weight of METH group).

(A)



(B)

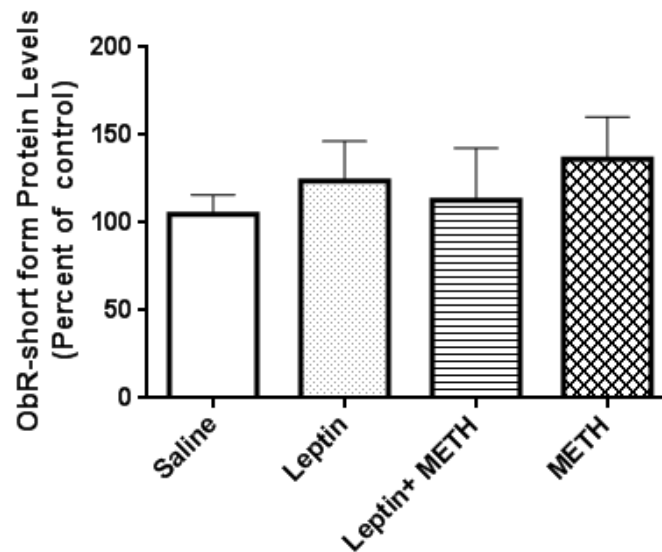
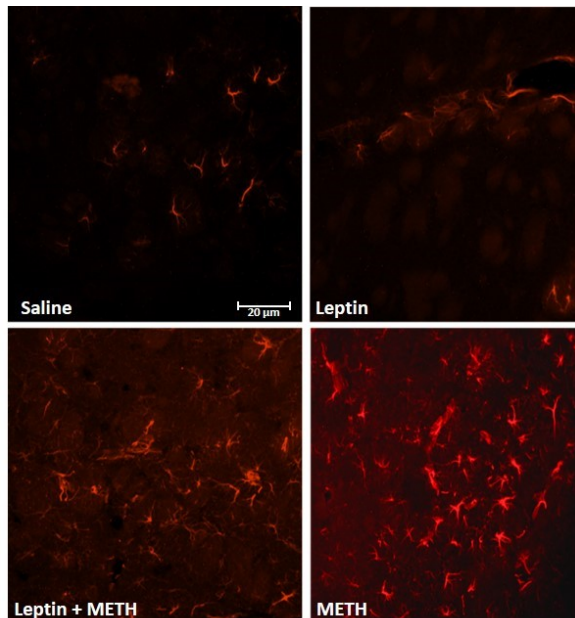


Figure 5 – 6: Leptin receptor expression in mouse striata: Both the short and long isoforms of the leptin receptor were expressed in the mouse striatum. **(A)** ObR protein levels were determined 24 hours post treatment of saline, leptin, leptin + METH and METH alone groups (n=6 animals per group). **(B)** Quantification of protein levels of both long (not shown) and short form indicates there are no differences between in ObR protein levels between the four groups post treatment.

5.2 Role of Leptin in Striatal Overactivation of Astrocytes and Microglia

Previously we have demonstrated that apoptosis peaks at 24 hours post METH treatment followed by increased activation of astrocytes and microglia (Zhu et al., 2005) by 72 hours. Identification of over activated astrocytes and microglia in the striatum was measured by immunohistochemistry using GFAP and Iba-1 staining respectively, post treatment of leptin (1 mg/kg) + METH (30 mg/kg), METH alone, leptin alone and saline. We assessed the number of reactive glia in the dorsal and ventral regions of the striatum in 6-8 coronal striatal sections per animal (n=6 per group). GFAP and Iba-1 immunohistofluorescence using specific antibodies detected the levels of activated signals. The level of activation of the saline group was set at zero in order to assess the activated astrocytes and microglia in the leptin alone, leptin+ METH and METH alone groups. One-way ANOVA (95% confidence level) with planned comparisons to METH alone group found a significant decrease in the over expression of astrocytes by 58% and microglia by 64% in the striatum in the leptin+ METH group (Figure 5 – 7 (a, b) and 5 – 8 (a, b)).

(A)



(B)

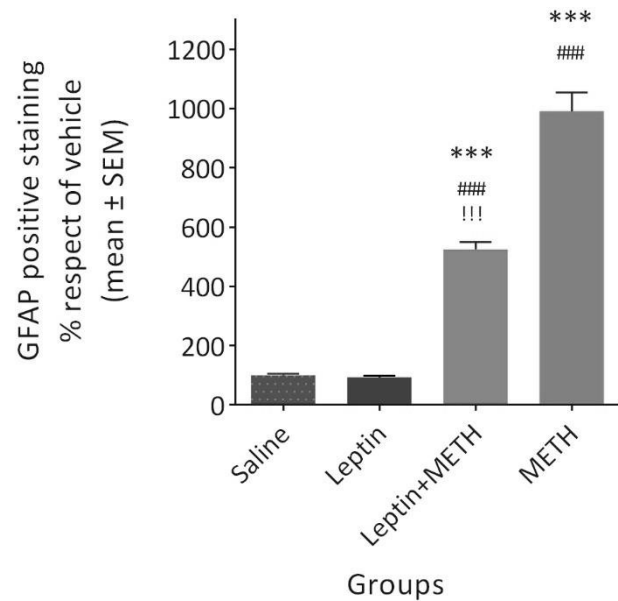
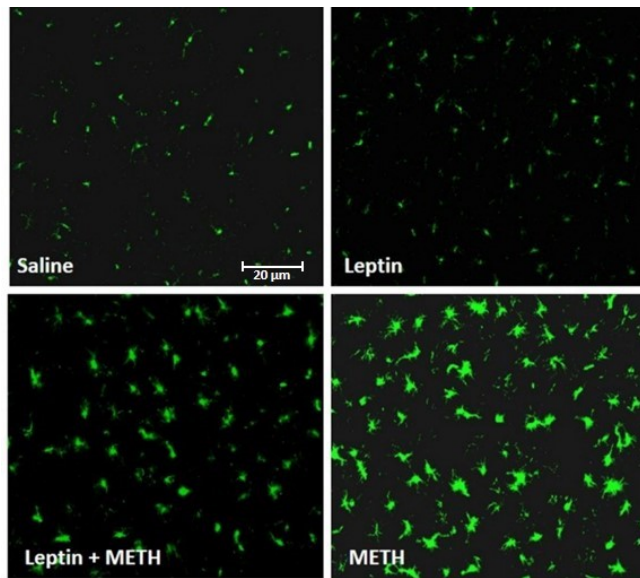


Figure 5 – 7: Activation of astrocytes measured using glial fibrillary acidic protein (GFAP): three days post injection of leptin (1 mg/kg) and METH (30 mg/kg) in mouse striatum. Coronal sections were processed for immunohistofluorescence with antibody against GFAP conjugated to the chromophore Cy3. **(A)** Micrographs of epifluorescent images of GFAP stained striatal tissue. **(B)** Percent of GFAP positive staining with respect to saline control shows that leptin attenuates the over activation of astrocytes (mean ± SEM). (***) $p < 0.001$ compare to saline, (###) $p < 0.001$ compare to leptin, (!!!) $p < 0.001$ compare to METH).

(A)



(B)

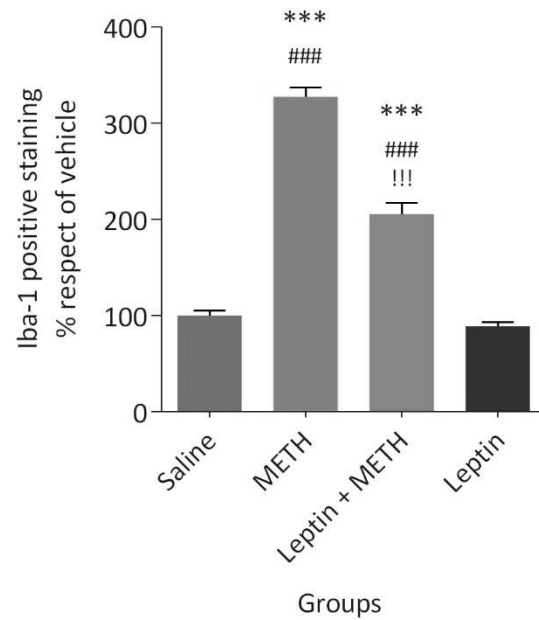


Figure 5 – 8 Activation of microglial cells in the mice striatum by METH is attenuated by leptin: (A) Microglia staining measured using immunohistofluorescence with antibody against Iba-1 three days post injection of leptin (1 mg/kg) and METH (30 mg/kg) in mouse striatum. (B) Percent Iba-1 positive staining with respect to saline control shows that leptin attenuates the over activation of microglia (mean \pm SEM). (***) $p < 0.001$ compare to saline, (###) $p < 0.001$ compare to leptin, (!!!) $p < 0.001$ compare to METH).

5.3 Role of Leptin in Methamphetamine-Induced Striatal Nitric Oxide and Caspase-3 Activation.

METH causes a significant depletion of DA and mostly affects the striatal DA system. The toxicity imposed by METH includes the release of ROS as well as glutamate-induced NO production (Dawson and Dawson 1996). Research have shown that there is a relationship between METH-induced toxicity and the over production of NO. Furthermore, nNOS knockout mice are spared from METH-induced DA toxicity (Itzhak et al., 1998). In another study METH treatment caused significant increase in nNOS expression in the striatum and hippocampus at one and 24 hours post treatment, further supporting the idea that NO plays a neurotoxic role in METH toxicity (Deng and Cadet, 1999). Researchers have reported high levels of nNOS and iNOS to be present in patients with PD in the SN (Hunot et al., 1996) and in animal models of these diseases (Liberatore et al., 1999). In the striatum, NO is involved in the regulation of striatal dopamine neurotransmission (West et al., 2002). Increased levels of NO also impose a neurotoxic effects in the SNc dopaminergic neurons (Przedborski et al., 1996; Zhang et al., 2000).

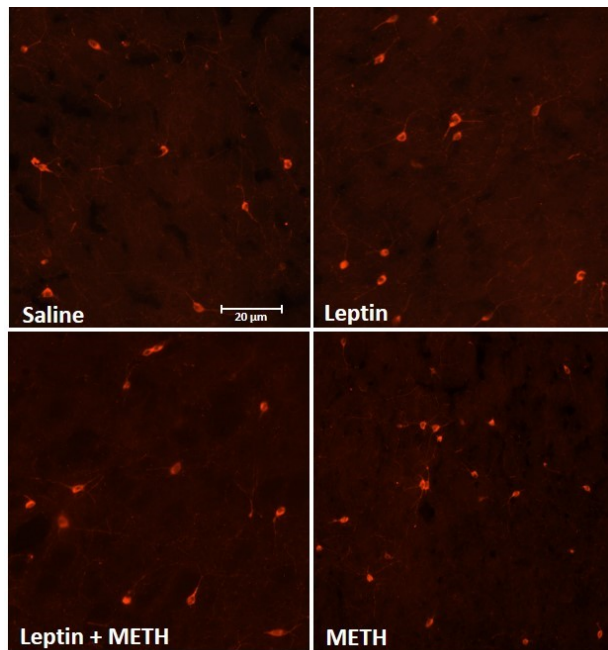
Nitric oxide is a small gaseous signaling molecule in the nerve terminals with a transitory life span, produced on demand to maintain neurosecretion, thermoregulation, and plasticity among other cellular homeostasis. Uncontrolled formation of NO plays an important factor in neurotoxicity and neurodegenerative disorders. It mainly acts by diffusing from neuron to neuron to directly act on intracellular mechanisms. The synthesis of NO in the brain is regulated by its synthase, NOS, the three types being neuronal NOS, endothelial NOS and inducible NOS. In neuronal cells NO is produced by the catalytic conversion of amino acid L-arginine to NO and L-citrulline by nNOS that gets activated when cellular levels of Ca^{2+} increases and the Ca^{2+} /calmodulin complex binds (Itzhak et al., 2000). NO diffuses from the neuronal cells into the extracellular space where it tightly binds and activates GC (also known as NO-activated GC or soluble GC). This activated GC converts its substrate, GTP to

second messenger, cGMP. Therefore an increase in cGMP level activates several cascading signal transduction pathways (Alderton et al., 2001). With increasing NO, more GC is activated and cellular cGMP level rises and the cGMP pathway is stimulated. The key to controlling NO lies in regulation of its synthesis.

We aimed to better understand NO production and striatal apoptosis upon METH administration and to see if leptin may attenuate such increased levels of nNOS and cGMP expression. The goal of this experiment was to know if leptin could decrease the over activation of nNOS 24 hours post treatment of leptin and METH as this time point was identified as the peak activation of nNOS (Deng and Cadet, 1999). Since NO affects the target cell by diffusion through the intracellular space and crosses the cell membrane of nearby target cell. We wanted to know if the production of cGMP is also decreased upon leptin treatment. Our lab previously characterized the cellular expression of cGMP in striatal neurons and found that peak expression happens at 8 hours after METH (Yarosh and Angulo, 2012). Therefore, we used the established time course in the current experiment. nNOS and cGMP levels were assessed by immunohistochemistry. Increase of nNOS and cGMP are reliable indicator of NO toxicity. Furthermore, METH-induced apoptosis is caused by multiple pathways along with activated caspase death pathways. Activated caspase-3 is an early marker of apoptosis which is also expressed highly after METH administration and peaks at 8 hours post treatment (Yarosh and Angulo, 2012). We assessed the expression of activated- caspase 3 at the above mentioned time point. Brain tissue was collected by perfusion methods after METH and leptin administrations as previously described (see above). Serial coronal tissue sections were collected, stained and imaged by Leica confocal microscopy and Leica imaging software. Differences between groups were analyzed by one-way ANOVA with appropriate post-hoc test (95% confidence level). As shown in figure 5-8 (A, B), leptin treatment reduced the expression of nNOS positive neurons in the leptin+ METH treated animals compared to METH alone group. As expected both saline and leptin groups did not show a significant increase in nNOS expression. cGMP expression also diminished 8 hours post treatment in

the leptin + METH group compared to METH group, similarly, saline and leptin alone did not show a significant increase (Figure 5-9, (A, B)). Finally, leptin also attenuated the over activation of caspase-3 in the leptin + METH group compared to METH alone group (Figure 5 – 10 (A, B)).

(A)



(B)

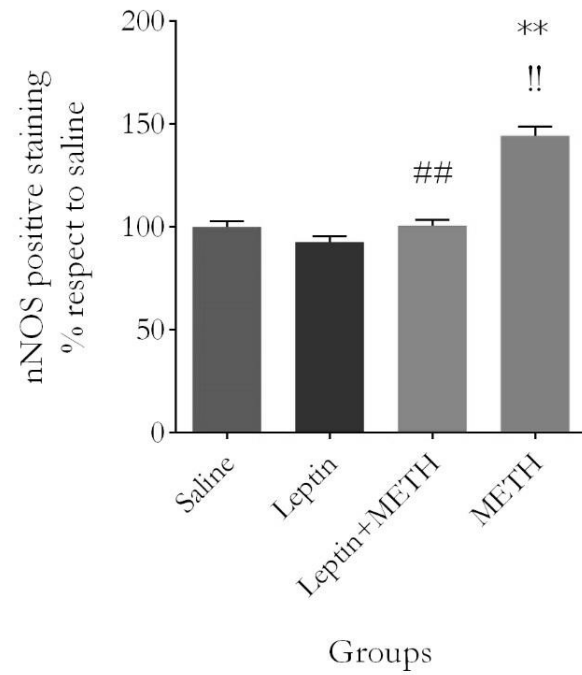
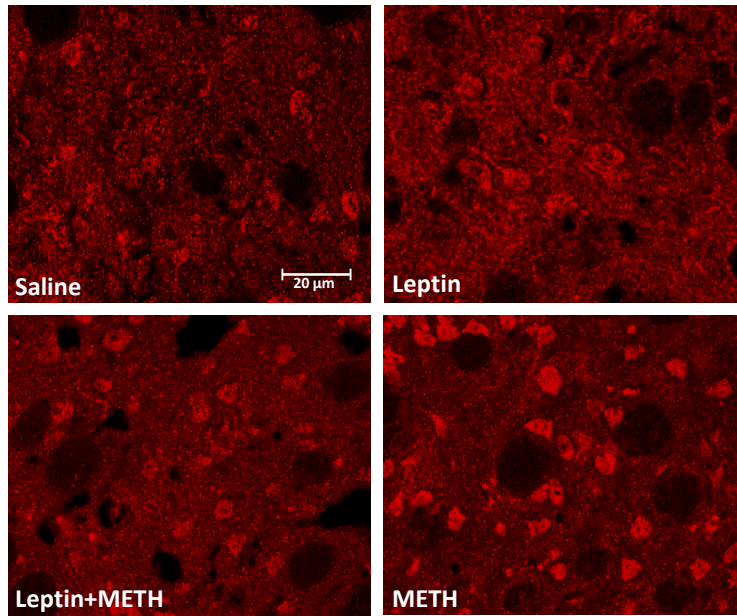


Figure 5 – 9: photomicrographs of nNOS stained striatal sections. (A) Animals treated with saline, leptin, leptin + METH and METH were sacrificed at 24 hrs. **(B)** METH caused a significant increase in the number of nNOS positive cells whereas saline and leptin alone did not increase nNOS expression and leptin + METH group showed a slight reduction of nNOS expression at 24 hours compared to METH group. (** $p < 0.01$ compare to saline, !!! $p < 0.01$ compare to leptin, ## $p < 0.01$ compare to METH).

(A)



(B)

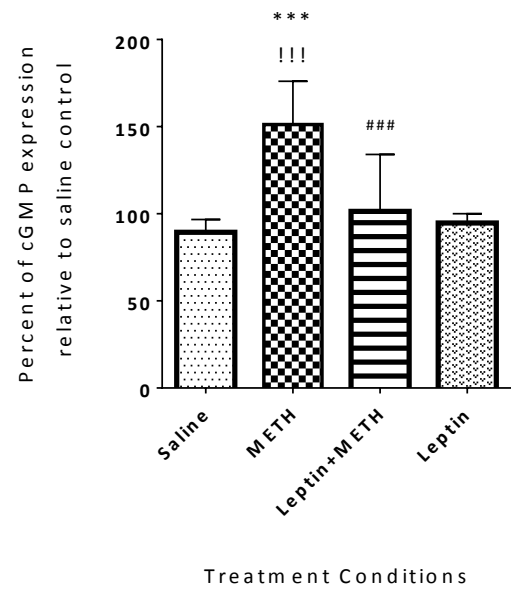


Figure 5 – 10: Cyclic guanosine monophosphate (cGMP) expression in all cell types in the mouse striata. Treatment with leptin (1 mg/kg), METH, leptin + METH or Saline, animals sacrificed 8 hours post treatment. **(A)** Micrographs of striatal tissue stained with cGMP antibody conjugated to Cy3 in saline, leptin, leptin + METH and METH animals (6-8 animals per group) show leptin reduced cGMP production when compared to METH animals. **(B)** Analysis of cGMP production relative to saline control. (***) $p < 0.001$ compared to saline, (!!!) $p < 0.001$ compared to leptin, (###) $p < 0.001$ compared to METH).

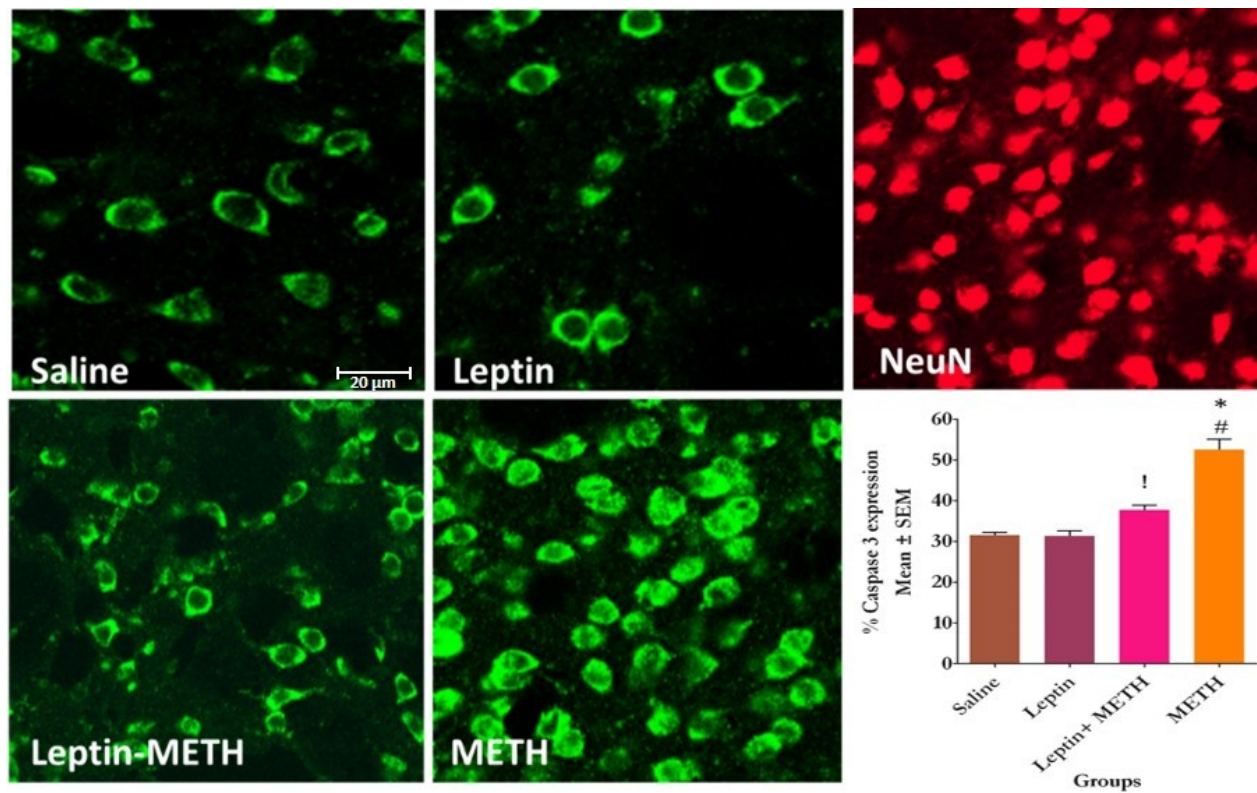


Figure 5 – 11: photomicrograph of activated caspase-3 stained striatal sections. The animals were treated with saline, leptin alone, leptin+METH or METH alone and sacrificed 8 hours post treatment. Leptin caused a significant decrease in active caspase-3 expression at 8 hours post drug administration compared to METH alone group and saline (as baseline activation) (* $p < 0.001$ compared to saline, # $p < 0.001$ compared to leptin, ! $p < 0.001$ compared to METH).

5.4 Discussion

In the present study we demonstrated the protection of striatal neurons by leptin treatment from METH-induced apoptosis. Results described above provide evidence that, administration of an established acute-high dose of METH (30 mg/kg by body weight) and the determined optimal dose of leptin (1 mg/kg by body weight) shows attenuation from cell death. Leptin alone in various doses did not induce any toxicity. In addition, we found attenuation of apoptosis in the leptin + METH group across all five doses when compared to METH alone group. METH caused about 25% of the striatal neurons to undergo apoptosis. However, leptin treatment protected apoptosis by 18%. It is significant to note that even low dose of leptin (0.25 mg/kg) provided some reduction in cell death within the striatum. However, our data demonstrate that leptin failed to protect against striatal dopamine terminal toxicity.

The effect of leptin on METH-induced hyperthermia was measured to delineate if neuroprotection from apoptosis was a result of leptin preventing hyperthermia. We measured METH-induced hyperthermia and found that leptin does not reduce the rise in core body temperature. One reason leptin may not reduce hyperthermia is because it is an anorexigenic peptide and causes animals to increase activity and energy expenditure. Leptin + METH does not show a significant reduction of weight compared to METH alone group.

METH-induced toxicity leads to release of harmful toxins like NO, which is formed by different isoforms of the enzyme NOS, such as neuronal NOS. nNOS is the primary source of NO in METH-induced toxicity. nNOS expression is increased upon METH administration. In order to measure if nNOS is contributing to the METH-induced production of NO we tested if leptin can protect striatal NO production. When leptin was administered prior to METH administration, animals showed a slight reduction of nNOS expression compared to animals in the METH group. With increasing NO, more GC is activated and cellular cGMP level rises and the cGMP pathway is stimulated. The key to controlling NO is regulating its' synthesis. So we measured cGMP levels in animals pretreated with

saline, leptin alone, leptin + METH and METH alone and found that leptin treatment reduced cGMP production when compared to METH animals.

We also found that a single dose of leptin treatment (1 mg/kg) attenuates the ubiquitous over activation of the astrocytes and microglia caused by METH toxicity and decreases the activation of pro-apoptotic protein signal. A plethora of evidence demonstrate that METH induces neural damage in the striatum and other parts of the brain. Our contribution to this area of research is the finding that peripheral hormone, leptin, can protect degeneration caused by METH in the striatum.

CHAPTER 6

LEPTIN-MEDIATED STRIATAL NEUROPROTECTION BY MODULATING GLUTAMATE TRANSMISSION.

6.1 Contribution of Leptin in NMDA-Induced Striatal Apoptosis and Dopamine

Terminal Toxicity

High densities of glutamate receptor are concentrated in various parts of the brain including the cortex, hippocampus and striatum. It is now well established that prolonged stimulation or over activation of NMDA receptors damages and kills target neurons via excitotoxicity. Excess glutamate acting via the NMDA receptors mediates cell death in glutamate neurotoxicity and has been linked to neurodegenerative diseases like Huntington's disease. NMDA receptor is a multifaceted molecular unit with numerous distinct recognition sites for endogenous and exogenous signals each with its own binding domains. Researchers have identified six pharmacologically distinct sites used by compounds to alter NMDA receptor activity. NMDA receptors can easily interact with other membrane and cytoplasmic proteins. Studying these interactions between proteins with NMDA receptors can allow researchers to find therapeutic use of glutamatergic drugs.

One way the NMDA receptor activity induces glutamate toxicity is via the activation of NMDA receptors and increase in intracellular calcium levels. In the current, study we tested the hypothesis that leptin can attenuate excessive glutamate toxicity through the striatal NMDA receptor. We treated two groups of animals (6-7 per condition) for each experiment (TUNEL and TH levels). The first group (control) received IP injection of vehicle. The second group received leptin (1 mg/kg) 30 minutes prior to intrastriatal infusions. Intrastriatal infusion of 1 μ L NMDA (20 nM) on one side and artificial

cerebrospinal fluid (aCSF) on contralateral side of the striatum was injected (see general methods for experimental procedures). In order to see leptin attenuation of NMDA-mediated cell loss, mice were euthanized and brain tissue was collected by perfusion and processed using TUNEL immunohistofluorescence 24 hours post-NMDA infusion. Values from the injected side were compared with those of the contralateral control side by ANOVA (95% confidence level) followed by post-hoc Tukey's test. For NMDA-induced dopamine terminal toxicity by leptin, fresh frozen brain tissue was collected 3 days after NMDA treatment, by decapitation and TH levels were assessed by Western blots. Protein expression was normalized to β -actin levels.

Leptin attenuated form NMDA-induced apoptosis in the striatum. Figure 6-1 demonstrates significant cell loss in the brain with NMDA infusion alone compared to NMDA-infusion + peripheral leptin injection. However, NMDA- mediated dopamine terminal toxicity was not prevented by leptin (Figure 6-2).

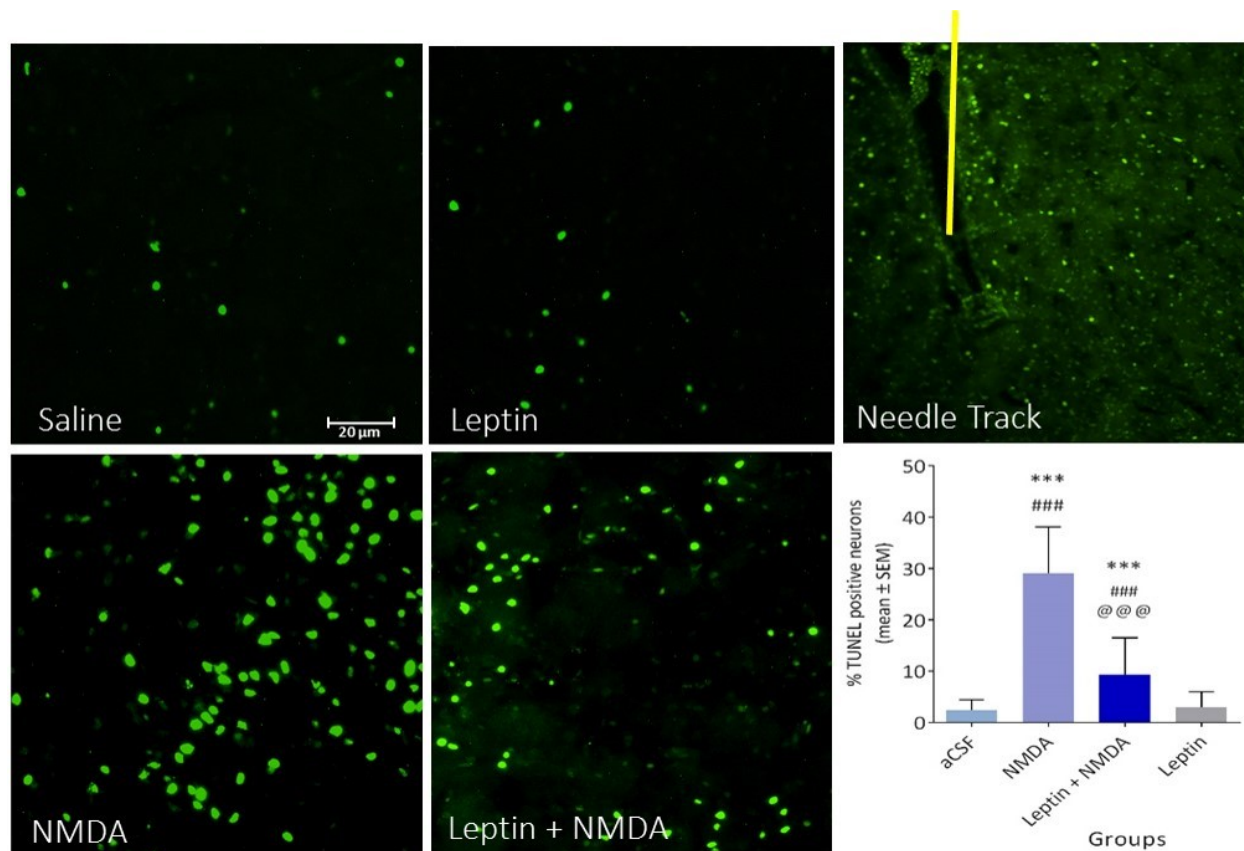


Figure 6 – 1: Leptin attenuation of NMDA-mediated apoptosis in striatum. Pretreatment with leptin caused a significant reduction in NMDA-induced apoptotic cell death measured with TUNEL histochemistry. Mice were IP injected once with leptin (at the 1 mg/kg dose) or with saline (n=10 per group) 30 minutes prior to intrastriatal infusion of NMDA (20 nM). Animals sacrificed 24 hours post-NMDA treatment. Striatal tissue was processed for TUNEL immunohistochemistry. **(A)** micrographs of striatal tissue stained with TUNEL with FITC-conjugated dUTPs in saline, leptin, NMDA or leptin+NMDA groups. **(B)** Quantification of TUNEL-positive neurons computed by percent TUNEL positive nuclei in the striatum quantified using ImageJ for dorsal and ventral striatal region (mean ± S.E.M.). Cell counts analyzed by one-way ANOVA, normalized to control group (saline and aCSF). NMDA-mediated cell loss was attenuated by leptin signaling. *** $p < 0.001$ compare to saline, ### $p < 0.01$ compare to leptin, @@@ $p < 0.001$ compare to NMDA.

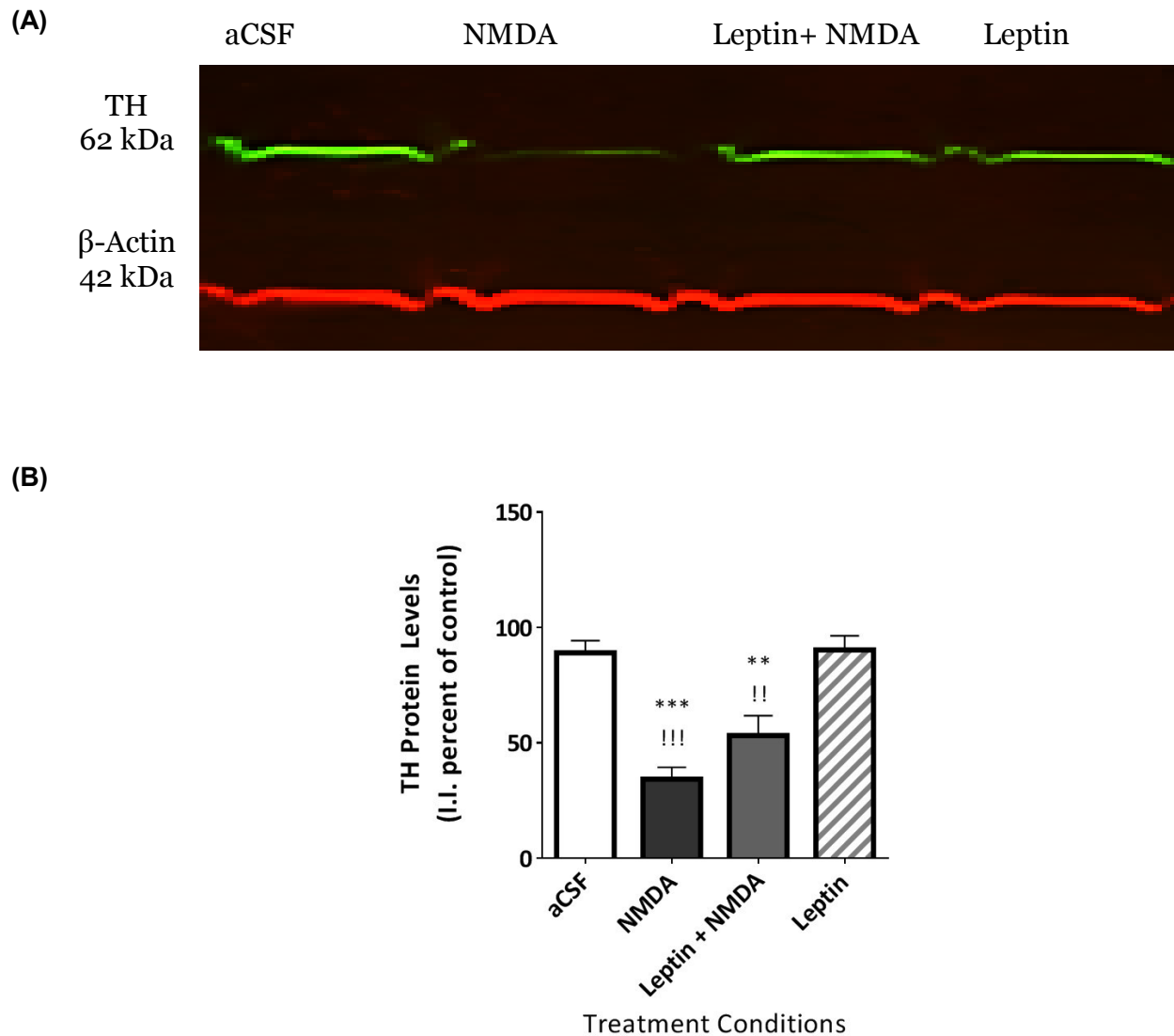


Figure 6 – 2: Leptin does not protect dopamine terminals from NMDA toxicity. (A) Western blot analysis was used to measure striatal tyrosine hydroxylase (TH) protein levels and thus determine dopamine terminal viability. (B) Pretreatment with leptin failed to protect from NMDA-induced dopamine terminal degeneration. Mice (n=10 per group) received IP of saline or leptin (1 mg/kg) 30 minutes prior to NMDA and aCSF infusion. Animals were sacrificed 72 hours after treatment. (** $p < 0.001$ and ** $p < 0.05$ as compared to aCSF group, !!! $p < 0.001$ and !! $p < 0.05$ as compared to leptin group).

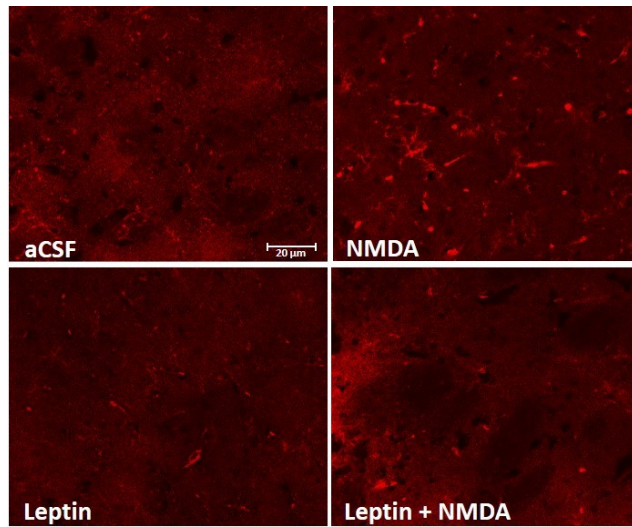
6.2 Role of Leptin in NMDA-Induced Formation of Nitric Oxide

Our lab has previously studied METH-induced excess NO production and the activation of other pro-toxic neuropeptides (Wang et al., 2008; Wang and Angulo, 2011). Glutamate excitotoxicity has an essential role in METH-mediated neural injury. Glutamate innervation into the striatum mainly signals via NMDA receptors (Garside and Mazurek, 1997). The activation of NMDA receptors and the increase in intracellular calcium levels initiates glutamate neurotoxicity. Since NOS is a calcium-dependent enzyme, activation of NOS could be involved in NMDA neurotoxicity which can increase NO production. When NO is generated in high amounts, a neurotoxic cascade is initiated. Cytotoxic pathways that get activated following increased levels of NO are stimulated by peroxynitrite (the reaction product NO and O₂).

We hypothesized that leptin is able to attenuate the NMDA-induced formation of NO. We tested the effects of leptin on NO production via glutamate signaling. Therefore, intrastratial microinjection of NMDA (as described in the methods section) in the absence of METH should result in increase of 3-nitrotyrosine (3-NT) levels (an indirect measure of NO synthesis (Schulz et al., 1995). However, systemic administration of leptin (1 mg/kg) should reduce NMDA-induced 3-NT production. NO production after METH peaks at 24 hours post treatment, as established by our lab previously (Zhu et al., 2009; Afanador et al., 2011). Therefore, we followed this time course. If the assumption is that NO mediated oxidative stress is mainly responsible for cell loss then the bulk of NO synthesis should occur by the peak of striatal apoptosis, which occurs at 24 hours (Zhu et al., 2005). Animals (10 per group) received NMDA-infusion following IP leptin or saline injection. Brain tissues were collected and processed with 3-NT immunohistochemistry and imaged by Leica scanning confocal microscope and Leica imaging software to measure 3-NT staining intensity.

Figure 6 – 3 shows that leptin treatment significantly reduced 3-NT staining within 24-hours in the leptin + NMDA infusion group compared to animals that received only NMDA infusion and a peripheral saline injection.

(A)



(B)

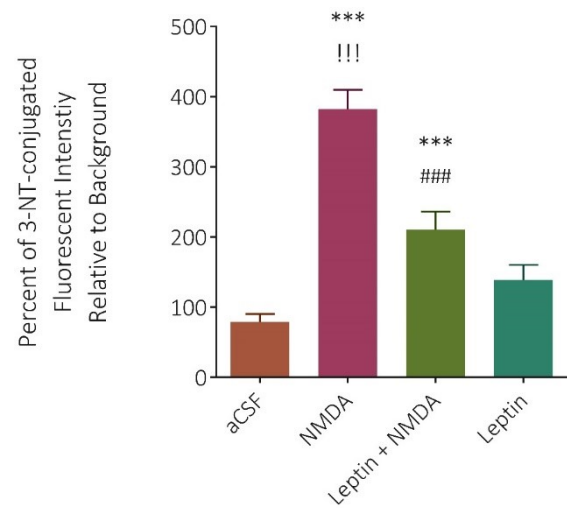
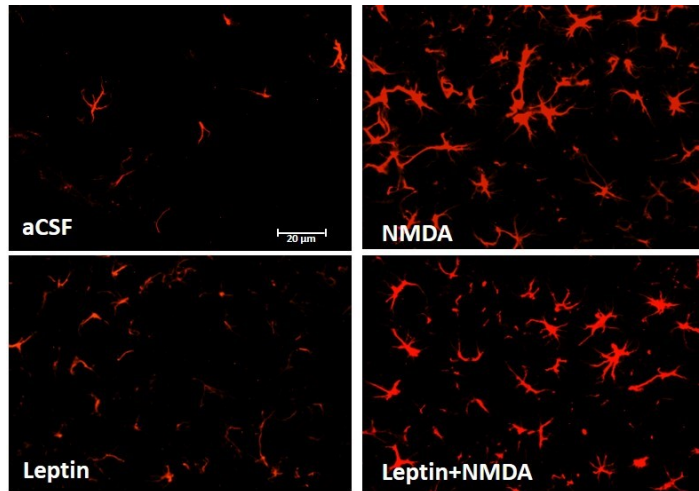


Figure 6 – 3: NMDA-mediated striatal NO attenuated by leptin. NMDA striatal infusion resulted in an increase in 3-nitrotyrosine production (3-NT) (10 animals per group). 3-NT is an indirect measure of nitric oxide synthesis, identifier of cell damage and inflammation which peaks at 24 hours. Animals were sacrificed 24 hours after NMDA infusion and leptin or saline IP injection. NMDA agonist caused a significant augmentation in 3-NT production but leptin caused a significant decrease in 3-NT production at 24 hours post treatment. (** $p < 0.001$ as compared to aCSF group, !!! $p < 0.001$ compared to leptin group, ### $p < 0.001$ compare to NMDA.)

6.3 Role of Leptin in Striatal Expression of Astrocytes and Microglia upon NMDA-Infusion

NMDA infusion also induces gliosis (astrocytic and microglial activation) that is similar to METH-induced activation as a response to neurotoxic injury. Excessive activation of glial cells is known to be harmful in the striatum and can release ROS (Krasnova and Cadet, 2009). Therefore we hypothesize that leptin should block excessive astrocytic and microglial activation and show protection from NMDA-induced increase. Intrastratial microinjection of NMDA (as described above) should increase glial activation. However, systemic administration of leptin (1mg/kg) should reduce NMDA-induced glial activation. Injection, intrastratial infusions, tissue collection, and analysis followed the paradigm described above; except mice were euthanized 3 days post NMDA infusion and leptin/saline injections. Immunohistofluorescence using GFAP (for astrocytes) and Iba-1 (for microglia) was done according to the procedures described above. Images taken using Leica scanning confocal microscope and ImageJ was used to analyze overactivation of GFAP and Iba-1 staining. Peripheral leptin injection did not significantly reduce NMDA-induced striatal activation of astrocytes and microglia (figure 6-4 and 6-5). Pretreatment with leptin prior to NMDA microinjection shows a very slight decrease in the overactivation with is not statically significant.

(A)



(B)

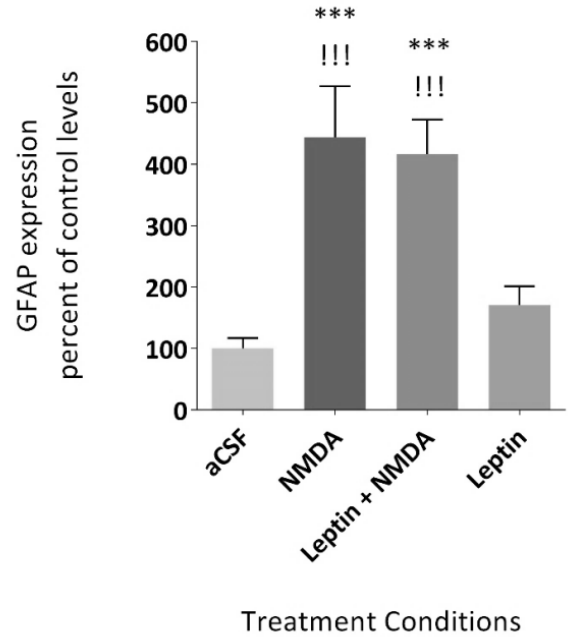
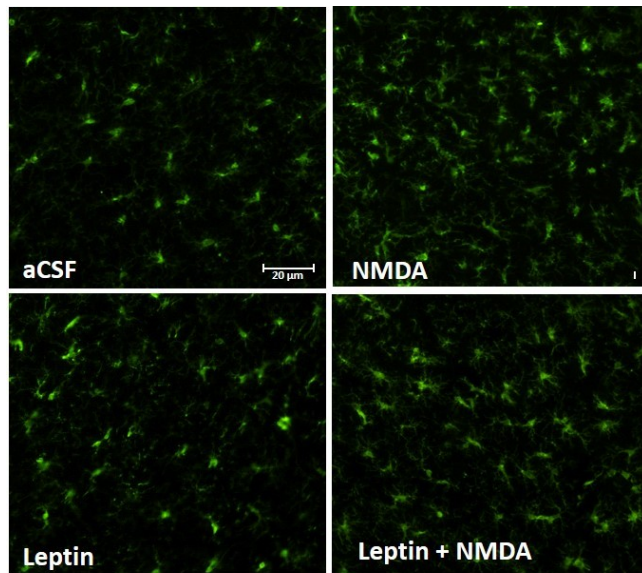


Figure 6 – 4: NMDA-induced activation of astrocytes measured using glial fibrillary acidic protein (GFAP). Animals were sacrificed three days post injection of leptin (1 mg/kg) and NMDA infusion in mouse striatum. Collected coronal sections were processed for immunohistochemistry with antibody against GFAP conjugated to the chromophore Cy3. **(A)** Micrographs of epifluorescent images of GFAP stained striatal tissue. **(B)** Percent of GFAP positive staining with respect to aCSF control shows that leptin did not attenuate the overactivation of astrocytes (mean \pm SEM). (***) $p < 0.001$ compare to saline, !!! $p < 0.001$ compare to leptin).

(A)



(B)

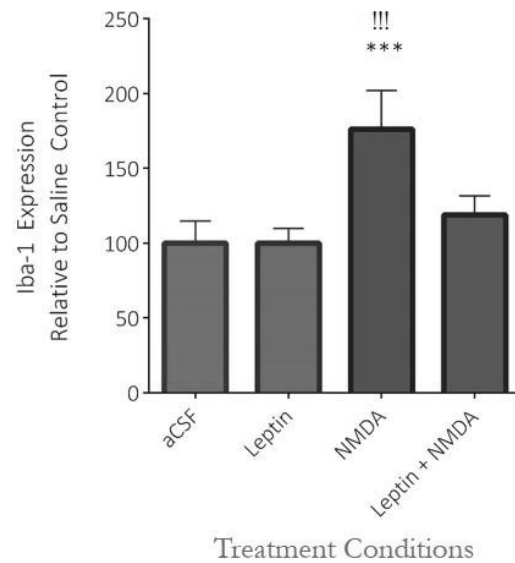


Figure 6 – 5: Activation of microglial cells in the mice striatum by NMDA-infusion is not attenuated by leptin: (A) Microglia staining measured using immunohistofluorescence with antibody against Iba-1 three day post injection of leptin (1 mg/kg) or saline followed by infusion of NMDA in to the mouse striatum. (B) Percent Iba-1 positive staining with respect to saline control shows that leptin does not significantly attenuate the overactivation of microglia (mean ± SEM). *** $p < 0.001$ compared to aCSF, !!! $p < 0.001$ compared to leptin.

6.4 Discussion

METH-induced striatal toxicity resulted in elevated concentration of both DA and glutamate release and an increased binding of glutamate to its NMDA receptor. Nucleus accumbens and prefrontal cortex show excessive DA overflow but little GLU overflow. In the CPu METH-induced overflow of both DA and glutamate is high (Stephans and Yamamoto, 1994). In the second aim, we tested the hypothesis that leptin can attenuate excessive glutamate toxicity through striatal NMDA receptor. First, our results indicate that NMDA-induced apoptosis is attenuated by leptin 24 hours post intrastriatal infusion of NMDA. However, dopamine terminal toxicity was not prevented by leptin treatment upon NMDA-induced toxicity. Neurotoxic administration of METH or NMDA results in continued release of glutamate, which is associated with oxidative stress. We know that increased NO signaling leads to neurodegeneration, so we tested NMDA-mediated NO production since the glutamate/NO cascade plays a key role in degeneration of striatal areas. We found that leptin is able to attenuate NMDA-induced formation of NO by reducing 3-NT expression in NMDA infused animals upon leptin treatment. Finally, elevated gliosis was not reduced by leptin treatment prior to NMDA infusion.

CHAPTER 7

CONCLUSION

To recapitulate, use of METH can cause reduction of DA terminals, and cell body, along with significant decreases in TH activity, DAT, DA and its' metabolites DOPAC, finally a decreased level of VMAT2. In addition to TH loss, which indicates fiber damage, METH causes 20 to 25% loss of dopaminergic neurons in the SNc (Hirata and Cadet, 1997; Sonsalla et al., 1996). Furthermore, long-term abstinence from METH does not entail full recovery of TH fibers in the striatum which is understood to be a result of loss of DA neurons in the SNc. This type of loss is similar to what occurs in Parkinson's disease. Furthermore, METH toxicity and neurodegenerative disorders like Parkinson's disease show commonality in the malfunction in the modulatory role between DA and glutamate input in the striatum. In the current study we sought to identify if and how leptin, an endogenous hormone can be responsible for mediating protection. If protection is offered by this hormone, we tried to delineate the mechanism behind it.

Classically, cells releasing dopamine entail characteristics of neurons as neuromodulators, glutamate as excitatory and GABA as inhibitory signal. A current accepted view is that neurons also release other classical neurotransmitter different from the one they are typically associated and which may have opposing effects. The activity of midbrain DA neurons has an intricate and specific role during addictive behaviors that has been studied and shown to be enhanced under dopaminergic signaling. The precise molecular and synaptic mechanisms by which these neurons behave as neuromodulators and innervate the cortex, the basal ganglia and other targets remains elusive. They mediate goal-directed and reward-driven behavior which are better understood when diagnosed with

Parkinson's disease or under METH abuse/addiction patients display severe psychomotor deficits. It is now believed that synaptic transmission between dopaminergic and striatal neuron population is not only complex but ranges beyond its respective neurotransmitter release.

In the present study we demonstrated the protection of striatal neurons by leptin treatment from METH-induced apoptosis. Administration of an established acute-high dose of METH (Zhu et al., 2006) and optimal dose of leptin (1 mg/kg by body weight) showed attenuation from apoptotic death and protect striatal brain tissue. Leptin alone does not induce any toxicity. It is significant to note that even low dose of leptin (0.25 mg/kg) can provide some reduction in cell death within the striatum. METH caused about 25% of the striatal neurons to undergo apoptosis and leptin treatment protected apoptosis by 18%. Leptin did not prevent METH-induced hyperthermia or weight loss. Leptin may not reduce hyperthermia because it is an anorexigenic peptide and causes animals to increase activity and energy expenditure. Leptin treatment attenuated the ubiquitous over activation of the astrocytes and microglia caused by METH toxicity. METH-induced oxidative stress was attenuated by leptin. Furthermore we investigated the role of leptin in NMDA mediated glutamate transmission and NO production. We eliminated METH and narrowed in on NMDA transmission in a model that can be applied to METH toxicity. Leptin treatment prior to NMDA-mediated striatal cell loss resulted in a decrease in cell death. In addition, analysis of NMDA-mediated NO synthesis was attenuated by leptin treatment.

The traditional notion of neurotransmitter release by neurons was thought to be fixed meaning, one type of neuron releases only its respective chemical defining its' identity. A modern understanding has been the notion of transmitter switching defined as replacing one neurotransmitter for another by one type of neuron which depends on the cellular environment imposed (Hnasko et al., 2010; Lavin et al., 2005). This idea is known as a new form of plasticity which is prevalent during development, maturation and changes in the adult brain of humans and rodents. It may also provide new avenues for finding therapeutics and treatments of neurological and addictive disorders.

In METH-induced degeneration, in order to identify the effect of DA depletion on extracellular glutamate release, the notion of transmitter switching may come into play. Cytoplasmic glutamate can be transported into vesicles by the vesicular glutamate transporters (vGLUTs). VGLUTs were discovered in 1994 even though glutamate was discovered in the late 1950s (Chiosa and Gane, 1956, Ni et al., 1994). Three isoforms of vGLUTs (vGLUT1, vGLUT2, vGLUT3) have been discovered throughout the years with high immunoreactivity of vGLUT1 in the neocortex, striatum, hippocampus, thalamus and cerebellum; high affinity of vGLUT2 in the olfactory bulb, nucleus accumbens, hypothalamus. Medium and low expression of vGLUT3 in the striatum, hippocampus, nucleus accumbens, cerebellum, hippocampus among other areas. It is important to note that all three VGLUTs are expressed in most CNS areas either with a strong or weak level of expression. In the striatum and NAc, vGLUT1 and 2 have opposing immunoreactivity. Presynaptic glutamate neurons harbors vGLUTs and their functionality includes packaging glutamate into vesicles before exocytosis, mediated by the electrochemical gradient (Takamori, 2006). vGLUT 1 and 2 are found glutamatergic neurons but, vGLUT3 is expressed in cholinergic, serotonergic and GABAergic neurons (Gras et al., 2002; Fremeau et al., 2002). Cortical projections to the striatum uses vGLUT1 mainly (Raju et al., 2008).

Another interesting way dopaminergic neurons impact the METH induced toxicity is that it has the ability to inhibit striatal projection neurons by co-releasing GABA (Hnasko et al., 2010). Similarly, there is evidence showing that stimulation of VTA dopaminergic neurons in rats show glutamatergic postsynaptic activation in the PFC and nucleus accumbens neurons (Chuhma et al., 2009, Lavin et al. 2005). Also, glutamate corelease from the DA neurons activates postsynaptic glutamate receptors which indicates that DA neurons is capable of harboring the machinery necessary for releasing glutamate. vGLUTs are necessary for cytoplasmic release and signifies one way glutamate is being utilized as a transmitter besides its role in other cellular function such as protein synthesis and metabolism (Takamori, 2006). This may be one possible way how leptin is mediating neuroprotection. As we and others have found both long and short form of leptin receptors (ObR-l, ObR-s) to be

expressed in the striatum including the nucleus accumbens (Figure 5-6) (Zhang et al. 2007). The administration of leptin attenuated the METH and NMDA induced neuronal death in mice. Typically, glutamate uptake increases the pH across the synaptic vesicles, and dopamine uptake by VMAT-2 is mainly dependent upon passive diffusion and changes in the internal pH. Transmitter switching implies that transport of glutamate into the same vesicles might be expected to stimulate DA uptake. METH may cause the loss of vGLUT2 which may be one way to account for the reduction of DA content within the striatum. Also, METH compromises the balance and changes the pH of VMAT-2 and causes excessive release. Perhaps one way leptin is attenuating METH-induced toxicity is by playing a role in the transmitter switching mechanism. One possibility is that dopamine neurons that co-release glutamate, mediated by vGLUT2, may show a reduction of vesicular storage of DA in the striatum. Also glutamate may stimulate vesicular DA transport. Leptin receptors may be co-localized in these same neurons and may prevent the stimulation of vGLUT2 in these DA neurons and thus provide protection from METH toxicity.

Additionally, recent studies have shown that glutamate corelease by mesostriatal DA neurons regulate a plethora of behavioral activation. However how glutamate release by DA neurons might play this role still remains to be elucidated. There is little research available regarding functional significance of glutamate corelease by DA neurons of the CNS. However, it's important to note that many CNS areas coexpress one of several types of the vGLUTs, therefore, it might have a greater significance than what we know so far. Also, downregulation of vGLUT2 in DA neurons of mice was shown to decrease DA release in the striatum (Hnasko et al., 2010). With respect to the current study, applying the notions of transmitter switching within the nigrostriatal pathway suggests that transport of glutamate into the vesicles can stimulate DA uptake as well. Typically Glutamate uptake increases pH across the vesicles and since DA uptake depends on passive diffusion and pH balance a homogeneous status is maintained. Under METH which can cause a loss of vGLUTs along with its known loss of DAT, VMAT2, and DA content. Additionally it changes the pH balance of VMAT2

initiating excessive release which can't be restored by vGLUTs. Leptin may be attenuating METH-induced neurotoxicity, by using the transmitter switching mechanism via the DA neurons that coreleases glutamate. Past research have shown that nigrostriatal neurons harbor leptin receptors. Thus, these DA neurons corelease glutamate and contains ObRb. Upon METH administration along with leptin treatment the vGLUTs are becoming activated and possibly restoring the reduction of glutamate and by vesicular synergy also restoring DA in these neurons affording attenuation.

Lastly, the field of leptin research within the extra-hypothalamic areas is in great need of further exploration. Years of work on molecular biology of leptin yielded a host of insights regarding the intricate functions of its activity. However, an integrated understanding of leptin activity will require a complete understanding of how this peptide interacts with other physiological functions. The mechanisms by which leptin intrinsically alters the expression or activity of other neuropeptides remain elusive. Currently, leptin's ability to attenuate METH-induced toxicity in the striatum was investigated. Hopefully, new methods of determination of protein interactions and co-expression will pave the way for further breakthroughs.

REFERENCES

- Afanador L., Mexhitaj I., Diaz C., Ordonez D., Baker L., Angulo, J.A. (2013). The role of the neuropeptide somatostatin on methamphetamine and glutamate-induced neurotoxicity in the striatum of mice. *Brain Res.*, 1510, 38 – 47
- Albers D.S. and Sonsalla, P.K. (1995). Methamphetamine-induced hyperthermia and dopaminergic neurotoxicity in mice: pharmacological profile of protective and nonprotective agents. *J Pharmacol Exp Ther.*, 275, 1104 – 1114.
- Albertson, T.E., Derlet, R.W., Van Hoozen, B.E., (1999). Methamphetamine and the expanding complications of amphetamines. *West. J. Med.*, 170, 214 – 219.
- Alderton, W.K. Cooper, C.E., Knowles, R.G. (2001). Nitric oxide synthases: structure, function and inhibition. *Biochem J*, 357, 593 – 615.
- Aldrich, M. (2000). Parkinsonism. In: Principles and practice of sleep medicine (Kryger, M.H., Roth, T., Dement, W.C., eds), pp 1051–1057. Philadelphia: Saunders.
- Ali, S.F. and Itzhak, Y. (1998). Effects of 7-nitroindazole, an NOS inhibitor on methamphetamine-induced dopaminergic and serotonergic neurotoxicity in mice. *Ann. N. Y. Acad. Sci.* 844, 122 – 130.
- Ali, S.F., Newport, G.D., Holson, R.R., Slikker, Jr W., Bowyer, J.F. (1994). Low environmental temperatures or pharmacologic agents that produce hypothermia decrease methamphetamine neurotoxicity in mice. *Brain Res.*, 658, 33 – 38.
- American Psychiatric Association. (2013). *Diagnostic and statistical manual of mental disorders* (5th ed.). Washington, DC.
- Antle, M.C. and Silver, R. (2009). Neural basis of timing and anticipatory behaviors. *Eur J Neurosci.*, 30(9), 1643 – 1649.
- Axt, K.J. and Molliver, M.E. (1991). Immunocytochemical evidence for methamphetamine-induced serotonergic axon loss in the rat brain. *Synapse* 9(4), 302 – 313.
- Banks, W.A., Kastin, A.J. Huang, W. Jaspan, J.B., Maness, L.M. (1996). Leptin enters the brain by a saturable system independent of insulin. *Peptides*, 17, 305 – 311.
- Betarbet, R. and Greenamyre, J.T. (1999). Differential expression of glutamate receptors by the dopaminergic neurons of the primate striatum. *Exp Neurol.* 159, 401 – 408.

- Berthoud, H.R. (2007). Interactions between the “cognitive” and “metabolic” brain in the control of food intake. *Physiol Behav*, 91, 486 – 498.
- Bjorbaek, C., Elmquist, J.K., Michl, P., Ahima, R.S., Van Bueren, A., McCall, A.L., Flier, J.S. (1998). Expression of leptin receptor isoforms in rat brain microvessels. *Endocrinology*, 139, 3485 – 3491.
- Brecht, M.L., O'Brien, A., Von Mayrhauser, C., Anglin, M.D. (2004). Methamphetamine use behaviors and gender differences. *Addictive Behaviors*, 29(1), 89 – 106.
- Brien, J.F., Kitney, J.C., Peachey, J.E., Rogers, B.J. (1978). Methamphetamine-induced behavioural effects and brain concentrations of methamphetamine and its metabolite amphetamine in mice. *Res Commun Chem Pathol Pharmacol*, 22, 313 – 328.
- Boden, G., Chen, X., Mozzoli, M., Ryan, I. (1996). Effect of fasting on serum leptin in normal human subjects. *J Clin Endocrinol Metab*, 81, 3419 – 3423.
- Bowyer, J.F., Tank, A.W., Newport, G.D., Slikker Jr., W., Ali, S.F., Holson, R.R. (1992). The influence of environmental temperature on the transient effects of methamphetamine on dopamine levels and dopamine release in rat striatum. *J. Pharmacol. Exp. Ther.* 260, 817 – 824.
- Bowyer, J.F., Davies, D.L., Schmued, L., Broening, H.W., Newport, G.D., Slikker, Jr. W., Holson, R.R. (1994). Further studies of the role of hyperthermia in methamphetamine neurotoxicity. *J. Pharmacol Exp Ther.*, 268(3), 1571 – 1580.
- Bowyer, J.F., Holson, R.R., Miller, D.B., O'Callaghan, J.P., (2001). Phenobarbital and dizocilpine can block methamphetamine-induced neurotoxicity in mice by mechanisms that are independent of thermoregulation. *Brain Res.*, 919, 179 – 183.
- Brecher, Edward M. and the editors of Consumer Reports. (1972). Licit and Micit Drugs. The Consumers Union Report on Narcotics, Stimulants, Depressants, Inhalants, Hallucinogens, and Marijuana—including Caffeine, Nicotine, and Alcohol. Boston: Little, Brown and Company.
- Broening, H.W., Morford, L.L., Vorhees, C.V. (2005). Interactions of dopamine D1 and D2 receptor antagonists with D-methamphetamine-induced hyperthermia and striatal dopamine and serotonin reductions. *Synapse*, 56, 84 – 93.
- Brown, J.M., Quinton, M.S., Yamamoto, B.K. (2005). Methamphetamine-induced inhibition of mitochondrial complex II: roles of glutamate and peroxynitrite. *J. Neurochem.* 95, 429 – 436.
- Brunswick, D.J., Benmansour, S., Tejani-Butt, S.M., Hauptmann, M. (1992). Effects of high-dose methamphetamine on monoamine uptake sites in rat brain measured by quantitative autoradiography. *Synapse*, 11(4), 287 – 293.
- Burrows, K.B., Gudelsky, G., Yamamoto, B.K. (2000). Rapid and transient inhibition of mitochondrial function following methamphetamine or 3,4-methylene –dioxy-methamphetamine administration. *Eur. J. Pharmacol.* 398, 11 – 18.

- Cadet, J.L., Ali, S.F., Epstein, C. (1994). Involvement of oxygen-based radicals in methamphetamine-induced neurotoxicity: evidence from the use of CuZnSOD transgenic mice. *Ann. N. Y. Acad. Sci.*, 738, 388 – 391.
- Cadet, J.L. and Brannock, C., (1998). Free radicals and the pathobiology of brain dopamine systems. *Neurochem. Int.* 32, 117 – 131.
- Cadet J.L., Jayanthi S., Deng X. (2003). Speed kills: cellular and molecular basis of methamphetamine-induced nerve terminal degeneration and neuronal apoptosis. *FASEB J*, 17, 1775 – 1788.
- Cass, W.A. and M.W. Manning (1999). Recovery of presynaptic dopaminergic functioning in rats treated with neurotoxic doses of methamphetamine. *J. Neurosci* 19(17), 7653 – 7660.
- Cepeda C., Buchwald N.A., Levine, M.S. (1993). Neuromodulatory actions of dopamine in the neostriatum are dependent upon the excitatory amino acid receptor subtypes activated. *Proc Natl Acad Sci USA* 90, 9576 – 9580
- Cervia, D., Martini, D., Ristori, C., Timperio, A.M., Bagnoli, P., and Casini, G. (2008) Modulation of the neuronal response to ischaemia by somatostatin analogues in wild-type and knock-out mouse retinas. *J. Neurochem.*, 106, 2224 – 2235.
- Chang, L., Alicata, D., Ernst, T., Volkow, N. (2007). Structural and metabolic brain changes in the striatum associated with methamphetamine abuse. *Addiction* 102 (Suppl. 1), 16 – 32.
- Cheng, A., Wang, S., Yang, D., Xiao, R., Mattson, M.P. (2003). Calmodulin mediates brain-derived neurotrophic factor cell survival signaling upstream of Akt kinase in embryonic neocortical neurons. *J Biol Chem.*, 278(9), 7591 – 7599.
- Cho, A.K., Melega, W.P., Kuczenski, R., Segal, D.S. (2001). Relevance of pharmacokinetic parameters in animal models of methamphetamine abuse. *Synapse*, 39, 161 – 166.
- Chiosa, L. and Gane, P. (1956). Action of glutamic acid on the higher nervous activity of small laboratory animals (rats); considerations on the relations between the biochemical substratum and the higher nervous activity. *Rev Sci Med*, 1, 165 – 171.
- Chuhma, N., Choi, W.Y., Mingote, S., Rayport, S. (2009). Dopamine neuron glutamate cotransmission: frequency-dependent modulation in the mesoventromedial projection. *Neuroscience* 164, 1068 – 1083.
- Cook, C.E., Jeffcoat, A.R., Sadler, B.M., Hill, J.M., Voyksner, R.D., Pugh, D.E. ... Perez-Reyes, M. (1992). Pharmacokinetics of oral methamphetamine and effects of repeated daily dosing in humans. *Drugs Metab Dispos.*, 20(6), 856 – 862.
- Cook, C.E., Jeffcoat, A.R., Hill, J.M., Pugh, D.E., Patetta, P.K., Sadler, B.M.... Perez-Reyes, M. (1993). Pharmacokinetics of methamphetamine self-administered to human subjects by smoking S-(1)- methamphetamine hydrochloride. *Drug Metab Dispos.*, 21, 717 – 723.

- Coleman, D.L. (1973). Effects of parabiosis of obese with diabetes and normal mice. *Diabetologia* 9(4), 294 – 298.
- Coleman, D. L. (2010). A historical perspective on leptin. *Nat Med*, 16(10), 1097 – 1099.
- Cubells, J.F., Rayport, S., Rajendran, G., Sulzer, D. (1994). Methamphetamine neurotoxicity involves vacuolation of endocytic organelles and dopamine-dependent intracellular oxidative stress. *J Neurosci*, 14(4), 2260 – 2271.
- Cuesta, M., Aungier, J., Morton, A.J. (2011). The methamphetamine-sensitive circadian oscillator is dysfunctional in a transgenic mouse model of Huntington's disease. *Neurobiol Dis*, 45, 145 – 155.
- Dafters, R.I. (1995). Hyperthermia following MDMA administration in rats: effects of ambient temperature, water consumption, and chronic dosing. *Physiol Behav.*, 58, 877 – 882.
- Dawson, V.L. and Dawson TM. (1995). Physiological and toxicological actions of NO in the central nervous System. *Adv Pharmacol*, 34, 323 – 42.
- Dawson, V.L., and Dawson, T. M. (1996). Nitric oxide neurotoxicity. *J. Chem. Neuroanatomy*, 10, 179 – 190.
- Dawson, V.L., Kizushi, V.M., Huang, P.L., Snyder, S.H., Dawson, T.M. (1996). Resistance to neurotoxicity in cortical cultures from neuronal nitric oxide synthase-deficient mice, *J.Neurosci.*, 16, 2479 – 2487.
- Deng, X., and Cadet, J.L. (1999). Methamphetamine administration causes overexpression of nNOS in the mouse striatum. *Brain Research*, 851, 254 – 257.
- Di Monte, D.A., Royland, J.E., Jakowec, M.W., Langston, J.W. (1996). Role of nitric oxide in methamphetamine neurotoxicity: protection by 7-nitroindazole, an inhibitor of neuronal nitric oxide synthase. *J. Neurochem.* 67, 2443 – 2450.
- Eisch, A.J., Gaffney, M., Weihmuller, F.B., O'Dell, S.J., Marshall, J.F. (1992). Striatal subregions are differentially vulnerable to the neurotoxic effects of methamphetamine. *Brain Res* 598(1-2), 321 – 326.
- Ellinwood, E.H., Jr. (1971). Effect of chronic methamphetamine intoxication in Rhesus monkeys. *Biol Psychiatry* 3(1), 25-32.
- Ellison, G. and Switzer, R.C. 3rd. (1993). Dissimilar patterns of degeneration in brain following four different addictive stimulants. *Neuroreport* 5(1), 17 – 20.
- Elmqvist, J.K., Bjorbaek, C., Ahima, R.S., Flier, J.S., Saper, C.B. (1998). Distributions of leptin receptor mRNA isoforms in the rat brain. *J. Comp Neurol.*, 395, 535 – 547.
- Elmqvist, J.K., Coppari, R., Balthasar, N., Ichinose, M., Lowell, B.B. (2005). Identifying

- hypothalamic pathways controlling food intake, body weight, and glucose homeostasis. *J. Comp Neurol.* 493, 63 – 71.
- Escalante, O.D. and Ellinwood, Jr. E.H. (1970). Central nervous system cytopathological changes in cats with chronic methedrine intoxication. *Brain Res.*, 21(1), 151-155.
- Fei, H., Okano, H.J., Li, C., Lee, G.H., Zhao, C., Darnell, R., Friedman, J.M. (1997). Anatomic localization of alternatively spliced leptin receptors (Ob-R) in mouse brain and other tissues. *Proc Natl Acad Sci USA*, 94, 7001–7005.
- Fibiger, H. C., and McGeer, E. G. (1971). Effect of acute and chronic methamphetamine treatment on tyrosine hydroxylase activity in brain and adrenal medulla. *Eur. J. Pharm.*, 16, 176 – 180.
- Figlewicz, D.P., Evans, S.B., Murphy, J., Hoen., M. Baskin, D.D. (2003). Expression of receptors for insulin and leptin in the ventral tegmental area/substantia nigra (VTA/SN) of the rat. *Brain Res.*, 964(1), 107 – 115.
- Forloni, G., Lucca, E., Angeretti, N., Chiesa, R., Vezzani, A. (1997) Neuroprotective effect of somatostatin in nonapoptotic NMDA-induced neuronal death: role of cyclic GMP. *J. Neurochem.*, 68, 319 – 327.
- Fornai, F., Lenzi, P., Gesi, M., Ferrucci, M., Lazzeri, G., Busceti, C.L.... Paparelli, A. (2003). Fine structure and biochemical mechanisms underlying nigrostriatal inclusions and cell death after proteasome inhibition. *J. Neurosci.* 23, 8955 – 8966.
- Francis, S.H. and Corbin, J.D. (2005). Phosphodiesterase-5 inhibition: the molecular biology of erectile function and dysfunction. *Urol Clin North Am* 32:419–429.
- Freneau, R.T., Burman, J., Qureshi, T., Tran, C.H., Proctor, J., Johnson, J....Edwards, R.H. (2002). The identification of vesicular glutamate transporter 3 suggests novel modes of signaling by glutamate. *Proc. Natl. Acad. Sci. USA.* 99, 14488 – 14493.
- Frey, K., Kilbourn, M., Robinson, T. (1997). Reduced striatal vesicular monoamine transporters after neurotoxic but not after behaviorally-sensitizing doses of methamphetamine. *Eur J Pharmacol*, 334(2-3), 273 – 279.
- Fuller, R.W., Hemrick-Luecke, S.K., Ornstein, P.L. (1992). Protection against amphetamine-induced neurotoxicity toward striatal dopamine neurons in rodents by LY274614, an excitatory amino acid antagonist. *Neuropharmacology*, 31, 1027 – 1032.
- Fulton, S., Woodside, B., Shizgal, P. (2000). Modulation of brain reward circuitry by leptin. *Science*, 287, 125 – 128.
- Fulton, S., Pissios, P., Manchon, R.P., Stiles, L., Frank, L., Pothos, E.N.... Flier, J.S. (2006). Leptin regulation of the mesoaccumbens dopamine pathway. *Neuron*, 51, 811 – 822.
- Fowler, J.S., Volkow, N.D., Logan, J., Alexoff, D., Telang, F., Wang, G.J...Apelskog, K. (2008). Fast

- uptake and long-lasting binding of methamphetamine in the human brain: comparison with cocaine. *Neuroimage*, 43, 756 – 763.
- Gao, Q., and Horvath, T.L. (2008). Cross-talk between estrogen and leptin signaling in the hypothalamus. *Am J Physiol Endocrinol Metab.*, 294, E817 – E826.
- Gao, Q., and Horvath, T.L. (2007). Neurobiology of feeding and energy expenditure. *Annu. Rev. Neurosci.* 30, 367 – 398.
- Garside, S., and Mazurek, M.F. (1997). Role of glutamate receptor subtypes in the differential release of somatostatin, neuropeptide Y, and substance P in primary serum-free cultures of striatal neurons. *Synapse*, 27, 161 – 167.
- Gautier-Sauvigne, S., Colas, D., Parmantier, P., Clement, P., Gharib, A., Sarda, N., Cespuglio, R. (2005). Nitric oxide and sleep. *Sleep Med Rev.* 9(2), 101 – 113.
- Gerfen, C.R., Engber, T.M., Mahan, L.C., Susel, Z., Chase, T.N., Monsma, Jr. F.J., Sibley, D.R. (1990). D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. *Science*, 250(4986), 1429 – 1432.
- Ginawi, O.T., Al-Majed, A.A., Al-Suwailem, A.K. (2005). Ondansetron, a selective 5-HT₃ antagonist, antagonizes methamphetamine-induced anorexia in mice. *Pharmacol Res*, 51, 255 – 259.
- Giros, B., Jaber, M., Jones, S.R., Wightman, R.M., Caron, M.G. (1996). Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature*, 379(6566), 606 – 612.
- Giovanni, A., Liang, L.P., Hastings, T.G. and Zigmond, M.M. (1995). Estimating hydroxyl radical content in rat brain using systemic and intraventricular salicylate: impact of methamphetamine. *J. Neurochem*, 64, 1819 – 1825.
- Grill, H.J. (2006). Distributed neural control of energy balance: contributions from hindbrain and hypothalamus. *Obesity (Silver Spring)* 14 [Suppl5], 216S – 221S.
- Grinspoon, L. and Hedblom, P. (1975). Chapter 1: The contemporary Amphetamine Scene in Speed Culture: Amphetamine use and abuse in america. Cambridge, Harvard University Press , pp 11-28
- Gras, C., Herzog, E., Bellenchi, G.C., Bernard, V., Ravassard, P., Pohl, M....El Mestikawy, S. (2002). A third vesicular glutamate transporter expressed by cholinergic and serotonergic neurons. *J. Neurosci.*, 22, 5442 – 5451.
- Grinspoon, L. and Hedblom, P. (1975). Chapter 1: The contemporary Amphetamine Scene in Speed Culture: Amphetamine use and abuse in america. Cambridge, Harvard University Press , pp 11-28

- Gonzalez, R., Rippeth, J.D., Carey, C.L., Heaton, R.K., Moore, D.J., Schweinsburg, B.C... Grant, I. (2004). Neurocognitive performance of methamphetamine users discordant for history of marijuana exposure. *Drug and Alcohol Dependence*, 76(2), 181 – 190.
- Guo, Z., Jiang, H., Xu, X., Duan, W., Mattson, M.P. (2007). Leptin-mediated cell survival signaling in hippocampal neurons mediated by Jak /Stat3 and mitochondrial stabilization. *J Biol Chem.*, 282(3), 1754 – 63.
- Hagan, M.M., Havel, P.J., Seeley, R.J., Woods, S.C., Ekhardt, N.N., Baker... Geraciotti, T.D. (1999). Cerebrospinal fluid and plasma leptin measurements: covariability with dopamine and cortisol in fasting humans. *J Clin Endocrinol Metab* 84, 3579 – 3585.
- Halaas, J.L., Gajiwala, K.S., Maffei, M., Cohen, S.L., Chait, B.T., Rabinowitz, D... Friedman, J.M. (1995). Weight-reducing effects of the plasma protein encoded by the obese gene. *Science*, 269(5223), 543 – 546.
- Harvey, J., Hardy, S.C., Irving, A.J., Ashford, M.L. (2000). Leptin activation of ATP-sensitive K⁺ (KATP) channels in rat C RI-G1 insulinoma cells involves disruption of the actin cytoskeleton. *J Physiol*, 527, 95 – 107.
- Harvey, J. (2007). Leptin regulation of neuronal excitability and cognitive function. *Current opinion in pharmacology*, 7(6), 643 – 647.
- Hashimoto, K., Tsukada, H., Nishiyama, S., Fukumoto, D. Kakiuchi, T., Shimizu, E., Iyo, M. (2004) protective effects of N-acetyl-L-cysteine on the reduction of dopamine transporters in the striatum of monkeys treated with methamphetamine. *Neuropsychopharmacology*, 29, 2018 – 2023.
- Heinz, S., and Tseng, K. (2010). Handbook of Basal Ganglia Structure and Function: A Decade of Progress (Handbook of Behavioral Neuroscience). Academic Press, Elsevier, Burlington, MA.
- Hileman, S.M., Pierroz, D.D., Masuzaki, H., Bjorbaek, C., El-Haschimi, K., Banks, W.A., Flier, J.S. (2002). Characterization of short isoforms of the leptin receptor in rat cerebral microvessels and of brain uptake of leptin in mouse models of obesity. *Endocrinology* 143, 775 –783.
- Hirata, H., and Cadet, J.L. (1997). p53-knockout mice are protected against the long-term effects of methamphetamine on dopaminergic terminals and cell bodies. *J. Neurochemistry*, 69(2), 780 –790.
- Hnasko, T.S., Chuhma, N., Zhang, H., Goh, G.Y., Sulzer, D., Palmiter, R.D., Rayport, S., Edwards, R.H. (2010). Vesicular Glutamate Transport Promotes Dopamine Storage and Glutamate Corelease In Vivo. *Neuron*, 65(5), 643 – 656.
- Hogan, K.A., Staal, R.G., Sonsalla, P.K. (2000). Analysis of VMAT2 binding after methamphetamine or MPTP treatment: disparity between homogenates and vesicle preparations. *J. Neurochemistry*, 74(5), 2217 – 2220.

- Homer, B.D., Solomon, T.M., Moeller, R.W., Mascia, A., DeRaleau, L., Halkitis, P.N. (2008). Methamphetamine abuse and impairment of social functioning: a review of the underlying neurophysiological causes and behavioral implications. *Psychological Bulletin*, 134(2), 301 – 310.
- Hommel, J.D., Trinko, R., Sears, R.M., Georgescu, D., Liu, Z.W., Gao, X.B... DiLeone, R.J. (2006). Leptin receptor signaling in midbrain dopamine neurons regulates feeding. *Neuron*, 51, 801 – 810.
- Hosoi, T., Okuma, Y., Nomura, Y. (2000). Expression of leptin receptors and induction of IL-1beta transcript in glial cells. *Biochem Biophys Res Commun.*, 273, 312 – 315.
- Hotchkiss, A.J., Morgan, M. E., Gibb, J.W. (1979). The long-term effects of multiple doses of methamphetamine on neostriatal tryptophan hydroxylase, tyrosine hydroxylase, choline acetyltransferase and glutamate decarboxylase activities. *Life Sci*, 25(16), 1373 – 1378.
- Hotchkiss, A.J. and Gibb, J.W. (1980). Long-term effects of multiple doses of methamphetamine on tryptophan hydroxylase and tyrosine hydroxylase activity in rat brain. *J. Pharmacol Exp Ther.*, 214(2), 257 – 262.
- Hunot, S., Boissière, F., Faucheux, B., Brugg, B., Mouatt-Prigent, A., Agid, Y., Hirsch, E.C. (1996). Nitric oxide synthase and neuronal vulnerability in Parkinson's disease. *Neuroscience*, 72(2), 355 – 63.
- Imam, S.Z., Itzhak, Y., Cadet, J.L., Islam, F., Slikker Jr., W., Ali, S.F., (2001). Methamphetamine-induced alteration in striatal p53 and bcl-2 expressions in mice. *Brain Res. Mol. Brain Research*, 91, 174 – 178.
- Itzhak, Y., and Ali, S.F. (1996). The neuronal nitric oxide synthase inhibitor, 7-nitroindazole, protects against methamphetamine-induced neurotoxicity in vivo. *J. Neurochemistry*, 67, 1770 – 1773.
- Itzhak, Y., Martin, J. L., Ali, S. F. (1999). Methamphetamine- and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced dopaminergic neurotoxicity in inducible nitric oxide synthase-deficient mice, *Synapse*, 34, 305–312.
- Jayanthi, S., Deng, X., Noailles, P.A., Landenheim, B., Cadet, J.L (2004). Methamphetamine induces neuronal apoptosis via cross-talk between endoplasmic reticulum and mitochondria-dependent death cascades. *FASEB J*, 18, 238 – 251.
- Johnson, M., G. R. Hanson, R., Gibb, J.W. (1989). Effect of MK-801 on the decrease in tryptophan hydroxylase induced by methamphetamine and its methylenedioxy analog. *Eur J. Pharmacology*, 165(2-3), 315 – 318.
- Kandel ER., Schwartz JH., Jessell TM., (2000). Principles of neural science - New York. McGraw-Hill, Health Professions Division- 4th Ed.

- Kavanaugh, M.P. (1998). Neurotransmitter transport: models in flux. *Proc Natl Acad Sci USA*, 95(22), 12737 – 12738.
- Kawaguchi, Y., Wilson, C.J., Augood, S.J., Emson, P.C. (1995). Striatal interneurons: chemical, physiological and morphological characterization. *Trends Neurosci.*, 18(12), 527 – 535.
- Kobeissy, F.H., Jeung, J.A., Warren, M.W., Geier, J.E., Gold, M.S. (2008). Changes in leptin, ghrelin, growth hormone and neuropeptide-Y after an acute model of MDMA and methamphetamine exposure in rats. *Addict Biol.*, 13, 15 – 25.
- Koob, G., LeMoal, M. (1997). Drug abuse: hedonic homeostatic dysregulation. *Science*, 278, 52-58.
- Kovachich, G. B., Aronson, C. E., Brunswick, D. (1992). Effect of repeated administration of antidepressants on serotonin uptake sites in limbic and neocortical structures of rat brain determined by quantitative autoradiography. *Neuropsychopharmacology*, 7(4), 317 – 324.
- Krasnova I.N., Landenheim, B., Jayanthi, S., Oyler J., Moran, T.H., Huestis, M.A., Cadet, J.L (2001). Amphetamine-induced toxicity in dopamine terminals on CD-1 and C57BL/6J mice: complex roles for oxygen based species and temperature regulation. *Neuroscience*, 107(2), 265 – 274.
- Krasnova, I.N. and Cadet, J.L. (2009). Methamphetamine toxicity and messengers of death. *Brain Research Reviews*, 60(2), 379 – 407.
- Krasnova, I.N., Justinova, Z., Ladenheim, B., Jayanthi, S., McCoy, M.T., Barnes, C... Cadet, J.L. (2010). Methamphetamine self-administration is associated with persistent biochemical alterations in striatal and cortical dopaminergic terminals in the rat. *PLoS, One* 5(1), e8790.
- Krueger, J.M., Rector, D.M., Roy, S., Hans, P.A., Dongen, V., Belenky, G., Panksepp, J. (2008). Sleep as a fundamental property of neuronal assemblies. *Nature Reviews Neurosci.* 9, 910 – 911.
- Kumar, U. (2008) Somatostatin in medium-sized aspiny interneurons of striatum is responsible for their preservation in quinolinic acid and N-methyl-D-aspartate-induced neurotoxicity. *J. Mol. Neurosci.*, 35, 345 – 354.
- Lamb, R.J., and Henningfield, J.E. (1994). Human d-amphetamine drug discrimination: methamphetamine and hydromorphone. *J Exp Anal Behav*, 61, 169 – 180.
- Larsen, K.E, Fon, E.A., Hastings, T.G, Edwards, R.H., and Sulzer, D. (2002). Methamphetamine-induced neurotoxicity degeneration of dopaminergic neurons involves autophagy and upregulation of dopamine synthesis. *Jur. Neurosci*, 22, 8952 – 8960.
- Lavin, A., Nogueira, L., Lapish, C.C., Wightman, R.M., Phillips, P.E., Seamans, J.K. (2005). Mesocortical dopamine neurons operate in distinct temporal domains using multimodal signaling. *J Neuroscience*, 25, 5013 – 5023

- LaVoie, M.J., Hastings, T.G. (1999). Dopamine quinine formation and protein modification associated with striatal neurotoxicity of methamphetamine: evidence against the role for extracellular dopamine. *J. Neuroscience*, 19, 1484 – 1491.
- Lee, G.H., Proenca, R., Montez, J.M., Carroll, K.M., Darvishzadeh, J.G., Friedman, J.M. (1996). Abnormal splicing of the leptin receptor in diabetic mice. *Nature*, 379(6566), 632 – 635.
- Leininger, G.M., Jo, Y.H., Leshan, R.L., Louis, G.W., Yang, H., Barrera, J.G... Myers, M.G. (2009). Leptin acts via leptin receptor-expressing lateral hypothalamic neurons to modulate the mesolimbic dopamine system and suppress feeding. *Cell Metab*, 10, 89 – 98.
- LeSauter, J., Hoque, N., Weintraub, M., Pfaff, D.W., Silver, R. (2009). Stomach ghrelin- secreting cells as food-entrainable circadian clocks. *Proc Natl Acad Sci USA*, 106(32), 13582 – 13587.
- Leshan, R.L., Louis, G.W., Jo, Y.H., Rhodes, C.J., Munzberg, H., Myers, M.G. Jr (2009) Direct innervation of GnRH neurons by metabolic- and sexual odorant-sensing leptin receptor neurons in the hypothalamic ventral premammillary nucleus. *J. Neuroscience*, 29, 3138 – 3147.
- Liberatore, G.T., Jackson-Lewis, V., Vukosavic, S., Mandir, A.S., Vila, M., McAuliffe, W.G... Przedborski, S. (1999). Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease. *Nat Med*, 5(12), 1403 – 1409.
- Licinio, J., Mantzoros, C., Negrao, A.B., Cizza, G., Wong, M.L., Bongiorno, P.B....Flier, J.S., Gold, P.W. (1997). Human leptin levels are pulsatile and inversely related to pituitary- adrenal function. *Nat Med*, 3(5), 575 – 579.
- Licinio, J., Negrao, A.B., Mantzoros, C., Kaklamani, V., Wong, M.L., Bongiorno, P.B... Gold, P.W. (1998). Sex differences in circulating human leptin pulse amplitude: clinical implications. *J. Clin Endocrinol Metab*, 83, 4140 – 4147.
- Liechti, M.E. and Vollenweider, F.X. (2000). The serotonin uptake inhibitor citalopram Reduces acute cardiovascular and vegetative effects of 3,4 methylenedioxymethamphetamine ('Ecstasy') in healthy volunteers. *J. Psychopharmacology*, 14(3), 269 – 274.
- Liu, M. (2004). Leptin: a piece of the obesity pie. *The Science Creative Quarterly. University of British Columbia*.
- Lu, X.Y., Kim, C.S., Frazer, A., Zhang, W. (2006) Leptin: a potential novel antidepressant. *Proc Natl Acad Sci USA*, 103, 1593 – 1598.
- Madej, T., Boguski, M.S., Bryant, S.H. (1995). Threading analysis suggests that the obese gene product may be a helical cytokine. *FEBS Lett*, 373, 13 – 18.
- Maffei, M., Fei, H., Lee, G.H., Dani, C., Leroy, P., Zhang, Y... Friedman, J.M. (1995). Increased expression in adipocytes of ob RNA in mice with lesions of the hypothalamus and with mutations at the db locus. *Proc Natl Acad Sci, USA* 92(15): 6957 – 6960.

- Maffei, M., Halaas, J., Ravussin, R.E., Pratley, G.H., Lee, Y., Zhang, H... Friedman, J.M. (1995). Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat Med*, 1(11), 1155 – 1161.
- Mantzoros, C.S., and Moschos, S.J. (1998). Leptin: in search of role(s) in human physiology and pathophysiology. *Clin Endocrinol (Oxf)*, 49, 551 – 567.
- Mark, K.A., Soghomonian, J.J., Yamamoto, B.K. (2004). High-dose methamphetamine acutely activates the striatonigral pathway to increase striatal glutamate and mediate long-term dopamine toxicity. *Journal of Neuroscience*, 24(50), 11449 – 11456.
- Marshall, J.F., O'Dell, S.J., Weihmuller, F.B. (1993). Dopamine-glutamate interactions in methamphetamine-induced neurotoxicity. *J. Neural Transm Gen Sect*, 91(2-3), 241 – 254.
- Mattson M. P., Perry T., Greig N. (2003) Learning from the gut. *Nat. Med*, 9, 1113 – 1115.
- McCann, U.D., Wong, D.F., Yokoi, F., Villemagne, V., Dannals, R.F., Ricaurte G.A. (1998). Reduced striatal dopamine transporter density in abstinent methamphetamine and methcathinone users: evidence from positron emission tomography studies with [¹¹C]WIN-35,428. *J. Neurosci*. 18, 8417 – 8422.
- McCann, U.D., and Ricaurte, G.A. (2004). Amphetamine neurotoxicity: accomplishments and remaining challenges. *Neurosci Biobehav Rev*, 27(8), 821 – 826.
- Melega, W.P., Williams, A.E., Schmitz, D.A., DiStefano, E.W., Cho, A.K. (1995). Pharmacokinetic and pharmacodynamic analysis of the actions of D-amphetamine and D-methamphetamine on the dopamine terminal. *J. Pharmacol Exp Ther*, 274, 90 – 96.
- Melega, W.P., Raleigh, M.J., Stout, D.B., Lacan, G., Huang, S.C., Phelps, M.E. (1997). Recovery of striatal dopamine function after acute amphetamine- and methamphetamine-induced neurotoxicity in the vervet monkey. *Brain Res*, 766, 113 – 120.
- Meredith, C.W., Jaffe, C., Ang-Lee, K., Saxon, A.J. (2005). Implications of chronic methamphetamine use: a literature review. *Harv. Rev. Psychiatry*, 13, 141 – 154.
- Morton G.J., Cummings D.E., Baskin D.G., Barsh G.S., and Schwartz M.W. (2006). Central nervous system control of food intake and body weight. *Nature*, 443, 289 – 295.
- Miller, M.A., and Hughes, A.L., (1994). Epidemiology of amphetamine use in the United States. In: Cho, A.K., Segal, D.S. (Eds.), Amphetamine and its Analogs. *Academic Press*, San Diego, p. 503.
- Mistlberger, R.E. (1994). Circadian food-anticipatory activity: formal models and physiological mechanisms. *Neurosci Biobehav Rev*, 18, 171 – 195.
- Myers, M.G., Jr, Munzberg, H., Leininger, G.M. and Leshan, R.L. et al. (2009). The geometry of leptin action in the brain: more complicated than a simple ARC. *Cell Metab*, 9, 117 – 123.

- Najib, S., Sanchez-Margalet, V. (2002). Human leptin promotes survival of human circulating blood monocytes prone to apoptosis by activation of p42/44 MAPK pathway. *Cell. Immunol*, 220, 143 – 149.
- Nakayama, M., Koyama, T., Yamashita, I. (1993). Long-lasting decrease in dopamine uptake sites following repeated administration of methamphetamine in the rat striatum. *Brain Res*, 601(1-2), 209 – 212.
- Nash, J.F. and Yamamoto, B.K. (1992). Methamphetamine neurotoxicity and striatal glutamate release: comparison to 3,4-methylenedioxymethamphetamine. *Brain Res*, 581(2), 237-243.
- National Drug Intelligence Center (NDIC), Department of Justice (2011). National methamphetamine threat assessment 2011 report document accessed.
- National institute on drug abuse (NIDA) (2010). Drug Pages methamphetamine <http://drugabuse.gov/DrugPages/Methamphetamine.html>. (Retrieved May 15, 2013)
- Ni, B., Rosteck, P.R. Jr., Nadi, N.S., Paul, S.M. (1994). Cloning and expression of a cDNA encoding a brain-specific Na(+)- dependent inorganic phosphate cotransporter. *Proc Natl Acad Sci USA*, 91, 5607 – 5611.
- Nicosa, N., Pacula, R.L., Kilmer, B., Lundberg, R., Chiesa, J. (2005). Economic cost of methamphetamine use in the US, 2005, Santamonica, CA: Drug Policy Res. Cent., RAND corp. <http://www.rand.org/pubs/monographs/MG829.pdf>
- O'Dell, S.J., Weihmuller, F.B., Marshall, J.F. (1992). MK-801 prevents methamphetamine-induced striatal dopamine damage and reduces extracellular dopamine overflow, *Ann. N.Y. Acad. Sci*, 648, 317 – 319.
- O'Neil, M.L., Kuczenski, R., Segal, D.S., Cho, A.K., Lacan, G., Melga, W.P. (2006). Escalating dose pretreatment induces pharmacodynamic and not pharmacokinetic tolerance to a subsequent high-dose methamphetamine binge. *Synapse*, 60, 465 – 473.
- Pacher, P., Beckman, J.S., Liaudet, L. (2007). Nitric oxide and peroxynitrite in health and disease. *Physiol. Rev.*, 87, 315 – 424.
- Pendergast, J.S., Oda, G.A., Niswender, K.D., Yamazaki, S. (2012). Period determination in the food-entrainable and methamphetamine-sensitive circadian oscillator(s). *Proc Natl Acad Sci USA*, 109, 14218 – 14223.
- Perschak, H. and Cuenod, M. (1990). In vivo release of endogenous glutamate and aspartate in the rat striatum during stimulation of the cortex. *Neuroscience*, 35(2). 283 – 287.
- Przedborski, S., Jackson-Lewis, V., Yokoyama, R., Shibata, T. Dawson, V.L., Dawson, T.M. (1996). Role of nitric oxide in 1-methyl-4-phenyl-1,2,3,6-tetrapyridine (MPTP)-induced dopaminergic neurotoxicity. *J. Neurosci.*, 12, 1658 – 1667.

- Pu, C. and C. V. Vorhees (1993). Developmental dissociation of methamphetamine-induced depletion of dopaminergic terminals and astrocyte reaction in rat striatum. *Brain Res Dev Brain Res.*, 72(2), 325 – 328.
- Pu, C., Zhu, K., Wang, G. (1995). Complications in multiple systems resulting from acute cerebrovascular diseases. *Chinese journal of internal medicine*, 34(8), 525 – 527.
- Raju, D.V., Ahern, T.H., Shah, D.J., Wright, T.M., Standaert, D.G., Hall, R.A., Smith, Y. (2008). Differential synaptic plasticity of the corticostriatal and thalamostriatal systems in an MPTP-treated monkey model of Parkinsonism. *Eur. J. Neurosci.*, 27, 1647 – 1658.
- Rasley, A., Bost, K.L., Olson, J.K., Miller, S.D., Marriot, I. (2002). Expression of functional NK-1 receptors in murine microglia, *Glia*, 37, 258 – 267.
- Ricaurte, G.A., Guillery, R.W., Seiden, L.S., Schuster, C.R. (1984). Nerve terminal degeneration after a single injection of D-amphetamine in iprindole-treated rats: relation to selective long-lasting dopamine depletion. *Brain Res.*, 291(2), 378 – 382.
- Ricaurte, G.A., Guillery, R.W., Seiden, L.S., Schuster, C.R., Moore, R.Y. (1982). Dopamine nerve terminal degeneration produced by high doses of methylamphetamine in the rat brain. *Brain Res.*, 235(1), 93 – 103.
- Ricaurte, G.A., Schuster, C.R., Seiden, L.S. (1980). Long-term effects of repeated methylamphetamine administration on dopamine and serotonin neurons in the rat brain: a regional study. *Brain Res.*, 193(1), 153 – 163.
- Ricaurte, G.A., Seiden, L.S., Schuster, C.R., (1984). Further evidence that amphetamines produce long-lasting dopamine neurochemical deficits by destroying dopamine nerve fibers. *Brain Res.*, 303(2), 359 – 364.
- Riddle, E.L., Fleckenstein, A.E., Hanson, G.R. (2006). Mechanisms of methamphetamine-induced neurotoxicity. *AAPSJ*, 8, E413 – E418.
- Rocha, B.A., Fumagalli, F., Gainetdinov, R.R., Jones, S.R., Ator, R., Giros, B.... Caron, M.G. (1998). Cocaine self-administration in dopamine-transporter knockout mice. *Nat Neurosci.*, 1(2), 132 – 137.
- Rodrigues, T.B., Granado, N., Ortiz, O., Cerdán, S., Moratalla, R. (2007). Metabolic interactions between glutamatergic and dopaminergic neurotransmitter systems are mediated through D(1) dopamine receptors. *J. Neurosci Res.*, 85, 3284 – 3293.
- Salisbury, J.J., and Wolgin, D.L. (1985). Role of anorexia and behavioral activation in amphetamine-induced suppression of feeding: implications for understanding tolerance. *Behav Neurosci.*, 99, 1153 – 1161.
- Salo, R., Nordahl, T.E., Possin, K., Leamon, M., Gibson, D.R., Galloway, G.P... Sullivan, E.V. (2002). Preliminary evidence of reduced cognitive inhibition in methamphetamine-dependent individuals. *Psychiatry Research*, 111, 65 – 74.

- Santolaria, F., Perez-Cejas, A., Aleman, M.R., Gonzalez-Reimers, E., Milena, A., De la Vega, M.J... Gomez-Rodriguez, M.A. (2003). Low serum leptin levels and malnutrition in chronic alcohol misusers hospitalized by somatic complications. *Alcohol Alcoholism*, 38(1), 60 – 66.
- Schmued, L.C., Albertson, C., Slikker, W. (1997). Fluoro-Jade: a novel fluorochrome for the sensitive and reliable histochemical localization of neuronal degeneration. *Brain Res.*, 751(1), 37 – 46.
- Schulz, J.B., Matthews, R.T., Jenkins, B.G., Ferrante, R.J., Siwek, D., Henshaw, D.R... Beal, M.F. (1995). Blockade of neuronal nitric oxide synthase protects against excitotoxicity in vivo. *J. Neurosci.*, 15, 8419 – 8429.
- Schepers, R.J., Oyler, J.M., Joseph Jr., R.E., Cone, E.J., Moolchan, E.T., Huestis, M.A. (2003). Methamphetamine and amphetamine pharmacokinetics in oral fluid and plasma after controlled oral methamphetamine administration to human volunteers. *Clin. Chem.*, 49, 121 – 132.
- Schindler, C., and Darnell, J.E. (1995). Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. *Annu Rev Biochem.*, 65, 621 – 51.
- Schneider, C. A.; Rasband, W. S. & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature methods* 9(7): 671-675, [PMID 22930834](#).
- Scott, J.C., Woods, S.P., Matt, G.E., Meyer, R.A., Heaton, R.K., Atkinson, J.H., Grant, I. (2007). Neurocognitive effects of methamphetamine: a critical review and meta-analysis. *Neuropsychol Rev.*, 17(3), 275 – 297.
- Scott, M.M., Lachey, J.L., Sternson, S.M., Lee, C.E., Elias, C.F., Friedman, J.M., Elmquist, J.K. (2009). Leptin targets in the mouse brain. *J. Comp Neurol.*, 514, 518 – 532.
- Segal, D.S., Kuczenski, R., O'Neil, M.L., Melega, W.P., Cho, A.K. (2005). Prolonged exposure of rats to intravenous methamphetamine: behavioral and neurochemical characterization. *Psychopharmacology*, 180(3), 501 – 512.
- Seiden, L. S. (1975). Brain monoamines and behavior. *Psychopharmacol Bull.*, 11(2), 60 – 61.
- Seiden, L.S., Commins, D.L. et al. (1988). Neurotoxicity in dopamine and 5-hydroxytryptamine terminal fields: a regional analysis in nigrostriatal and mesolimbic projections. *Ann NY Acad Sci.*, 537, 161 – 172.
- Sekine, Y., Iyo, M., Matsunaga, T., Tsukada, H., Okada, H., Yoshikawa, E.... Mori, H. (2001). Methamphetamine-related psychiatric symptoms and reduced brain dopamine transporters studied with PET. *Am J. Psychiatry*, 158(8), 1206 – 1214.
- Sekine, Y., Minabe, Y., Ouchi, Y., Takei, N., Iyo, M., Nakamura, K... Mori, N., (2003). Association of dopamine transporter loss in the orbitofrontal and dorsolateral prefrontal cortices with methamphetamine-related psychiatric symptoms. *Am. J. Psychiatry* 160(9), 1699 – 1701.

- Sekine, Y., Ouchi, Y., Takei, N., Yoshikawa, E., Nakamura, K., Futatsubashi, M... Mori, N. (2006). Brain serotonin transporter density and aggression in abstinent methamphetamine abusers. *Arch Gen Psychiatry* 63(1), 90 – 100.
- Sekine, Y., Minabe, Y., Ouchi, Y., Takei, N., Iyo, M., Nakamura, K.... Mori, N. (2003). Association of dopamine transporter loss in the orbitofrontal and dorsolateral prefrontal cortices with methamphetamine-related psychiatric symptoms. *Am. J. Psychiatry* 160, 1699 – 1701.
- Shanley, L.J., Irving, A.J., Harvey, J. (2001). Leptin enhances NMDA receptor function and modulates hippocampal synaptic plasticity. *J. Neurosci*, 21, RC186.
- Shanley, L.J., O'Malley, D., Irving, A.J., Ashford, M.L., Harvey, J. (2002). Leptin inhibits epileptiform-like activity in rat hippocampal neurons via PI 3-kinase-driven activation of BK channels. *J. Physiol*, 545, 933 – 944.
- Shoblock, J.R., Sullivan, E.B., Maisonneuve, I.M., Glick, S.D. (2003). Neurochemical and behavioral differences between d-methamphetamine and d-amphetamine in rats. *Psychopharmacology*, 165, 359 – 369.
- Silva, A.P., Pinheiro, P.S., Carvalho, A.P., Carvalho, C.M., Jakobsen, B., Zimmer, J., Malva, J.O. (2003). Activation of neuropeptide Y receptors is neuroprotective against excitotoxicity hippocampal slice cultures. *FASEBJ*, 17, 1118 – 1120.
- Silva, A.P., Xapelli, S., Grouzmann, E., Cavadas, C., (2005). The putative neuroprotective role of neuropeptide Y in the central nervous system. *Curr. Drug Targets CNS Neurol. Disord.*, 4(4), 331 – 347.
- Spiegelman, B.M. and Flier, J.S. (1996). Adipogenesis and obesity: rounding out the big picture. *Cell*, 87(3), 377 – 389.
- Sonsalla, P.K., Gibb, J.W., Hanson, G.R. (1986). Roles of D1 and D2 dopamine receptor subtypes in mediating the methamphetamine-induced changes in monoamine systems. *J. Pharmacol Exp Ther.*, 238, 932 – 937.
- Sonsalla, P.K., Nicklas, W.J., Heikkila, R.E., (1989). Role for excitatory amino acids in methamphetamine-induced nigrostriatal dopaminergic toxicity. *Science* 243, 398 – 400.
- Sonsalla, P., Jochnowitz, N.D., Zeevalk, G.D., Oostveen, J.A., Hall E.D. (1996). Treatment of mice with methamphetamine produces cell loss in the substantia nigra. *Brain Res.* 738(1), 172 – 175.
- Steiner, H., and Tseng, K.Y. (2010). Handbook of basal ganglia structure and function. Amsterdam: Elsevier/Academic Press.
- Stephans, S.E. and Yamamoto, B.Y. (1995). Effect of repeated methamphetamine administrations on dopamine and glutamate efflux in rat prefrontal cortex. *Brain Res.*, 700(1-2), 99 – 106.

- Substance Abuse and Mental Health Services Administration (SAMHSA). Results from the (2011) National Survey on Drug Use and Health.
- Sulzer, D., Sonders, M.S. Poulsen, N.W., Galli, A. (2005). Mechanisms of neurotransmitter release by amphetamines: a review. *Prog Neurobiol*, 75(6), 406 – 433.
- Takamori, S. (2006). VGLUTs: 'exciting' times for glutamatergic research? *Neurosci. Res.*, 55, 343 – 351.
- Tartaglia, L.A. (1997). The leptin receptor. *J. Biol Chem.*, 272, 6093 – 6096.
- Terui, K., Enosawa, S., Haga, S., Zhang, H.Q., Kuroda, H., Kouchi, K... Ozaki, M. (2004). Stat3 confers resistance against hypoxia/reoxygenation-induced oxidative injury in hepatocytes through upregulation of Mn-SOD. *J. Hepatology*, 41(6), 957 – 965.
- Thermos, K., Bagnoli, P., Epelbaum, J., Hoyer, D. (2006). The somatostatin sst1 receptor: An autoreceptor in brain and retina? *Pharmacol. Ther.*, 110, 455 – 464.
- Thiriet, N., Deng, X., Solinas, M., Ladenheim, B., Curtis, W., Goldberg, S.R... Cadet, J.L. (2005). Neuropeptide Y protects against methamphetamine-induced neuronal apoptosis in the mouse striatum. *J. Neurosci.*, 25, 5273 – 5279.
- U.S. Drug Enforcement Administration. (2015, January 24). Drug schedules. Retrieved from <http://www.dea.gov/druginfo/ds.shtml>.
- United Nations Office on Drugs and Crime (UNODC). World Drug Report Annual overview 2014. http://www.unodc.org/documents/wdr2014/World_Drug_Report_2014 web.pdf. (Accessed 4 December 2014).
- Villemagne, V., Yuan, J., Wong, D.F. Dannals, R.F., Hatzidimitriou, G., Mathews, W.B... Ricaurte, G.A. (1998). Brain dopamine neurotoxicity in baboons treated with doses of methamphetamine comparable to those recreationally abused by humans: evidence from [11C]WIN-35,428 positron emission tomography studies and direct in vitro determinations. *J. Neurosci.*, 18(1), 419 – 427.
- Volkow, N.D., Chang, L., Wang, G.J., Fowler, J.S., Leonido-Yee, M., Franceschi, D... Miller, E.N. (2001). Association of dopamine transporter reduction with psychomotor impairment in methamphetamine abusers. *Am J Psychiatry*, 158(3), 377 – 382.
- Volkow, N.D., Fowler, J.S., Gatley, S.J., Dewey, S.L., Wang, G.J., Logan, J... King, P. (1999). Comparable changes in synaptic dopamine induced by methylphenidate and by cocaine in the baboon brain. *Synapse*, 31(1), 59 – 66.
- Volkow, N.D., Wang, G., Fowler, J.S., Logan, J., Gerasimov, M., Maynard, L.... Franceschi, D. (2001). Therapeutic doses of oral methylphenidate significantly increase extracellular dopamine in the human brain. *J. Neurosci.*, 21(2), RC121.

- Wagner, G.C., Ricaurte, G.A., Seiden, L., Schuster, C.R., Miller, R.J., Westley, J. (1980). Long-lasting depletions of striatal dopamine and loss of dopamine uptake sites following repeated administration of methamphetamine. *Brain Res.*, 181(1), 151 – 160.
- Wang, J., Xu, W., Ali, S.F., Angulo, J.A. (2008). Connection between the striatal neurokinin-1 receptor and nitric oxide formation during methamphetamine exposure. *Annals of the New York Academy of Sciences*, 1139, 164 – 171.
- Wang, J. and Angulo, J.A. (2011). Methamphetamine induces striatal neurokinin-1 receptor endocytosis primarily in somatostatin/NPY/NOS interneurons and the role of dopamine receptors in mice. *Synapse*, 65, 300 – 308.
- Warren, M.W., Kobeissy, F.H., Liu, M.C., Hayes, R.L., Gold, M.S., Wang, K.K. (2005). Concurrent calpain and caspase-3 mediated proteolysis of alpha II-spectrin and tau in rat brain after methamphetamine exposure: a similar profile to traumatic brain injury. *Life Sci.*, 78, 301 – 309.
- West, A.R., Galloway, M.P., Grace, A.A. (2002). Regulation of striatal dopamine neurotransmission by nitric oxide: effector pathways and signaling mechanisms. *Synapse*, 44, 227 – 245.
- Winslow, B. T., Voorhees, K.I., Pehl, K.A. (2007). Methamphetamine abuse. *Am Fam Physician*, 76(8), 1169 – 1174.
- Wisor, J.P., Nishino, S., Sora, I., Uhi, G.H., Mignot, E., Edgar, D.M. (2001). Dopaminergic role in stimulant-induced wakefulness. *J. Neurosci.*, 21, 1787 – 1794.
- Woods, S.P., Rippeth, J. D., Conover, E., Gongvatana, A., Gonzalez, R., Carey, C. L... HIV Neurobehavioral Research Center Group. (2005). Deficient strategic control of verbal encoding and retrieval in individuals with methamphetamine dependence. *Neuropsychology*, 19(1), 35 – 43.
- Wu, W., Liuzzi, F.J., Schinco, F.P., Depto, A.S., Li, Y., Mong, J.A... Snyder, S.H. (1994). Neuronal nitric oxide synthase is induced in spinal neurons by traumatic injury. *Neurosci.*, 61, 719 – 726.
- Xu, W., Zhu, J.P., Angulo, J.A., (2005). Induction of striatal pre- and postsynaptic damage by methamphetamine requires the dopamine receptors. *Synapse*, 58, 110–121.
- Yarosh, H.L., Angulo, J.A. (2012). Modulation of methamphetamine- induced nitric oxide production by neuropeptide Y in the murine striatum. *Brain Res.*, 1483, 31 – 38.
- Yamamoto, B.K., Zhu, W. (1998). The effects of methamphetamine on the production of free radicals and oxidative stress. *J. Pharmacol. Exp. Ther.*, 287, 107 – 114.
- Yamamoto, B. K., Gudelsky, G. A. and Stephans, S. E. (1998). Amphetamine neurotoxicity: Roles for dopamine, glutamate and oxidative stress, in *Neurochemical Markers of Degenerative Nervous Diseases and Drug Addiction* (Qureshi G. A., ed), pp-223-244. VSP Press, Utrecht, Netherlands.

- Zhang, F., Wang, S., Signore, A., Chen, J. (2007). Neuroprotective effects of letpin against ischemic injury induced by oxygen-glucose deprivation and transient cerebral ischemia. *Stroke*, 38, 2329 – 2336.
- Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., Friedman, J.M. (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature* 372(6505), 425 – 432.
- Zhang, Y., Dawson, V.L., Dawson T.M. (2000). Oxidative stress and genetics in the pathogenesis of Parkinson's disease. *Neurobiol. Dis.*, 7, 240 – 250.
- Zhu, J.P.Q., Xu, W. and Angulo, J.A. (2005). Disparity in the temporal appearance of methamphetamine-induced apoptosis and depletion of dopamine terminal markers in the striatum of mice, *Brain Res.*, 1049, 171-181.
- Zhu, J.P.Q., Xu, W. and Angulo, J.A. (2006a) Distinct mechanisms mediating methamphetamine- induced neuronal apoptosis and dopamine terminal damage share the neuropeptide substance P in the striatum of mice. *Ann. N.Y. Acad. Sci.*, 1074, 135 – 148.
- Zhu, J.P.Q., Xu, W. and Angulo, J. A. (2006b). Methamphetamine-induced cell death: Selective vulnerability in neuronal subpopulations of the striatum of mice. *Neurosci.*, 140, 607 – 622.
- Zhu, J.P.Q., Xu, W., Wang, J., Ali, S., and Angulo, J.A. (2009). The neurokinin-1 receptor modulates the methamphetamine-induced striatal apoptosis and nitric oxide formation in mice. *J. Neurochem.*, 111, 656 – 668.