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The Role of the Striatal Neuropeptide Neurotensin in the Methamphetamine-induced Neural Injury in Mice

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The Role of the Striatal Neuropeptide Neurotensin in the Methamphetamine-induced Neural Injury in Mice

By

Qingkun Liu

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The City University of New York

2014
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ABSTRACT

The Role of the Striatal Neuropeptide Neurotensin in the Methamphetamine-induced Neural Injury in Mice

By
Qingkun Liu

Advisor: Professor Jesus A. Angulo

Methamphetamine (METH) is a widely abused psychostimulant that induces neurotoxicity to several brain regions, including the striatum. Similar to dopamine (DA) in chemical structure, METH can be transported into DA pre-synaptic terminals, evoking neurodegeneration in DA terminals and post-synaptic striatal neurons. Despite the critical role of DA in METH-induced neurodegeneration, no pharmaceutical therapeutics has been approved for these conditions. It is therefore essential to investigate endogenous factors regulating the dopaminergic system. The neuropeptide neurotensin has emerged as a potential modulator of METH-induced striatal neurodegeneration mainly due to its intimate interactions with dopamine in the striatum.

In this study, we investigated the role of the neuropeptide neurotensin on METH-induced striatal neurodegeneration in mice. We observed that a single injection of METH (30 mg/kg, ip) induced the loss of approximately 15% of striatal neurons. An agonist of the neurotensin receptor 1 (PD149163, ip) attenuated the METH-induced striatal neuron apoptosis in a dose-dependent manner, while exerted no effect on METH-induced dopamine terminal degeneration. Utilizing quantitative Real Time PCR, we showed that METH also up-regulated neurotensin gene expression by 96% in stratal neurotensin mRNA. These data demonstrate that neurotensin modulates METH-induced striatal apoptosis through neurotensin receptor 1 (NTR1) in the striatum. In addition, NTR1 agonist attenuated METH-induced hyperthermia and can also attenuate the striatal apoptosis independent of such body temperature regulatoin. To further investigate the corresponding mechanisms, we assessed its effect on glial cell activation, nitric oxide accumulation and DARPP32 phosphorylation in the striatum, which are all believed to
aggravate METH-induced neurodegeneration. We observed that the NTR1 agonist attenuated the effects of METH on each of these three biomarkers. Our results also show that the NTR1 agonist alone caused decrease in phosphorylation of DARPP32 at Thr34, while NTR1 antagonist (SR48692) per se increased the phosphorylation of DARPP32 at Thr34. Since the DARPP32 phosphorylation pathway is responsible for interpreting signals to striatal projection neurons, neurotensin possibly modulates its phosphorylation through regulation of DA and glutamate neurotransmission. Finally, the agonist of neurotensin, PD149163, may be considered as a potential therapeutic for treatment of METH-induced neurotoxicity. (Supported by R01 DA020142 from NIDA/NIH)
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LIST OF ABBREVIATIONS

3-NT: 3-Nitrotyrosine
5-HT: Serotonin
AMPH: Amphetamine
APAF-1: Apoptotic Protease Activating Factor
Ca: Calcium
cAMP: Cyclic Adenine Monophosphate
cGMP: Cyclic Guanine Monophosphate
CNS: Central Nervous System
Cy3: Cyanine 3
D1R: Dopamine-1 Receptor
D2R: Dopamine-2 Receptor
DA: Dopamine
DARPP-32: Dopamine and Adenosine 3’,5’-monophosphate-regulated phosphoprotein (32 kilodaltons)
DAT: Dopamine Transporter
Dk: Donkey
ER: Endoplasmic Reticulum
eNOS: Endothelial Nitric Oxide Synthase
FITC: Fluorescein Isothiocyanate
GABA: Gamma-aminobutyric acid
GC: Guanylyl Cyclase
Gt: Goat
GTP: Guanine Nucleotide Triphosphate
iNOS: Inducible Nitric Oxide Synthase
IF: Immunofluorescence
L-DOPA: L-dihydroxyphenylalanine
MAO: monoamine oxidase
METH: Methamphetamine
NK-1R: Neurokinin-1 Receptor
NMDA: N-methyl-D-Aspartate Receptor
nNOS: Neuronal Nitric Oxide Synthase
NOS: Nitric Oxide Synthase
NO: Nitric Oxide
NPY: Neuropeptide Y
PARP: Poly (ADP-ribose) polymerase
PC-12 cells: Pheochromocytoma cells
PD: Parkinson’s disease
PFA: Paraformaldehyde
PFC: Prefrontal Cortex
Rb: Rabbit
ROS: Reactive Oxygen Species
RNS: Reactive Nitrogen Species
TH: Tyrosine Hydroxylase
TUNEL: Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
Tx-PBS: Triton-X 100 Phosphate Buffered Saline
VMAT 2: Vesicular Monoamine Transporter 2
VTA: Ventral Tegmental Area
SPECIFIC AIMS

Role of the Striatal Neuropeptide Neurotensin on the Methamphetamine-induced Neural Injury in Mice

Aim 1: To investigate the hypothesis that neurotensin attenuates the METH-induced striatal apoptosis and neurotoxicity of the dopamine terminals.

Aim 1a: To assess effect of PD149163 on METH-induced striatal neuron apoptosis

Hypothesis: We expect that a neurotensin agonist will attenuate the METH-induced apoptosis of some striatal neurons in a dose-dependent manner.

Rationale: We used the Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay to detect the apoptosis level of striatal neurons. TUNEL detects the early apoptotic process, not cascade-3, a later marker for apoptosis, which may not appear during the early stages of apoptosis.

Aim 1b: To investigate whether activation of neurotensin receptor 1 by PD149163 could attenuate striatal dopamine terminal degeneration induced by METH.

Hypothesis: We expect that a neurotensin agonist would attenuate depletion of tyrosine hydroxylase induced by METH.

Rationale: We assessed the level of tyrosine hydroxylase with western blot. Tyrosine hydroxylase acts as biomarker for neurotoxicity of dopamine terminals along with dopamine content and dopamine transporter in both METH abuser and rodent.
Aim 2: To investigate the mechanisms by which neurotensin protect against METH-induced striatal neuron apoptosis

Aim 2a: To testify the hypothesis that neuroprotection by neurotensin results in part from inhibition of glial cell activation.

**Hypothesis:** We predict that a neurotensin agonist will attenuate or prevent the METH-induced activation of striatal microglia and astrocytes.

**Rationale:** Since our preliminary data have shown that neurotensin dramatically attenuates METH-induced apoptosis of striatal neurons, we propose to explore the precise protective mechanism of neurotensin. Since both microglial and astrocyte cells contribute to METH-induced neurodegeneration through activation and neuron damage is among several contributing factors for activation of glial cells, we will test whether neurotensin attenuates their activation.

Aim 2b: To test the hypothesis that neurotensin attenuates the METH-induced accumulation of reactive nitrogen species.

**Hypothesis:** We expect that a neurotensin agonist will increase the METH-induced accumulation of nitric oxide, and that a neurotensin antagonist will produce the opposite effect.

**Rationale:** Nitric oxide and reactive oxygen species production are both induced by METH administration and interact to produce mass generation of reactive nitric species (RNS). Damage by RNS is one reason for METH-induced neurotoxicity. Since neurotensin attenuates METH-induced striatal apoptosis and NO contributes to this neurotoxicity, we will test whether neurotensin exerts neuroprotection by inhibiting NO overproduction induced by METH with level of cGMP, a biomaker for NO in vivo.
Aim 2c: To investigate the effect of neurotensin on the phosphorylation of DARPP-32.

**Hypothesis:** We hypothesize that a neurotensin receptor agonist will attenuate the METH-induced phosphorylation of DARPP-32, while NTR1 antagonist augment this phosphorylation in the striatum.

**Rationale:** Since neurotensin regulates dopamine and glutamate neurotransmission and DARPP-32 plays a central role in dopamine signaling inside striatal MSNs, we hypothesize that neurotensin regulates the DARPP-32 phosphorylation pathway through its effect on dopamine. This hypothesis is supported in part by our discovery that neurotensin pre-treatment prevents METH-induced striatal apoptosis, which is regulated by DARPP-32.

Aim 2d: To investigate the effect of METH and neurotensin on levels of preproneurotensin mRNA in striatal tissue.

**Hypothesis:** We hypothesize that METH will increase the levels of preproneurotensin mRNA in striatal tissue.

**Rationale:** Peptidergic systems of the striatum have been shown to be highly dynamic, displaying changes in response to various stimuli affecting dopaminergic, glutamatergic and GABAergic transmissions (Greengard P, 2001). Since delivery of the neurotensin receptor agonist prevents apoptosis of striatal neurons, and neurotensin regulates both dopamine and glutamate neurotransmissions, the main METH targets inside the striatum, we predict that METH administration will decrease the intracellular neurotensin peptide pool. Consequently, preproneurotensin mRNA levels will increase to restore the peptide pool following METH administration. Sixteen animals will be required for this experiment.
Aim 2e: To investigate whether activation of neurotensin receptor attenuates the METH-induced hyperthermia

**Hypothesis:** We hypothesize that neurotensin receptor 1 agonist would induce low body temperature and thereby attenuate METH-induced hyperthermia.

**Rationale:** Recent literatures support that METH-induced hyperthermia contributes to neurodegeneration. For example, blockage of such hyperthermia by environmental condition attenuates METH-induced striatal apoptosis, dopamine terminal neurodegeneration and also glial cell activation in mice. Activation of neurotensin receptor 1 has been reported to induce hypothermia instead, which may counter the increase of body temperature induced by METH. We detected the body core temperature of mice with a BAT-12 thermometer coupled to RET-3 mouse rectal probe.

Aim 2f: To investigate whether activation of neurotensin receptor attenuates the METH-induced apoptosis independent of body temperature regulation

**Hypothesis:** we proposed that neurotensin receptor 1 agonist, PD149163, attenuate METH-induced striatal apoptosis independent of its body temperature regulation.

**Rationale:** in the brain, hypothalamus but not striatum controls the body temperature and neurotensin exert the effects to regulate body temperature through neurotensin receptor 1 which also locates in the hypothalamus. Therefore it is possible to disable function of neurotensin receptor 1 on body temperature regulation through intrastriatal infusion. And since neurotensin receptor 1 can regulate dopamine and glutamate neurotransmission in the striatum, it is potential that it attenuates the METH-induced striatal apoptosis independent of body temperature
regulation.
CHAPTER 1. INTRODUCTION

Methamphetamine (N-Methyl-O-phenylisopropylamine, “METH”) is an analog of amphetamine (alpha-Methyl-phenethyl-amine) used as a psychostimulant with potent sympathetic and CNS action (Davidson et al., 2001; Sulzer et al., 2005). Although both molecules are similar, METH is more lipophilic and crosses the blood-brain barrier more readily, making the molecule more potent and toxic than its parent compound (Meredith et al., 2005).

1. Epidemiology of Methamphetamine abuse
As a synthetic psychostimulant, METH can be injected, smoked, snorted, ingested or transrectally administered (Anglin et al., 2000; Cantrell et al., 2006). The current METH use epidemic in the U.S. began in the mid-1980s on the West Coast and has since spread across the western states to the Midwest, southern states, and the East Coast (Rose and Grant, 2008). While METH use has been reported on every continent, the vast majority of METH users reside in East and South-East Asia as well as North America (United Nations Office on Drugs and Crime, 2013). In China, the largest METH market in the world, the prevalence of METH use among new drug users is estimated at 5.6%, which is surpassed only by heroin use among new users (Lu et al., 2008). In North America, Mexico has become the largest producer of METH as a result of restrictions on precursor chemicals in the U.S. and Canada (Ritsner, 2011), but the west coasts of the U.S. and Canada have also been greatly affected by METH use (Maxwell and Rutkowski, 2008; National Drug Intelligence Center, 2006). The estimated annual prevalence rate in the general U.S. population for METH use was 1.4% in 2006 (Maxwell and Rutkowski, 2008).

2. Social and financial costs of Methamphetamine abuse
METH use poses significant public health challenges and economic burdens. In a recent economic assessment, METH use cost the United States $23.4 billion in 2005 alone (Gonzales,
2010). This expenditure included costs associated with morbidity and mortality, criminal justice and social welfare services, environmental damage from METH chemical production, and, most significantly, lost productivity and reduced quality of life (Nicosia, 2009). Tremendous social harms associated with METH are experienced by neighborhoods, including METH production, trafficking and dealing, and related systemic violence. The manufacture and production of METH require chemicals that are explosive, corrosive, and flammable and that can result in burns, respiratory ailments, poisoning, and fires (Gonzales et al., 2010). Further, manufacturing of METH in automobiles increases the likelihood of spilling toxic wastes and chemicals into the environment and polluting ground water sources (Holton, 2001).

3. METH-induced neurodegeneration

Compounding these challenges, a considerable body of evidence indicates that METH abuse can lead to serious and persistent cognitive, psychiatric, and neurological dysfunction in the user, and can negatively affect the development of children exposed to METH in utero and the well-being of children raised by METH-addicted parents (Chandler, 2010). Despite severe METH abuse in the U.S. and worldwide, we do not fully understand the mechanisms through which METH triggers neurodegeneration and dysfunction of the central nervous system. Such understanding could contribute to the development of medications to treat the consequences of neurotoxicity.

Although METH abuse leads to dysfunction in many other areas of the brain, including the prefrontal cortex and hippocampus (Graham et al., 2008; Kuczenski et al., 2007), this project focused on the striatum, the major component of the basal ganglia, given its psychomotor behavioral control function. The striatum controls a broad range of behaviors including learning, motivation, habit-forming, and addictive behaviors, and experiences severe neurodegeneration
with METH administration (Kawagushi, 1997; Koob and Le Moal, 1997; Balleine et al., 2007).

3.1 Striatal neurodegeneration due to METH use

3.1.1 Striatum structure and function

The striatum, also known as the neostriatum or striate nucleus, is a subcortical part of the forebrain. It is the major input station of the basal ganglia system. Most cortical areas provide inputs to the basal ganglia, which in turn provide outputs to the brain systems involved in the generation of behaviors. The vast majority of the striatal neurons are medium spiny projection neurons, the activity of which is stimulated as follows: 1) excitatory inputs from the cerebral cortex and thalamus with glutamate; and, 2) feedback pathways from the substantia nigra pars compacta (SNc) by dopaminergic neurons (Kawaguchi et al., 1997; DiFiglia et al., 1976; Wilson and Groves, 1980; Bishop et al., 1982; Kemp and Powell, 1971). Essentially all the functions ascribed to the striatum are intimately linked to their rich dopaminergic innervation, which originates in the midbrain SNc (Somogyi et al., 1981). In addition to the spiny projection neurons that comprise up to 97.7% of striatal neurons in rodents (Rymar et al., 2004), the striatum is composed of one type of cholinergic interneuron and electrophysiologically distinct GABAergic interneurons that extend axons within but not outside the striatum (Kawaguchi, 1993; Kawaguchi et al., 1995; Tepper and Bolam, 2004; Tepper et al., 2008). Striatal cholinergic neurons, which use acetylcholine as a neurotransmitter, constitute an important type of interneuron (Bolam et al., 1984; Wilson et al., 1990; Kawaguchi, 1993). Although acetylcholine release is clearly important to striatal function, the neuroanatomical substrates by which this is regulated have been difficult to identify (Paxinos, 2004).

The second major subtype of striatal interneurons is characterized morphologically as a medium-
sized aspiny neuron that uses GABA as the main neurotransmitter. Although few in number, striatal GABAergic interneurons play a predominant role in regulating spike timing in the spiny output neurons through feedforward inhibition (Tepper et al., 2004, 2008). The most abundant type of GABAergic interneurons expresses the calcium-binding protein parvalbumin (Gerfen et al., 1985; Cowan et al., 1990; Kita et al., 1990). A second class of GABAergic interneurons co-expresses somatostatin, neuropeptide Y, and nitric oxide synthetase (Vincent et al., 1983; Smith and Parent, 1986; Pasik et al., 1988; Dawson et al., 1991). In addition to the neurons, glial cells, mainly astrocytes and microglia, support and protect neurons as innate immune cells inside the striatum (Jesse et al., 1980; Gourine, et al., 2010).

Given its similar molecular structure to dopamine (DA), METH enters the dopamine axons of striatum via dopamine transporters (DAT) and passive diffusion (Iversen, 2006). Within these neurons, METH enters synaptic vesicles through VMAT-2 and causes DA release into the cytoplasm via changes in pH balance. By reversing transport of DA by DAT into the synaptic cleft from the cytoplasm, METH dramatically increases the concentration of DA between the synapses of DA terminals and striatal neurons (Sulzer et al., 2005).

3.1.2 Neuronal degeneration of striatum after METH abuse

The neurotoxic effects of METH on striatal dopaminergic neurons are suggested to be mediated by excess DA release (Stephans and Yamamoto, 1994). Numerous studies have confirmed this hypothesis and suggested that once DA is released from synaptic vesicles to the cytoplasm of DA terminals by METH, DA then auto-oxidizes into potentially toxic chemicals, including superoxide radicals, hydroxyl radicals, hydrogen peroxide and DA quinone (DAQ) (Acikgoz et al., 1998; Kita et al., 1999; LaVoie and Hastings, 1999; Lazzeri et al., 2007; Miyazaki et al.,
2006). These reactive oxygen species (ROS) in the DA terminals then diffuse from the dopamine terminals to the post-synaptic neurons (i.e. MSNs) and damage them by reacting with proteasomes, mitochondria and DNA, leading to protein misfolding, proteasomal and mitochondrial dysfunction and eventually programmed cell death (Halliwell, 1992; Andersen, 2004).

Our lab has discovered that 25% of the projecting neurons in the striatum experienced apoptosis after acute METH administration in mice. Although new neurons were generated after apoptosis, the function of these newly generated neurons is unknown (Tulloch et al., 2011). In addition, METH induces the neurotoxicity of dopamine terminals from the nigral-striatal pathway marked by a decrease of at least 50% in Tyrosine hydroxylase (TH) level, increased synapse DA level and reduced DAT after acute METH administration in mice (Yu et al., 2002; Zhu et al., 2005). These three biomarkers of the DA terminal contribute to the DA neurotransmission to striatal medium spiny neurons differentially. Tyrosine hydroxylase is the rate-limiting enzyme that catalyzes the biosynthesis of DA from tyrosine (Luvone et al., 1978; Daubner et al., 2011). Due to decreased levels of this enzyme, DA productivity is dramatically reduced as demonstrated by lower DA levels inside the striatum (Kogan et al., 1976; Krasnova et al., 2009). DAT normally transport the released DA from synapses back to the DA terminals (Kahlig et al., 2003). By doing this, DAT prevent the over excitation of the medium spiny projecting neurons. After the METH administration, instead of recycling the dopamine, DAT transports more DA outside the cytoplasm to over excite the striatal neurons (Wilson, 1996). As a result, DA as the neurotransmitter accumulates between the synapses, forming a nigral-striatal pathway.
3.2. Potential mechanisms of METH-induced neurodegeneration

DA neurotransmission may play a significant role in METH-induced neurodegeneration of DA terminals and striatal neuronal apoptosis. Other factors are also involved in this process, including hyperthermia, oxidative stress, nitric oxide, glial cell activation, and the DARPP32 (dopamine and cAMP-regulated phosphoprotein, molecular weight=32 kD) pathway.

3.2.1 Neurotransmission disruption by METH and DARPP32 pathway

METH interrupts several neurotransmitters in the striatum, including dopamine, glutamate and nitric oxide. The striatal medium spiny projecting neurons as main input and output neurons in the striatum adjust their function according to these three neurotransmitters through their receptors from SNc, the prefrontal cortex and interneurons of the striatum (Steiner and Tseng, 2010). After entering the neurons, METH increases release of both dopamine and nitric oxide into the striatum. The dopamine also increases the glutamate release, thereby causing signal transmission dysfunction of the striatum. METH increases the dopamine release by reversing the transmission of dopamine out of the cytoplasm via the dopamine transporter, which not only over-excites the striatal projecting neurons, but also up regulates the glutamate input from the prefrontal cortex, causing excitotoxic damage (Sulzer et al., 2005; Abekawa et al., 1994; Baldwin et al., 1993; Mark et al., 2004; Marshall et al., 1993). Both dopamine and glutamate manipulate function of striatal neurons through the DARPP32 pathway (Greengard, 2001). DARPP32 is highly concentrated in the striatum. The striatal medium spiny neurons that contain DARPP32 are the only neurons that convey an efferent pathway from this major brain region. These neurons interpret information entering the striatum from many other regions in the brain, and DARPP32 plays a central role in this integration process. Activation of dopamine receptors 1
by DA causes an increase in the level of cAMP, the activation of PKA, and the phosphorylation of threonine-34 of DARPP32. This pDARPP32 is activated to inhibit protein protease 1, which controls the state of phosphorylation and activities of numerous physiologically important substrates, including neurotransmitter receptors, voltage-gated ion channels, ion pumps, and transcription factors (Figure 1).
Figure 1. Signaling pathways in the neostriatum. Activation of the dopamine receptors D1 by dopamine stimulates the phosphorylation of DARPP-32 at Thr-34. This is achieved through a pathway involving the activation of adenylyl cyclase, the formation of cAMP, and the activation of PKA. Activation by dopamine of the D2 subclass of dopamine receptors causes the dephosphorylation of DARPP-32 through two synergistic mechanisms as indicated with green and orange arrows. In response to glutamate, activation of the AMPA and NMDA subclasses of glutamate receptor increases intracellular calcium and the activity of PP2B, and causes the dephosphorylation of DARPP-32 on Thr-34. All other neurotransmitters that have been shown to act directly to alter the physiology of dopaminoceptive neurons also alter the phosphorylation state of DARPP-32 on Thr-34 through the indicated pathways. Neurotransmitters that act indirectly to affect the physiology of these dopaminoceptive neurons also regulate DARPP-32 phosphorylation: e.g., neurotensin and cholecystokinin (CCK). (Adapted from Paul Greengard, 2001.)

Deletion of the DARPP-32 gene in animals has been shown to significantly reduce biological effects of all drugs of abuse (Byrne and Roberts, 2009). Drugs of abuse, all of which affect the physiology of these neurons, also regulate the state of DARPP-32 phosphorylation (Greengard, 2001). Since METH could up regulate the level of both dopamine and glutamate, which exert opposite regulations on this pathway, we hypothesized that the delicate balance of this DARPP-
32 phosphorylation and dephosphorylation would shift and trigger the neurodegeneration of the striatal neurons through protein phosphatase 1 (PP1).

Nitric Oxide (NO), a small molecular neurotransmitter, may also contribute to neurotoxicity by METH. The first evidence comes from the finding that after METH administration, the NO level indicated by 3-NT, an indirect marker for NO, has been increased by nearly seven times (Zhu et al., 2005). In addition, 7-nitroindazole and ILH, inhibitors of the NO-producing enzymes in neurons and microglia, respectively, could both significantly reduce the apoptosis of striatal neurons. The mechanism behind these phenomena may comprise three components. First, NO is found to increase DARPP-32 phosphorylation in striatal MSNs of brain slices through the NO-cGMP-PKG pathway (Tsou et al., 1993; Nishi et al., 2005), which may cause degeneration of striatal neurons.

Second, NO may exert its biological effect by regulating the glutamate neurotransmission. It is generally accepted that NO signaling can amplify excitatory neurotransmission at glutamatergic synapses in numerous brain regions (Garthwaite, 2008). Studies using reduced striatal preparations have shown that NO can inhibit glutamate transporters, leading to increases in extracellular glutamate concentrations. In vivo microdialysis studies in rats have also shown that intrastriatal infusion of both NO synthase (NOS) substrate and NO generators increased local glutamate efflux up to two-fold over basal levels (Guevara-Guzman et al., 1994; West and Galloway, 1997).

In addition, NO also contributes to regulation of dopamine release. Numerous studies performed in rats, mice and hamsters have demonstrated that endogenous NO produced via intrastriatal substrate (L-arginine) infusion facilitates DA efflux in vitro (Zhu and Luo, 1992; Liang and
Kaufman, 1998) and in vivo (Strasser et al., 1994; West and Galloway, 1997). The upregulation of dopamine release will undoubtedly augment the METH-induced dopamine overflow and thus cause further degeneration and over excitation of post-synaptic neurons, such as striatal medium spiny neurons.

3.2.2 METH-induced hyperthermia

METH-induced hyperthermia is well recognized as a significant contributing factor to neurodegeneration in rodents. In rats, temperature may reach above 40°C after each injection of amphetamine (Burrows and Meshul, 1999; Burrows et al., 2000; Kokoshka et al., 2000; Sandoval et al., 2000) where hyperthermia-induced death can occur. Chronic METH (20mg/kg/day×7 days) is also hyperthermic for the first 2 to 3 days, and can also result in death (Davidson et al., 2001). It has been shown that increased body temperature can induce oxidative stress in many systems (Lin et al., 1991; Omar et al., 1987; Skibba et al., 1991) in addition to neurodegeneration from cerebrovascular damage; this may be a mechanism whereby hyperthermia mediates neurodegeneration. METH-induced mild hyperthermia is an important component of METH-induced dopaminergic neurotoxicity (Evan L Riddle et al., 2006), as evidenced by the high correlation between the degree of changes in the DA system, or the degree of neurodegeneration, and the degree of hyperthermia (Bowyer et al., 1994; Sandoval et al., 2000; Davidson et al., 2001). In addition, prevention of hyperthermia attenuates METH-induced DA decrease and loss of DA uptake in the striatum (Bowyer et al., 1992; 1993; 1994). Due to the significant role of hyperthermia in METH-induced neurodegeneration, it is necessary to detect the effect of any drug candidate on body temperature regulation to rule out hyperthermia as a contributing factor to neurochemical and other changes.
3.2.3 Role of reactive oxygen species and nitric oxide (NO)

Reactive Oxygen Species (ROS) are chemically reactive molecules containing oxygen, which include superoxide, hydroxyl radical and peroxide (Devasagayam et al., 2004). ROS form as a natural byproduct through endogenous mechanisms such as mitochondrial respiration or via exogenous agents that augment glutamate activity and auto-oxidative processes in brain tissues (Bindoli et al., 1992; Cadet and Brannock, 1998). In several studies, overproduction of ROS by METH administration has been found to induce DA terminal neurodegeneration and striatal neuron apoptosis (Potashkin and Meredith, 2006; Fitzmaurice et al., 2006; Stone et al., 1989). METH-induced depletion of DA and 5-HT in the rat striatum are exacerbated by the superoxide dismutase (SOD) inhibitor, diethyldithiocarbamate (De Vito and Wagner, 1989). The role of ROS in METH-induced neurotoxicity is also supported by homozygous transgenic mice, which overexpress copper/zinc superoxide dismutase (CuZnSOD). These transgenic mice are nearly resistant to METH-induced neurotoxicity of the DA terminal, increase in caspase-3 activity and neuronal death (Deng and Cadet, 2000; Cadet et al., 1994; Hirata et al., 1996; Epstein et al., 1987).

Through reaction with ROS, nitric oxide also contributes to METH-induced neurodegeneration. There are three types of nitric oxide synthase, neuronal NOS (nNOS), immunological NOS (iNOS), and endothelial NOS (eNOS) (Nathan and Xie, 1994; Bredt and Snyder, 1994). Pharmacological inhibition of nNOS or deletion of this gene protects DA terminals against METH-induced toxicity (Itzhak and Ali, 1996; Imam et al., 2001a, 2001b). In addition, nNOS knockout mice (nNOS -/-) are protected from METH-induced dopaminergic neurotoxicity (Itzhak et al., 1998). These studies indicate the role of NO in METH-induced dopaminergic damage. Indeed, NO reacts with superoxide radicals (O2-) to produce Peroxynitrite (OONO-), a
potent oxidant (Radi et al., 1991). We have reported that the METH treatment that causes
dopaminergic neurotoxicity also induces a six-fold increase of 3-nitrotyrosine (3-NT), an in vivo
biomarker for OONO- production in the mouse striatum (Zhu et al., 2009). In addition, both
nNOS knockout mice and CuZnSOD transgenic mice resisted DA terminal damage induced by
METH. These results suggest the involvement of NO and ROS in METH-induced DA terminal
damage. There is also evidence showing that ROS induces neuronal apoptosis in vitro by
reacting with NO (Troy et al., 1996). Combining these findings, it is likely that ROS and NO are
closely correlated in their chemical reaction of producing peroxynitrite that may trigger METH-
induced neurotoxicity.

3.2.4 Glial cells contribute to neurodegeneration

Glial cells are non-neuronal cells that maintain homeostasis, form myelin, and provide support
and protection for neurons in the brain (Kristjan et al., 1980). There are two general types of glia
in the central nervous system: the macroglia class, which consists of astrocytes and
oligodendrocytes, and the microglia class, which consists mainly of microglia and has
macrophage-like properties (Haydon, 2001). Of the macroglia class, astrocytes are highly
numerous and are likely to have many divergent roles. microglia and astrocytes are the two most
abundant glial cells in the brain that monitor neurodamage and inflammation. Microglia are the
resident innate immune cells in the central nervous system and produce a barrage of factors (IL-
1, TNFα, NO, PGE2, superoxide) that are toxic to neurons. Evidence supports the activation of
microglia in response to environmental toxins, endogenous proteins and neuronal death, resulting
in the production of toxic factors that propagate neuronal injury (Gao et al., 2002; Wu et al.,
2005; Qin et al., 2002; Sanchez-Moreno et al., 2004).
An increasing number of studies indicate that activated glial cells may also contribute to neurotoxicity from METH. Using the PET technique, Yoshimoto and colleagues reported that METH causes microglial activation in the brains of human abusers (Yoshimoto et al., 2008). The LaVoie laboratory also indicated that microglial activation preceded the appearance of pathological changes in striatal DA fibers due to METH administration (LaVoie et al., 2004). The involvement of glial cells in METH toxicity is also supported by tolerance in mice to neurotoxic effects if the microglial activation is attenuated after METH administration (Thomas and Kuhn, 2005). Our lab has also shown that astrocytes are activated and peak their activation three days after METH administration (Zhu et al., 2005). Nevertheless, Sriram also showed that attenuation of microglial activity alone is not sufficient to protect against METH neurotoxicity, indicating the need for further study to explore the role of glial cells on METH-induced neurodegeneration (Sriram et al., 2006).

4. Neurotensin: candidates for neuroprotection

4.1 METH and neuropeptides

The central nervous system uses two main classes of chemical substances for signaling: small molecule transmitters such as dopamine and glutamate, and neuroactive peptides, which are short polymers of amino acids (Kandel et al., 2000). There are two types of neurons comprising the striatum; namely, interneurons and projection neurons. The striatal medium-sized spiny neurons, i.e., striatal projection neurons that comprise over 90% of striatal neurons, produce several neuropeptides as neurotransmitters such as substance P and neurotensin. Both striatal projection neurons and interneurons go through apoptosis after acute METH administration, except for somatostatin/nNOS/NPY interneurons, the only interneurons producing neuropeptides
neuropeptide Y and somatostatin (Zhu et al., 2006). Our laboratory has been investigating the role of endogenous striatal neuropeptides that modulate the METH-induced elevation of oxidative stress. Striatal neuropeptides are strategically placed to modulate striatal dopaminergic responses. The striatal neuropeptide substance P synergizes with METH in elevating the production of nitric oxide (Wang and Angulo, 2011a) and consequently striatal injury (Zhu et al., 2009). In particular, METH-induced DA overflow initially induces neuropeptide substance P production in striatal projection neurons, which then activate Somatostatin/nNOS/NPY interneurons, leading to nitric oxide overproduction, finally triggering neuronal apoptosis. Interestingly, two other striatal neuropeptides, somatostatin and neuropeptide Y, attenuate the METH-induced striatal production of nitric oxide (Yarosh and Angulo, 2012; Afanador et al., 2013).

4.2 Endogenous neuropeptide neurotensin

In addition to substance P, neuropeptide Y, and somatostatin, neurotensin may also play an essential role in METH-induced striatal neurodegeneration due to its intimate interactions with dopamine in the striatum. Neurotensin (NT) is a 13 amino acid peptide that was first isolated from the calf hypothalamus by Carraway and Leeman in 1973. NT derived its name from its location in the brain and its tendency to cause hypotension in animals when injected peripherally. The amino acid sequence of neurotensin is Pyr-Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH (Carraway and Leeman, 1975). In the brain, NT is particularly expressed within the nigrostriatal and mesolimbic dopamine system (Jennes et al., 1982; Leeman and Carraway, 1982) and most of its effects are mediated through the activation of two different G protein-coupled receptor subtypes: neurotensin receptor 1 (NTR1) and neurotensin receptor 2 (NTR2) (Vincent et al., 1999). Within the basal ganglia, NT is closely associated with the nigrostriatal
DA and striatopallidal GABA neurons. Its high affinity receptor (NTR1) is widely expressed (mRNA) on dopaminergic neurons in the substantia nigra while only small amounts of NTR2 are found in the striatum. In the striatum, NTR1 expression seems associated with the dopaminergic terminals from the nigral neurons and glutamatergic terminals from cortical inputs (Nicot et al., 1994; Alexander and Leeman, 1998). The close co-distribution between NT and DA systems together with the functional evidence that NT modulates dopaminergic signaling both in limbic and in striatal brain regions have suggested that NT may be implicated in the pathophysiology of various central nervous system disorders, including schizophrenia, Parkinson’s disease and drug abuse of cocaine and METH (Nemeroff, 1980; McMahon et al., 2002; Caceda et al., 2006; Kinkead and Nemeroff, 2006; St-Gelais et al., 2006).

Evidence of neurotensin participating in the psychostimulant-induced physiological phenomenon has also emerged. For example, early stereotactic experiments demonstrated that neurotensin produces psychostimulant-like effects following site-specific microinjection in the ventral tegmental area (VTA) (Kalivas et al., 1983; Kalivas et al., 1981). However, neurotensin administration in the NAc attenuates amphetamine-stimulated locomotor activity (Ervin et al, 1981; Robledo et al., 1993). These different effects most likely reflect differences in the neurons influenced by neurotensin signaling in these respective locations. Psychostimulants are also found to increase neurotensin expression in striatal D1-positive neurons and to elevate extracellular neurotensin levels in the striatum through a mechanism involving both D1 and NMDA receptor signaling (Wagstaff et al., 1997). This suggests that neurotensin is released from D1 positive neurons.
Neurotensin receptor 1 (NTR1) is the primary neurotensin receptor associated with dopamine neurons anatomically and also the major neurotensin receptor found in the striatum. Therefore, most research on neurotensin has emphasized NTR1 activity in the striatum. Several investigations have demonstrated that systemic delivery of NTR1 agonist PD1494163 or neurotensin analog NT69L attenuates amphetamine-induced hyperactivity or METH self-administration in rodents. In addition, NT injection into the nucleus accumbens reduces the responses to psychostimulants (Ervin et al., 1981; Robledo et al., 1993.). Mona Boules also reported that NT69L, neurotensin analog, blocks cocaine- and D-amphetamine-induced hyperactivity (Boules et al., 2001). In addition, NT69L blocked the acute locomotor effects of nicotine, as well as the initiation and expression of sensitization to this stimulant (Fredrickson et al., 2003. a, b).

The potential mechanism by which NT might be involved in amphetamine activation of D1 positive striatal neurons is shown in Figure 2, below. Psychostimulants have been shown to increase extracellular levels of striatal NT, and this release involves signaling through both dopamine D1 and NMDA receptors (Wagstaff et al., 1997). NTR-1 has been localized to both nigral dopamine and cortical projection neurons, but has not been localized to striatal neurons in the dorsal striatum (Boudin et al., 1996). NT has been shown to antagonize D2 receptor function and to enhance amphetamine-evoked increases in extracellular dopamine levels through antagonism of D2 auto-inhibition under certain circumstances (Diaz-Cabiale et al., 2002; Pehek, 1999). These findings indicate that NT in the striatum will augment dopamine and glutamate release through NTR1 as shown in figure 2.

Both glutamate and DA neurotransmissions affect DARPP32 phosphorylation pathway in the
pos-synaptic neurons, e.g. MSNs, which controls numerous physiological function including the programmed cell death (apoptosis). Glutamate release induced by neurotensin attenuates the DARPP32 phosphorylation (Figure 1), while dopamine release promotes such phosphorylation. Although neurotensin augments dopamine and glutamate neurotransmission modulating the phosphorylation of DARPP32, no information about the effect of neurotensin in DARPP32 phosphorylation in vivo is available. We intend to assess this effect by detecting the levels of phosphorylated DARPP32 and total DARPP32 affected by METH and NTR1 agonist in the striatum.
Figure 2. Neurotensin regulates dopamine and glutamate neurotransmission in the striatum. The release of neurotensin from D1-positive striatal neurons activates the NTR-1 in both substantia nigra dopamine neurons and corticostriatal neurons, which augment the release of dopamine and glutamate and attenuate D2 receptor activation to inhibit the dopamine and glutamate release (adapted from Dobner, 2001).

Besides regulation of DA and glutamate neurotransmission, Neurotensin also reduces body temperature by NTR1. Rise of body temperature induced by METH contributes to dopaminergic neurotoxicity (Riddle et al., 2006) in the striatum, with the evidence of the correlation between the changes in the DA system (the degree of neurodegeneration) and hyperthermia (Davidson et al., 2001). These findings show that neurotensin is likely to attenuate METH-induced hyperthermia through the NTR1 signaling. Despite this circumstantial information, no evidence indicates that neurotensin attenuates METH-induced hyperthermia through systemic delivery. One of the goals is to test the effect of NTR-1 on METH-induced hyperthermia.
As described above, METH administration also induces glial cell activation and Nitric oxide accumulation in the striatum, which causes neurodegeneration through neurotoxic pro-inflammatory factors and oxidative stress. Since METH-induced striatal apoptosis precedes the glial cell activation in the striatum, it is possible that glial cell activation is triggered by METH-induced neuron apoptosis. We assumed that neurotensin attenuates METH-induced glial cell activation and the corresponding over-production of NO from the glial cell activation.

To summarize, we assume that neurotensin probably attenuates METH-induced neurotoxicity, with characterization of reduced striatal neuron apoptosis through DARPP32 pathway, glial cell activation, NO production and body temperature regulation in the striatum.
CHAPTER 2. MATERIAL AND METHODS

1. Animal Care and Use

All procedures involving animal use were approved by the Institutional Animal Care and Use Committee of Hunter College, City University of New York (JAA-Neurotensin, approved 10/09/013) and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (2011). The Hunter College Animal Facility is certificated by the American Association for Accreditation of Laboratory Animal Care (AAALAC). 702 ICR male mice between 12 and 14 weeks old (Taconic; Germantown, NY) were housed individually on a 12-hour light/dark cycle with ad libitum access to food and water. The mice were habituated for at least two weeks prior to commencement of intraperitoneal (i.p.) drug administration.

2. Drug Preparation and Treatment

Neurotensin NTR1 receptor agonist, PD149163 (≥95%; Sigma; St. Louis, MO), a Neurotensin analog (8-13), Lys-(psiCH2NH)-Lys-Pro-Trp-Terleu-Leu-OEt, was dissolved in 10mM PBS to 0.4mg/ml concentration and administered to mice as 0.5mg/kg, 1mg/kg and 2mg/kg of body weight through intraperitoneal injection.

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

For perfusion, animals were fully anesthetized with ketamine (100mg/kg) and acepromazine (3mg/kg), and perfused through the heart with 20ml of PBS followed by 20 ml of 4% paraformaldehyde (PFA) in PBS. The brains were post-fixed overnight in 4% PFA at 4°C followed by incubation in 30% sucrose solution for 24 hours at 4°C for cryoprotection. The brains were then frozen at -80°C until used. Coronal sections 30µm in thickness were cut in a microtome at -20°C and collected serially from the striatum between bregma 0.14 and 1.54 mm. About 50 coronal sections were collected from each animal and stored in anti-freezing solution (30% glycerin solution in ethylene glycol) at -20°C until used. For intrastriatal surgeries, brain
tissue was nicked in the left dorsal cortex for orientation.

3. Intrastriatal Infusion

The surgical technique for intrastriatal infusion was performed as previously described (Wang and Angulo, 2011). In brief, animals were anesthetized by 2.5% isoflurane followed by shaving on the skull. The skin was then disinfected by iodine on a sterile cotton swab. After fixing the head in the stereotaxic instrument, a one-inch incision was made with a hand held razor blade above the injection site. A hole of 1millimeter diameter was drilled in the skull and a 25 gage 2µl Hamilton microinjection syringe was lowered into the striatum. The location of injection sites (bregma, 0.5 mm; lateral, 2.0mm; ventral, 2.5mm) was determined using a mouse brain atlas (Franklin and Paxinos, 1997). The needle was left in the position for five minutes before infusion of 1ul PD149163 (20µM)/SR48692 (20µM) into the right striatum (bregma: 2.0mm, lateral: 0.5mm, ventral: 2.5mm) and aCSF into the left striatum (bregma: 2.0mm, lateral: 0.5mm, ventral: 2.5mm). Thirty minutes after the intrastriatal infusion, 30 mg/kg METH were injected intraperitoneally into the mice. Intrastriatal infusion of PD149163 will eliminate its function to reduce the body temperature, making it possible to detect its role in METH-induced striatal apoptosis independent of body temperature regulation. Intrastriatal infusion of SR48692 will avoid the activation of neurotensin receptor 2 outside the striatum by SR48692.

4. Apoptosis detection: TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) histochemistry

The method is adapted from X. Deng, 2001 with minor modifications. In brief, the 30µm tissue coronal sections from each mouse were dissected and serially collected from the striatum between bregma 0.14 and 1.54mm into six adjacent wells. Five sections were randomly selected from one well of each of the adjacent wells and mounted on frog plus slides. The slides were washed three times in PBS for 10 minutes followed by heating in 0.4% Triton X-100 PBS for 40 min at 70°C. TUNEL reagents were then applied to sections and incubated at 37°C for four hours. The remaining reagents were then removed by cleansing with PBS and the slides were covered with Vectashield mounting medium H1000 and No. 1.5 coverslips. All images were
obtained from both hemispheres of each coronal section using a Leica SP2 Confocal microscope with TRICT filter (Leica Microsystems; Buffalo Grove, IL).

5. Neuronal Nuclei (NeuN) immunohistochemistry

Coronal sections of the striatum were washed with PBS for 10 minutes and then incubated in primary antibody of Anti-NeuN (rabbit polyclonal, 1:50, Millipore; Temecula, CA) for two hours at room temperature. After PBS wash, all sections were incubated in 1:200 Donkey anti-Rabbit secondary antibody (Millipore; Temecula, CA) and then covered with Vectashield mounting medium H1000. All images were obtained from both hemispheres of each coronal section using a Leica SP2 Confocal microscope with Cy3 filter (Leica Microsystems; Buffalo Grove, IL).

6. Cell counts and quantification

All coronal sections were taken from Bregma 0.14-1.54 mm. Cells were counted from 30µm-thick coronal sections in an area of 0.26 mm² for each region of the striatum (lateral and medial). For the sections collected from the microinjected mice, regions around the needle track were spared; the lateral and medial images were then counted. TUNEL and striatal neuronal cell counts were averaged from five 30µm serial sections per animal.

7. Presynaptic neurodegeneration: Immunoblot of Tyrosine Hydroxylase (TH)

All groups of mice were decapitated under full anesthesia with 2.5% isoflurane 72h after i.p. drug injection. All the brains were removed by dissection and kept in -80°C until further operation. A coronal section of the striatum 2mm thick was removed by using a brain blocker on ice and kept at -80°C for protein extraction. Each striatal homogenate in 200ul RIPA buffer was obtained by sonication with a Sonicator 3000 (QSonica; Newtown, CT) cup horn at 6 cycles of 30-second sonication with 60 seconds intermittent on ice. Only the soluble supernatant was collected after centrifuge at 5000rpm for 15 minutes as a protein sample for TH immunoblot. Concentration of each protein sample was detected by the Bradford Method (Bio-Rad; Hercules, CA) and 12µg protein of each sample was boiled for seven minutes at 95°C with a protein-loading buffer (Bio-Rad; Hercules, CA). 10% Tris-HCL SDS-PAGE gel (Bio-Rad; Hercules,
CA) was used to separate proteins only according to protein molecular weight. After transferring proteins to the PDVF membrane with iBlot transfer system (Invitrogen; Carlsbad, CA), the membrane was blocked with an Odyssey blocking buffer for one hour followed by incubation with polyclonal rabbit anti-TH primary antibody (1:5,000, Millipore; Temecula, CA) and monoclonal mouse anti β-actin primary antibody (1:20,000, Sigma; St. Louis, MO) in an Odyssey blocking buffer with 0.2% Tween 20 at 4 degrees overnight. The following day, after 0.1% Tween PBS wash, the membrane was incubated in a mixture of Odyssey IRDye secondary antibodies: donkey anti-rabbit 800CW (1:15,000, Licor; Lincoln, NE) and donkey anti-mouse 680LT (1:22500, Licor; Lincoln, NE) in an Odyssey blocking buffer with 0.2% Tween 20 at room temperature for one hour. After four final washes with 0.1% Tween PBS, the membranes were detected via the Odyssey infrared imager. Bands were quantified by Odyssey Imager analysis software and normalized against actin as internal standards.

8. Quantitative Real Time PCR (qRT-PCR) for Neurotensin and GAPDH in the striatum

Two groups of mice (each contains 6 animals) were decapitated under full anesthesia with 2.5% isoflurane. Brains were dissected and stored at -80°C for further treatment. The striatum was dissected and manually homogenized on ice, and RNA was extracted using RNeasy Mini kit (Qiagen; Valencia, CA). The RNA concentration levels were measured by Nanodrop 1000 (Thermo scientific; Waltham, MA). RNA template of 0.5ng, 5ng, 10ng and 25ng was applied to run the validation experiments for Comparative Ct method. And 10ng of RNA was chosen as the template for the following qRT-PCR. TaqMan Gene Expression Assay of Neurotensin (FAM probe, Life Technologies; Carlsbad, CA) and TaqMan Rodent GAPDH control reagent (VIC probe, Life Technologies; Carlsbad, CA) were used as primers in each qRT-PCR system with TaqMan RNA-to-Ct 1 step kit. Applied BioSystems 7500 Real-Time PCR System was used to operate the following cycles: 48°C for 15 minutes; 95°C for 10 minutes; followed by 40 cycles of 95°C for 15 seconds and 60°C for 1min. All Ct values were detected by 7500 Real-Time PCR System automatically and used for further statistical analysis by the Comparative Ct method.
9. Core body temperature detection

Core body temperature was determined using a BAT-12 thermometer coupled to RET-3 mouse rectal probe (Physitemp Instruments; Clifton, NJ). Individual mouse body temperatures were measured twice to obtain the average. Ambient room temperature was maintained at 20-22°C during the measurement period.

10. Astrocyte cell activation: GFAP immunohistochemistry

Three days after i.p. drug injection of four groups, mice were perfused and dissected as described above. Tissue sections of 30µm were then stored at -20°C until use. Five sections were randomly selected from one well of each six adjacent wells. Coronal sections were then washed with PBS and incubated in anti-GFAP primary antibody (rabbit polyclonal, 1:25, Millipore; Temecula, CA) for two hours at room temperature. After washing with PBS, the sections were mounted with Vectashield mounting medium H1000 and No. 1.5 coverslips. All images were obtained from both hemispheres of each coronal section using Leica SP2 Confocal microscope with TRICT filter (Leica Microsystems; Buffalo Grove, IL).

11. Microglial cell activation: Iba-1 immunohistochemistry

Coronal sections of the striatum were obtained as described in GFAP immunohistochemistry. All sections were then washed with PBS followed by blocking in 5% Donkey serum for 30 minutes. After blocking, all sections were incubated in anti Iba-1 primary antibody (rabbit polyclonal, 1:500, Millipore; Temecula, CA) overnight at 4°C. After PBS wash, the sections were incubated in 1:200 Donkey anti-rabbit secondary antibody (Millipore; Temecula, CA) and then covered with Vectashield mounting medium H1000. All images were obtained from both hemispheres of each coronal section using Leica SP2 Confocal microscope with TRICT filter.

12. Nitric oxide accumulation: cyclic GMP immunohistochemistry

Eight hours after i.p. injection of METH and NTR1 agonist, animals were perfused and coronal sections of the striatum were obtained as described in GFAP immunohistochemistry. All sections were washed with PBS and then blocked in 10% Donkey serum (Sigma; St. Louis, MO) in 0.1%
Tx-PBS for 30 minutes. After three PBS washes, the sections were incubated in anti-cGMP primary antibody (rabbit polyclonal, 1:200, Millipore; Temecula, CA) for 18 hours at 4°C. After another PBS wash, all sections were incubated in 1:200 Donkey anti-rabbit secondary antibody (Millipore; Temecula, CA) in 1% donkey serum (Sigma; St. Louis, MO) and then mounted. All dry sections were then covered with Vectashield mounting medium H1000 and No. 1.5 coverslips. Leica SP2 Confocal microscope with TRICT filter was used to obtain all images from both striata of each coronal section.

13. Immunoblot of DARPP32 and phosphorylated DARPP32 at Thr34 (p34 DARPP32)

To analyze the DARPP32 and p34 DARPP32 protein levels affected by the NTR1 agonist, PD149163 was i.p. injected into mice 30 minutes before METH administration. For analysis of protein levels in NTR1 antagonist experiment, SR49682 was infused into the striatum through stereotactic injection followed by METH administration 30 minutes later. One hour after METH injection, all animals were decapitated and a section of the striatum 2 mm thick was immediately dissected on ice. Each coronal section of the striatum was placed in 200µl RIPA buffer (Sigm; St. Louis, MO) with protease inhibitor cocktail (1:100, Sigma; St. Louis, MO), phosphatase inhibitor cocktail 2 (1:100, Sigma; St. Louis, MO) and phosphatase inhibitor cocktail 3 (1ml/500mg tissue, Sigma; St. Louis, MO). The striatum tissues were then subjected to sonication for six cycles of 30 seconds at one-minute intervals and centrifugation at 5000 rpm for 15 minutes to obtain the supernatant. Protein concentration of the supernatant was detected through the Bio-Rad protein assay. 35µg of protein was boiled for seven minutes with loading buffer followed by electrophoresis in 4-20% Tris-HCL SDS-PAGE gel (Bio-Rad; Hercules, CA). After transferring proteins to the PDVF membrane using the iBlot transfer system (Invitrogen; Carlsbad, CA), the membrane was blocked with an Odyssey blocking buffer for one hour at room temperature. Polyclonal rabbit anti-DARPP32 primary antibody (1:5,000, Millipore; Temecula, CA), or polyclonal rabbit anti-p34 DARPP32 primary antibody (1:1,000, Millipore; Temecula, CA) with monoclonal mouse anti β-actin primary antibody (1:20,000, Sigma, St. Louis, MO) were applied to the membrane in 10ml 0.2% Tween 20 Odyssey blocking buffer at 4 degrees overnight. After 0.1% Tween PBS wash, the membrane was incubated in a mixture of Odyssey IRDye secondary antibodies: donkey anti-rabbit 800CW(1:10,000, Licor; Lincoln, NE) and donkey anti-mouse 680LT(1:20,000, Licor; Lincoln, NE), in 15ml Odyssey blocking buffer.
with 0.2% Tween 20 at room temperature for one hour. After final washes with 0.1% Tween PBS, the membranes were detected via Odyssey infrared imager (Licor; Lincoln, NE). Bands were quantified by Odyssey Imager analysis software and normalized against β-actin as internal standards.

14. Statistical analysis

Analysis was performed from Means±SEM. Differences between two groups (Group 1 and Group 2 in Table 1) were analyzed by student t-test. Differences between four groups (As described in Table 1) were analyzed by one-way analysis of variance (ANOVA), followed by Tukey comparison. The p-value was set as 0.05. The null-hypothesis, indicating no significant difference between groups, was rejected when the p-value was less than 0.05.

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<td>Injection 1</td>
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<td>PD149163/SR48692</td>
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<td>Injection 2</td>
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<td>METH</td>
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Table 1. Treatment of four groups of mice
CHAPTER 3. RESULTS

1. The Neurotensin receptor 1 agonist (PD149163) attenuates METH-induced striatal apoptosis in a dose-dependent manner

To determine its effect on METH-induced striatal apoptosis, we delivered PD149163, the NTR1 agonist, through i.p. injection to facilitate its penetration of the brain blood barrier. Since it has been shown that i.p. injection of 1mg/kg and 2mg/kg of PD149163 exerts effects on NTR1 in mice (Feifel et al., 2004; Shilling et al., 2004), we used dosages of 0.5 mg/kg, 1 mg/kg and 2 mg/kg to examined the effects of NTR1 agonist on METH-induced striatal apoptosis and determine the optimal dosage.

Since METH-induced striatal apoptosis is limited to the striatal neurons (Zhu et al., 2006), we assessed the apoptotic ratio of striatal neurons by calculating the ratio of apoptotic cells to the number of total striatal neurons. To that end, we labeled the striatal apoptotic cells and striatal neurons by TUNEL and NeuN respectively. Terminal deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) can illustrate DNA fragmentation within cells, a late-stage hallmark of apoptosis in situ (Nagata, 2000). The DNA fragments inside the nucleus were labeled by Fluorescein (FITC) and emitted green fluorescence after excitement as shown in Figure 3. We also labeled striatal neurons with NeuN, a neuron nuclear biomarker, with red fluorescence (Cy3) through immunohistochemistry. We used confocal microscopy to obtain images of the striatum in four quarters (Ventral Medial, Ventral Lateral, Dorsal Medial, and Dorsal Lateral) and counted the apoptotic cells and total neurons in the striatum using Image J software. Data analysis indicated METH-induced TUNEL staining in 15.24±7% of striatal neurons compared
with 0.31% TUNEL positive neurons in the striatum of the control animals. The observation that METH induced significant apoptosis in the striatum is consistent with our laboratory's previous findings (Xu et al., 2005; Zhu et al., 2005). The NTR1 agonist, PD149163, affects METH-induced striatal apoptosis in a dose-dependent manner ranging from 0.5 mg/kg to 2 mg/kg. All three dosages of PD149163 affected the ratio of apoptotic neurons in the striatum, with 1mg/kg and 2mg/kg attenuating apoptosis significantly. In particular, pre-treatment of the PD149163 at 1 mg/kg and 2 mg/kg (i.p.) attenuated 86.2% and 96.7% of METH-induced striatal neuron apoptosis respectively. Due to its strongest protective effect of PD149163 at 2 mg/kg, we identified 2mg/kg as the optimal dosage and thus used 2mg/kg in all subsequent experiments. PD149163 treatment alone does not affect the apoptotic levels compared with control mice as shown Figure 3. Our findings also indicated that, as predicted, the neuropeptide neurotensin contributes to METH-induced striatal neuron apoptosis, and its agonist, PD149163, could become a potent drug candidate for treatment of METH-induced neurotoxicity.
Figure 3. NTR1 agonist attenuates METH-induced striatal neuron apoptosis. NTR1 agonist was i.p. injected into mice at 0.5mg/kg, 1mg/kg and 2mg/kg 30min before METH i.p. injection. (A) Scanning confocal micrographs of TUNEL staining and NeuN staining in striatum of mice. Arrows indicate TUNEL positive cell (green) and NeuN positive cell (red). (B) Percentage of striatal neurons displaying TUNEL staining. Data represent Means±SEM; all data were analyzed by one-way ANOVA followed by Tukey. *p≤0.05 compared with PBS control; †p≤0.05, ‡p≤0.01 compared with METH group. n≥6. Scale bar=75µm.
2. NTR1 agonist exerts no effect on METH-induced dopamine terminal degeneration

In addition to striatal neuron apoptosis, METH can also induce dopamine terminal degeneration characterized by depletion of dopamine, tyrosine hydroxylase, and dopamine transporter (Wilson et al., 1996). We assessed the level of tyrosine hydroxylase (TH), a dopamine terminal degeneration biomarker. TH is an enzyme that catalyzes the rate-limiting step of dopamine synthesis in the dopamine terminals. We quantified the TH levels in striatal homogenates by western blot with β-actin as the internal control for its constant expression in cells. Since METH has been shown to induce the lowest depletion of TH at 72 hours (Zhu et al., 2005), we used this as our time point and showed that METH reduced TH levels by 42% 72 hours after systemic administration. Systemic delivery of PD149163 thirty minutes prior to METH administration did not significantly affect the TH level, with an increase of 16% (Figure 4). Additionally, PD149163 alone did not alter TH levels significantly. These results suggest that, even at optimal dosage, PD149163 has no effect on METH-induced dopamine terminal degeneration.
Figure 4. Pretreatment of PD149163 has no effect on METH-induced tyrosine hydroxylase depletion. Mice in all four groups received all drugs through i.p. injection. 2mg/kg PD149163 was injected 30min before METH. (A) Scanning western blot image of TH and β-actin bands by Odyssey imager. (B) Corresponding level of tyrosine hydroxylase in striatum of four mice groups. Data represents Means±SEM; all data were analyzed by one-way ANOVA followed by Tukey. *p≤0.05, **p≤0.001 compared to PBS control group. n≥6. Note that no significant difference in tyrosine hydroxylase level was found between the METH group and the PD149163 pre-treated METH group.
3. METH up-regulates neurotensin gene expression in the striatum

Systemic delivery of the NTR1 agonist attenuating apoptosis of striatal neurons indicates that neurotensin may respond to METH administration by activating the NTR1. We hypothesized that METH administration would decrease the intracellular neurotensin peptide pool and thereby increase the preproneurotensin mRNA levels to restore the peptide pool.

To test this hypothesis, we delivered either METH (30 mg/kg) or PBS (phosphate buffered saline) as control through i.p. injection and measured the neurotensin mRNA level in the striatum six hours post administration in these two groups using quantitative Real-Time PCR. We chose six hours as the time point since Letter (Letter et al., 1987) showed that a single dose (15 mg/kg) of METH elevated neurotensin-like immunoactivity concentration starting from six to eighteen hours. The Taqman RT-PCR system (Life Technologies; Carlsbad, CA) was used to detect neurotensin mRNA levels in mouse striata. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used as an endogenous control due to its ubiquitous and stable expression. The comparative Ct method was chosen to interpret Ct values and qualified by verification experiments. 0.5ng, 5ng, 10ng and 25ng of total RNA from striata were extracted using RNeasy Kit (Qiagen; Venlo, Limburg) and used as RNA template for this validation experiments. Each reaction was repeated three times. After plotting the log of RNA concentration and the corresponding Ct values, we observed the slopes of standard curves for neurotensin and GAPDH only differed by 0.03(Figure 5). The efficiencies of these two RT-PCR systems are similar enough (˂0.1) to be interpreted by comparative Ct method (Schmittgen and Livak, 2008). The comparative Ct method then was used to interpret all Ct values for neurotensin and GAPDH RT-PCR system and showed that the neurotensin mRNA level reached up to 195±30% six hours after METH administration.
compared to the control as shown in Figure 6. This result suggests that METH induces neurotensin release from the striatal neurons and thereby triggers the up regulation of neurotensin gene expression as characterized by elevated neurotensin mRNA levels. These data also support our hypothesis that neurotensin responds to METH-induced pathology through activation of NTR1 in the striatum.
Figure 5. Validation of comparative Ct method for neurotensin and GAPDH RT-PCR system. Input total RNA amount ranged from 0.5ng to 25ng as shown above for each RT-PCR system. The absolute difference of these two slopes of the standard curves is $0.03 < 0.1$, which indicates that the efficiencies of these two RT-PCR systems are similar and the comparative Ct method is applicable.
Figure 6. METH up regulates neurotensin gene expression at six hours post administration. Animals received i.p. injection of either saline or METH (30mg/kg) and the total RNA from the striatum were extracted and used as RNA template for real-time quantitative PCR. Ct value was interpreted by comparative Ct method with Rodent GAPDH as internal control. Data were analyzed by student t-test. *p≤0.05 compared with PBS control animals. n≥6.
4. NTR1 agonist attenuates METH-induced hyperthermia

It has been shown that METH-induced hyperthermia plays a significant role in neurotoxicity (Sandoval et al., 2000; Bowyer et al., 1994); indeed, environmental suppression of hyperthermia attenuates METH-induced striatal neurodegeneration (Bowyer et al., 1992; Bowyer et al., 1993). Several phenomena have been observed as consequences of hyperthermia, including cerebrovascular damage, increased calcium entry, and oxidative stress (Davidson et al., 2001; Lin et al., 1991; Omar et al., 1987; Skibba et al., 1991), all of which may induce neurotoxicity. Due to the role of hyperthermia in METH-induced neurodegeneration, it has become necessary to consider body temperature response when studying the mechanisms of METH toxicity. We measured the core body temperature of animals using a rodent probe at four time points: 0 hours, two hours, four hours, and six hours post drug administration. It has been shown that METH-induced hyperthermia vanished at six hours post injection of acute METH (30 mg/kg) (Zhu et al., 2006). In this study, we observed an increase in body temperature from 37.5 °C to 39.6 °C at two hours post METH; this increase is temporary, as temperature drops to 36.5 °C at four hours (Figure 7). Systemic delivery of the NTR1 agonist attenuated hyperthermia and maintained core body temperature at 37 °C at two hours, followed by a decrease to 34.1°C at four hours post METH administration. The hypothermic effect of PD149163 was also observed in the PD149163 treatment only group; these mice displayed a sharp reduction of body temperature by 5.1°C (from 38.3 °C to 33.2 °C) at two hours post administration. These data suggest that systemic delivery of PD14963, the NTR1 agonist, attenuates METH-induced hyperthermia in mice at two hours post administration.
Figure 7. Systemic delivery of PD149163 attenuates METH-induced hyperthermia. All drugs were i.p. injected into mice of four groups. Core body temperatures were detected by thermometer probe for rodents at four time points post injection. Data represent Means±SEM; all data were analyzed by one-way ANOVA followed by Tukey. *p≤0.05, **p≤0.01, ****p≤0.0001 compared with PBS control, †p≤0.05, ‡‡‡‡p≤0.0001 compared with METH group. n≥6. Arrows indicate at 2 h post i.p. injection, METH induces body temperature increase while PD149163 causes reduction of body temperature.
5. NTR1 agonist attenuates striatal neuron apoptosis independent of body temperature regulation

To investigate whether NTR1 agonist can attenuate striatal apoptosis independent of body temperature regulation, we sought to bypass its hypothermic effect by stereotactic injection of PD149163 into the striatum. We microinjected 1 µL aCSF as vehicle into the left striatum and 20 µM PD149163 of 1 µL into the right striatum (stereotactic coordinates: 0.5 mm anterior, 2.0 mm lateral from bregma and 2.5 mm ventral). Thirty minutes post microinjection, mice received i.p. injection of METH or PBS, and then the core body temperatures were monitored at 0 hours and two hours post i.p. injection. PD149163 infused in the striatum exerted no effect on body temperature regulation, as evidenced by a 2.0 °C increase in core body temperature at two hours post PD149163 intrastrialat infusion and METH i.p. injection, as shown in Figure 8. The TUNEL staining shows METH-induced striatal apoptosis with large intra-animal variance; half of the METH-treated mice (n=4) showed a high degree of apoptosis while the other four mice showed a low degree of apoptosis. These findings, shown in Figure 9, are consistent with previous findings illustrated in Figure 3 and also shown by Zhu et al. (2006). Notably, while confocal micrographs showed 37.7% of the striatal neurons undergoing apoptosis after aCSF infusion and METH i.p. injection, the right striatum infused with PD149163 followed by METH i.p. injection showed only an average of 3.6% apoptosis (Figure 9A). As a comparison, less than 2% of striatal neurons experienced apoptosis in animals treated with aCSF or PD149163 infusion followed by PBS i.p. injection, which indicates that aCSF and PD149163 exert no effect on striatal apoptosis. These observations demonstrate that the NTR1 agonist, PD149163, can attenuate striatal apoptosis independent of its regulation of body temperature.
Figure 8. Intrastriatal infusion of PD149163 has no effect on METH-induced hyperthermia. Mice received 20 µM PD149163 in the right striatum (Coordinate: bregma 0.5mm; lateral 2.0; ventral 2.5mm) and vehicle aCSF in the left striatum (Coordinate: bregma 0.5mm; lateral 2.0; ventral 2.5mm) through stereotactic infusion. Followed by infusion, i.p. injection of 30 mg/kg METH was given to the mice 30 min post the PD 149163 infusion. Core body temperatures were detected 0 h and 2 h after the METH injection. All temperatures were detected twice. Data represent Means±SEM and were analyzed by student t-test. **p≤0.01 compared with core body temperature at 0 h post i.p. injection of METH. n=8.
Figure 9. Intrastriatal microinjection of NTR1 agonist attenuates METH-induced striatal apoptosis. Mice received 20uM PD149163 infusion in right striatum (Coordinate: bregma 0.5mm; lateral 2.0; ventral 2.5mm) and vehicle aCSF in left striatum (Coordinate: bregma 0.5mm; lateral 2.0; ventral 2.5mm). 30min post the infusion; mice receive METH or saline i.p. injection. (A) Scanning confocal micrographs of TUNEL staining. Scale bar= 715.48 µm. (B) Percentage of striatal neurons displaying TUNEL staining. Data represent Means±SEM; all data were analyzed by one-way ANOVA followed by Tukey. **** p≤0.0001 compared with aCSF infusion &Saline i.p. Control group, *** p ≤0.001 compared with aCSF infusion &METH i.p. Group.
6. NTR1 agonist attenuates activation of glial cells induced by METH in the striatum

The innate immune cells of the central nervous system, glial cells, are composed of microglia and astrocytes primarily in the brain (Sofroniew and Vinters, 2010; Stollg and Jander, 1999). Glial cells have been acknowledged to play a role in METH-induced neurotoxicity as evidenced by the fact that activations of both microglia and astrocytes are found in METH-treated rodents and METH abusers (Zhu et al., 2005; Sekine et al., 2008). Once reactivated, glial cells can produce several proinflammatory factors that are toxic to neurons such as IL-1, TNF-α and NO (Dinarello, 2000; Hanisch, 2002; Mark and Grinffin, 2005). These proinflammatory factors may drive neurodegenerative diseases by inducing cellular damage and even apoptosis (Liu and Hong, 2003; LaVoie et al., 2004; Wyss-Coray, 2006). To measure the effect of the NTR1 agonist, PD149163, on glial cell activation induced by METH, microglia were labeled with its biomarker, ionized calcium-binding adapter molecule 1 (Iba-1) (Imai et al., 1996), and astrocytes with glial fibrillary acidic protein (GFAP) (Eng et al., 2000; Bignami et al., 1972), through immunohistochemistry since activation of microglia and astrocytes lead to over-expression of Iba-1 and GFAP, respectively. Confocal microscopy was used to show the Iba-1 and GFAP staining in coronal sections of striatal tissues. Iba-1 staining showed that METH led to enlarged cell body and highlighted processes of microglia in addition to intense staining of Iba-1, which characterized the activation of microglia. In particular, METH induced microglia to form a hypertrophic, reactive shape as opposed to their resting, ramified shape. Also, the level of Iba-1 reached up to 185.3% of PBS control after METH treatment (Figure 10). Co-administration of PD149163 and METH attenuated such activation characterized by a resting ramified shape of the microglia and low levels of Iba-1, (86.97% of PBS control) (Figure 10). PD149163 treatment alone showed no effect on Iba-1 levels or microglial morphology as compared to PBS control.
Both morphology and Iba-1 levels indicate that the NTR1 agonist attenuated METH-induced microglial cell activation in the striatum.

GFAP immunohistochemistry showed METH treatment induced more GFAP staining in the astrocytes of the striatum as compared to PBS control. Due to the over expression of GFAP in the astrocytes, we observed approximately 11.3 times more astrocytes present in the striatum compared to the PBS control (Figure 11). PD149163 pretreatment attenuated such accumulation of astrocytes by 78% as shown in Figure 11. Again, PD149163 treatment alone had no effect on astrocyte activation as compared to the PBS control. This observation demonstrates that NTR1 agonist also attenuated activation of METH-induced astrocytes in the striatum.
Figure 10. NTR1 agonist attenuates METH-induced glial cell activation. (A) Scanning confocal micrographs of Iba-1 staining in the striatum. Scale bar=357.74 µm. Arrows indicate individual microglia. (B) Level of Iba-1 and GFAP in striatum. Data represent Means±SEM; all data were analyzed by one-way ANOVA followed by Tukey. **p≤0.01, ***p≤0.001 compared with PBS control; ""p≤0.01, """"p≤0.001 compared with METH group.
Figure 11. NTR1 agonist attenuates METH-induced astrocytes activation. (A) Scanning confocal micrographs of GFAP staining in the striatum. Scale bar=75 µm. Arrows indicate astrocytes. (B) Level of GFAP in striatum. Data represent Means±SEM; all data were analyzed by one-way ANOVA followed by Tukey. **p≤0.01, ***p≤0.001 compared with PBS control; "p≤0.01, ""p≤0.001 compared with METH group.
7. NTR1 agonist attenuates nitric oxide accumulation induced by METH in the striatum

The tiny molecule nitric oxide (NO), a reactive gas, can function both as a neurotransmitter and a killer molecule by activating the immune system in the brain (Änggård, 1994). Our laboratory has found that acute METH can induce over production of NO up to 32 hours post administration (Zhu et al., 2009) and pharmacological inhibition of nNOS (neuronal nitric oxide synthase) by 7-nitroindazole could attenuate METH-induced striatal neurotoxicity (Itzhak and Ali, 1996; Zhu et al., 2009). As a reactive free radical, Nitric Oxide has a short half-life and therefore is difficult to detect in situ (Nagano et al., 1999). Nevertheless, it is possible to quantify the byproducts of NO, such as cyclic guanosine monophosphate (cGMP) as its biomarker. The levels of cGMP in the striatum were assessed in situ using immunohistochemistry. The confocal micrographs of cGMP staining showed that METH (30mg/kg, i.p.) induced 157% levels of cGMP as compared to PBS control in the striatum (Figure 12). PD149163 pretreatment attenuated 70% of such accumulation (110% of the control) and PD149163 alone had no effect on cGMP levels as compared to the control. These data suggest that the NTR1 agonist significantly affects METH-induced NO accumulation. Furthermore, our lab has found that cGMP levels become elevated in all types of neurons in the striatum, including projection neurons and interneurons along with NO accumulation induced by METH in the striatum (Yarosh and Angulo, 2012). Based on this finding, the NTR1 agonist may attenuate METH-induced increase in cGMP levels in the striatal projection neurons as well as the striatal interneurons.
Figure 12. NTR1 agonist attenuates METH-induced cyclic GMP accumulation in the striatum. (A). Scanning confocal micrographs of cGMP staining in the striatum. (B). Quantification of cGMP level in the striatum. Data represent Means±SEM. All data were analyzed by one-way ANOVA followed by Tukey. **p≤0.01 compared to PBS group; 'p≤0.05, "p≤0.01 compared to METH group.
8. NTR1 modulates METH-induced phosphorylation of DARPP32 at Thr34 in the striatum

In the striatum, DARPP32 is highly concentrated in the medium-sized projection neurons and is found to play a central role in interpreting all neurotransmissions into the projection neurons (Greengard, 2001). The fact that DARPP32 knock-out mice showed little or no response to psychostimulant drugs at biochemical, behavioral and physiological levels indicates the role of DARPP32 in modulating such processes. DARPP32 exerts its effects through phosphorylation or dephosphorylation by multiple pathways including dopamine and glutamate neurotransmission. To investigate the effect of NTR1 on phosphorylation of DARPP32 at Thr34, DARPP32 and phospho-Thr34 DARPP32 levels were measured in mice striata using western blot. The NTR1 agonist was delivered intraperitoneally followed by METH i.p. injection. We also delivered PBS followed by PBS i.p. injection as the control group. The time point for the highest amount of phosphorylation of DARPP32 at Thr34 induced by METH was selected at one-hour post METH injection. At six and eight hours post METH, levels of DARPP32 and phospho-Thr34 DARPP32 were not affected compared to the control animals (Figure 13), which is consistent with previous findings (P-C Chen and J-C Chen, 2005). At one hour post administration, however, METH induced phosphorylation of DARPP32 at Thr-34 up to 140% of the control, while pretreatment of PD149163 attenuated such phosphorylation of DARPP32 to 51% of control (Figure 14). In contrast, the levels of total DARPP32 in the striatum were unaffected by any drug administration. Additionally, the NTR1 agonist alone also decreased levels of phosphorylation of DARPP32 at Thr34 by 54% compared to the control group. This suggests that PD149163 attenuates METH-induced DARPP32 phosphorylation in the striatum at one-hour post administration.
To further investigate the function of NTR1 on METH-induced DARPP32 phosphorylation, the effect of the NTR1 antagonist, SR48692, on levels of phosphorylated DARPP32 at Thr34 and DARPP32 were assessed one hour after METH i.p. injection following intrastriatal infusion of the NTR1 antagonist. The NTR1 antagonist was delivered through intrastriatal infusion to eliminate its function to activate NTR2 outside the striatum. Western blot data indicated that METH induced 224% of pThr34 DARPP32 compared to the control animals and the NTR1 antagonist augmented such phosphorylation of DARPP32 by 146% (370% of control animals) (Figure 15). It is also notable that SR48692 alone also promoted the phosphorylation of DARPP32 at Thr34 by 122% compared to the control group. The contrasting effects of NTR1 agonist and antagonist on METH-induced DARPP32 phosphorylation demonstrate that through regulating neurotransmission, NTR1 modulates phosphorylation of DARPP32 at Thr34.
Figure 13. METH does not affect levels of both DARPP32 and p34 DARPP32 in striatum 6h/8 h post drug. (A). Western blots of DARPP32, phosphor Thr34 DARPP32, and β-actin in striatum at six and eight hours post METH administration. (B) Quantification of DARPP32 and phosphor Thr34 DARPP32 in striatum with β-actin as internal control. Data represent Mean±SEM; all data are analyzed by one-way ANOVA followed by Tukey. n≥6.
Figure 14. NTR1 agonist attenuates METH-induced phosphorylation of DARPP32 at Thr 34 in striatum at one-hour post METH. (A). Western blots of DARPP32, phosphor Thr34 DARPP32, and β-actin in the striatum. (B) Quantification of DARPP32 and phosphor Thr34 DARPP32 in striatum with β-actin as internal control. Data represent Mean±SEM; all data are analyzed by one-way ANOVA followed by Tukey. n≥6. *p≤0.05, **p≤0.01 compared with PBS control animals, ****p≤0.0001 compared with METH-treated animals.
Figure 15. NTR1 antagonist augments METH-induced phosphorylation of DARPP32 at Thr 34 in the striatum at one-hour post METH. (A) Western blots of DARPP32, phosphor Thr34 DARPP32, and β-actin in the striatum. (B) Quantification of DARPP32 and phosphor Thr34 DARPP32 in striatum with β-actin as internal control. Data represent Mean±SEM; all data are analyzed by one-way ANOVA followed by Tukey. n≥6. *p≤0.05, **p≤0.01 compared with aCSF & saline treated animals, †p≤0.05, ‡p≤0.01 compared with SR48692 and METH-treated animals.
CHAPTER 4. DISCUSSION

METH has been shown to induce neural degeneration in several regions of the brain, including the striatum, in rodents (Green et al., 1992; Jason-Davis et al., 2003; Ladenheim et al., 2000). The toxicity of METH is thought to depend on its similar chemical structure to dopamine (DA) (Iversen, 2006). Due to this structural similarity, METH can enter dopamine terminals readily via DA transporters (Iversen, 2006), causing a premature release of DA inside the cytoplasm. The DA then rapidly auto-oxidizes, forming reactive oxygen species such as superoxide radicals, hydroxyl radicals, hydrogen peroxide and DA quinones (Acikgoz et al., 1998; Cubells et al., 1994; Kita et al., 1999; Larsen et al., 2002; LaVoie and Hastings, 1999). These reactive oxygen species may oxidize multiple cell components such as protein, lipid and nucleic acids, thus impairing their functions through oxidative stress (Coyle and Puttfarchen, 1993; Buttke and Sandstrom, 1994). In addition, METH reverses the function of DA transporters shuttling DA into synapses, resulting in heavy DA overflow between dopamine terminals and post-synaptic neurons in the striatum (O'Dell et al., 1993; Weihmuller et al., 1992). DA overflow induces neuron hyperactivity and neurotoxicity to post-synaptic neurons, mainly in striatal medium sized spiny projection neurons (MSNs), as DA projection from the midbrain to the striatum accounts for the bulk of DA innervation in the CNS. Because of the critical role of DA in METH-induced neurodegeneration, many studies have focused on this neurotransmitter. However, there are currently no therapeutic options available for treatment of METH-induced neurotoxicity. It is therefore essential to study the systems that regulate DA neurotransmission. Neurotensin is a tridecapeptide heterogeneously distributed in the central nervous system (CNS) and the gastrointestinal tract of mammals (Nemeroff et al., 1980). In the striatum, neurotensin is known to modulate both dopamine and glutamate neurotransmission via interactions with NTR1,
Dopamine receptor 1 and NMDA receptors (Dobner, 2005). Systemic delivery of neurotensin or a neurotensin analog antagonizes certain METH-induced behaviors (Hertel et al., 2001; Hanson et al., 2012), which supports the hypothesis that neurotensin participate in METH-induced pathology by interacting with the DA system in the CNS. Furthermore, psychostimulants such as methamphetamine and amphetamine have been shown to increase extracellular levels of striatal neurotensin (Wagstaff et al., 1996; Ervin et al., 1982; Robledo et al., 1993), which further suggests a role for neurotensin in psychostimulant-induced striatal neuronal activation. However, to date, no information is available on the neurochemical effect of neurotensin in METH-induced striatal neurodegeneration, let alone its corresponding mechanisms.

Our lab has been investigating the mechanisms of striatal neurodegeneration induced by acute METH in a mouse model. In this model, we utilize the ability of METH (30mg/kg, i.p.) to induce neurodegeneration in both presynaptic dopamine terminal and postsynaptic neurons in the striata of mice (Xu et al., 2005; Zhu et al., 2006). In particular, dopamine terminals from presynaptic neurons were shown to undergo neurodegeneration characterized by depletion of dopamine, tyrosine hydroxylase, and dopamine transporters (Wilson et al., 1996). Meanwhile, 20% of postsynaptic neurons, i.e., striatal medium-sized spiny neurons, were shown to experience apoptosis as a result of acute METH exposure.

Using this animal model, the present study shows that the striatal endogenous neuropeptide, neurotensin, modulates METH-induced striatal neuron apoptosis through neurotensin receptor 1. In particular, systemic delivery of an NTR1 agonist, PD149163, was shown to attenuate METH-induced striatal apoptosis in a dose-dependent manner ranging from 0.5mg/kg to 2mg/kg.
Among these dosages, 2 mg/kg is the optimal dose since at this dose, PD149163 attenuated the striatal apoptosis by 96.7%. This result suggests that NTR1 plays an important role in METH-induced striatal apoptosis and reveal the potential of PD149163 as a treatment for METH-induced neurotoxicity and damage.

In addition to striatal apoptosis, acute METH also triggers neurodegeneration in presynaptic neurons, e.g., dopamine neurons from SNC with tyrosine hydroxylase as a biomarker (Steiner and Tseng, 2010; Wilson et al., 1996). We applied western blot to quantify the tyrosine hydroxylase (TH) levels in the striatum in four different groups of animals (n≥6). Consistent with previous reports, METH induced approximately 50% depletion of TH compared to the control animals. Systemic delivery of NTR1 agonist at the optimal dosage of 2mg/kg did not significantly affect such depletion. These data imply that despite modulating METH-induced apoptosis, NTR1 does not participate in METH-induced dopamine terminal degeneration. And the reason may lie on the fact that although neurotensin receptor 1 has been found in dopaminergic terminals from the SNC (Quirion et al., 1985), no physiological interaction has been found between NTR1 and DA transporters that are responsible for the subsequent neurodegeneration by transporting METH into the dopamine terminal (Iversen, 2006).

To confirm the involvement of neurotensin in METH-induced pathology, we also examined neurotensin gene expression by quantifying neurotensin mRNA levels in the striatum. After analyzing the data from RT-PCR with comparative Ct method, we observed METH increased neurotensin mRNA level by 96% compared to the control. This set of data supports our hypothesis that METH administration increases the consumption of neurotensin, which induce...
the neurotensin gene expression in order to recover the neurotensin protein pool in the striatum. Ultimately, the protective effect of NTR1 agonist against striatal neuron apoptosis, in conjunction with METH-induced up-regulation of neurotensin gene expression, suggests that neurotensin plays an important role in METH-induced striatal apoptosis. The corresponding mechanisms may involve several pathways discussed in details as follows.

In the last four decades, investigations of METH-induced neurodegeneration have led to the discovery of distinct mechanisms mediating this process (Gibb and Kogan, 1979; Yamamoto and Bankson, 2005; Albers and Sonsalla, 1995; Bowyer et al., 1994; Escubedo et al., 1998; Guilarte et al., 2003; Cadet and Krasnova, 2007.). Following a review of the literature and our lab’s previous work, we hypothesized that hyperthermia, NO overproduction, glial cell activation, and the DARPP32 signaling pathway are involved in neurotensin modulating METH-induced neurodegeneration.

METH-induced hyperthermia plays a significant role in METH-induced neurodegeneration. In particular, systemic delivery of METH can increase core body temperature up to 40°C, which, in turn, can cause death in rodents (Molkov et al, 2014). Mild hyperthermia has also been found to contribute to METH-induced neurodegeneration (Albers and Sonsalla, 1995) as evidenced by the fact that environmental cooling or pharmacological manipulation, which directly attenuates hyperthermia, also abrogates the neural damage induced by METH in various brain regions such as the striatum (Ali et al., 1996; Albers and Sonsalla, 1995). Neurotensin is also shown to reduce body temperature through activation of neurotensin receptor 1 by many studies. For example, central administration of neurotensin can produce a marked dose-related decrease in body
temperature of mice and rats at an ambient temperature 25°C (Nemeroff et al., 1977). Further, targeted inactivation of the neurotensin receptor 1 revealed its role in body temperature control (Remaury et al., 2002). Thereby, we speculate that endogenous neuropeptide neurotensin attenuates METH-induced hyperthermia by lowering core body temperature dramatically through NTR1. In this study, we assessed the effect of the NTR1 agonist, PD149163 (2mg/kg, i.p. injection), on METH-induced hyperthermia. Our results corroborate the previous report on METH-induced hyperthermia (Xu et al., 2005), in which acute METH dosage induced an average increase of 2 to 3 °C in core temperature. Furthermore, systemic delivery of PD149163 reduced body temperature sharply by 6 °C, similar to a 1977 report by Nemeroff and colleagues. Correspondingly, pretreatment of PD149163 attenuated METH-induced hyperthermia to normal body temperature at two hours post i.p. injection. These data indicate that systemic delivery of NTR1 agonist attenuates METH-induced hyperthermia through its function of core body temperature regulation in mice. Hyperthermia may cause neuron damage through multiple pathways involving cerebrovascular damage, increasing oxidative stress and even glial cell activation as summarized in the introduction. As a result, prevention of hyperthermia at least partially protects neurons from neurodegeneration.

In addition to body temperature regulation, we suspect there are other pathways involved in protection of the NTR1 agonist against METH-induced striatal apoptosis based on previous findings. In particular, certain pharmacological and genetic manipulations, which do not influence core body temperature, also prevent METH-induced toxicity. For example, interleukin-6 knockout mice displayed METH-induced hyperthermia, yet were resistant to METH-induced axonal degeneration of dopamine and 5-HT neurons, as well as cell death and microgliosis
In addition, nNOS inhibitors attenuated METH-induced dopamine terminal degeneration while exerted no effect on METH-induced hyperthermia (Itzhak et al., 2000). To test this hypothesis, we eliminated the NTR1 agonist’s ability to lower body temperature through intrastriatal infusion of the NTR1 agonist. This allowed delivery of the agonist into the striatum but not the hypothalamus, which regulates body temperature, in the diencephalon of the CNS (Hammel et al., 1963; Hammel and Pierce, 1968). Infusion of the NTR1 agonist had no effect on body temperature two hours post METH i.p. injection (Figure 8). While lacking the hypothermic effect, infusion of the NTR1 agonist still attenuated striatal apoptosis induced by METH in the striatum by 90%. These results support our hypothesis that NTR1 can modulate METH-induced striatal apoptosis independent of body temperature regulation. Based on this result, we further hypothesize that NTR1 modulates METH-induced striatal apoptosis through several factors that contribute to METH-induced neurodegeneration including glial cell activation, nitric oxide accumulation and DARPP32 phosphorylation pathway.

METH-induced glial cell activation is supported to contribute to neurodegeneration by a considerable body of evidences. As innate immune cells in the CNS, glial cells have long been overlooked as merely supportive elements in the nervous system (Haydon, 2001; Sofroniew and Vinters, 2010). Recently, glial cells have drawn more attention, which resulted largely from the finding that glia can integrate neuronal inputs and modulate synaptic activity (Haydon, 2001; Sofroniew and Vinters, 2010). Microglia and astrocytes are found to be both activated by METH in rodents or human METH abusers, as shown by confocal microscopy and PET imaging (Sekine et al., 2008; Zhu et al., 2005). Our lab has also found that acute METH administration (30mg/kg,
i.p.) triggers the activation of astrocytes in the striatum, with a measured increase in GFAP levels starting one-day post administration and peaking at three days (Zhu et al., 2005). Additionally, increasing evidence suggests the potential role of immune response as an early stage biomarker that can be targeted pharmaceutically for treatment of neurodegenerative diseases. For example, Watson and colleagues demonstrated that the onset of inflammation in the striatum precedes the development of nigrostriatal dysfunction in Line 61 mice, a transgenic mouse model of Parkinson’s disease (Watson et al., 2013). LaVoie and colleagues (2004) also observed that microglial activation precedes dopamine terminal pathology in METH-induced neurotoxicity. Gill and colleagues further observed that direct brain infusion of glial cell line-derived neurotrophic factor increases striatal DA storage by 28%, which indicates the direct involvement of glia in psychostimulant abuse (Gill et al., 2003). These investigations support the role of glial cell activation on METH-induced neurodegeneration.

To assess the activation of glial cells in situ, we chose to label the glial cells by immunohistochemistry of Iba-1 (ionized calcium-binding adapter molecule 1) and GFAP (glial fibrillary acid protein) in the striatum. Expression of GFAP has long been a prototypical marker for immunohistochemical identification of astrocytes (Bignami et al., 1972; Sofroniew and Vinters, 2010). Iba-1, a calcium-binding protein specifically expressed in microglia, was also labeled by immunohistochemistry (Imai et al., 1996; Ito et al., 1998; Ito et al., 1998). GFAP and Iba-1 can also reveal the activation of astrocytes and microglia with increased level and morphological changes. The present study showed that systemic delivery of METH induced glial cell activation in figures 10 and 11, with characterization of more processes, swelled cell bodies and higher levels of Iba-1 and GFAP staining in the striatum. Pretreatment of NTR1 agonist with
METH administration attenuated such activation with lower levels of GFAP and Iba-1, similar to those of the control animals. Although it appears the population of astrocytes and microglial cells in the striata of control animals and NTR1 agonist pretreated mice is similar, this is unlikely given that there are five times as many glial cells as neurons in the CNS. It is more probable that the antibodies for GFAP and Iba-1 fell below the threshold for detection in these groups of mice. These results of Iba-1 and GFAP immunohistochemistry suggest that the NTR-1 agonist suppressed METH-induced glial cell activation in the striatum.

The NTR1 agonist may attenuate METH-induced glial cell activation through reducing METH-induced apoptosis in the striatum. There are various factors that can trigger the activation of glial cells, such as reactive oxygen species, glutamate accumulation in the synapse, environmental toxins and infections, and neuron death (Crews, 2012; Sofroniew and Vinters, 2010). Since striatal apoptosis peaks at one-day post METH administration, which precedes glial cell activation by three days, it is possible that the induced apoptosis subsequently triggers glial cell reactivation. Thereby it is possible that through attenuating striatal apoptosis, NTR1 agonist inhibited the subsequent glial cell activation. The activation of glial cells may exert its neurotoxicity through the production of pro-inflammatory cytokines to further promote the METH-induced striatal neurodegeneration. Cytokines comprise a significant portion of immune- and neuro-modulatory messengers that can be released by active glial cells (Hanisch, 2002). Some cytokines promote inflammation (pro-inflammatory) while others reduce inflammation and promote healing (anti-inflammatory) (Dinarello, 2000). There are 18 cytokines under the interleukin (IL) class (Dinarello, 2000). Other cytokines are identified due to their biological functions, as in the case of tumor necrosis factor (TNF). During the process of inflammation,
genes coding the small mediator molecules are up regulated by inflammatory products, especially IL-1 and TNF. IL-1 and TNF can then trigger the inflammatory cascade. For example, they induce chemokine synthesis, iNOS activation, COX-2 followed by NO production, leukotriene synthesis, and activation of neutrophils. IL-1 also appears to be common in cellular activity leading to apoptosis. In addition, TNF-α has direct toxic effects on neuronal structures and myelin, and high levels of TNF-α activation have destructive outcomes (Hanisch, 2002). Based on the above discussion, we speculated that METH-induced striatal neurotoxicity triggers the activation of glial cells, which promote the production of pro-inflammatory cytokines that may further augment neurotoxicity in the striatum. The NTR1 agonist may indirectly attenuate METH-induced glial cell activation and its subsequent toxic proinflammatory factors through inhibiting the striatal neuron apoptosis.

In addition to the role of glia in neurotoxicity, Nitric Oxide, the minuscule gas molecule in the CNS, may also exert function in METH-induced neurotoxicity. NO was first recognized as a messenger molecule in the CNS in 1988 (Garthwaite et al., 1988). Produced from the amino acid L-arginine by the members of the NO synthase (NOS) (Calabrese et al., 2007), NO could disperse from its production site to influence neurotransmission, regulation of blood-vessel tone and immune responses (Garthwaite and Boulton, 1995). The NOS consists of three isoforms: neuronal NOS (nNOS); endothelial NOS (eNOS); and inducible NOS (iNOS) (Guix et al., 2005; Bredt et al., 1999; Dawson et al., 1994). iNOS and nNOS have been found in glial cells and neurons respectively in the CNS. For example, in the striatum, Somatostatin/nNOS/NPY interneurons express nNOS (Figueroed-Cardenas et al., 1996), which can be induced by activation of such interneurons. Levels of iNOS in the CNS are low, but iNOS can be induced in
activated glial cells (Peruzzi et al., 2004). A great number of investigations indicate Nitric Oxide played a significant role in METH-induced neurodegeneration. For example, our lab found that overproduction of NO induced by endogenous neuropeptides modulated the METH-induced striatum neurodegeneration. In particular, substance P mediates METH-induced striatal apoptosis through excessive production of NO by nNOS in Somatostatin/nNOS/NPY interneurons (Zhu et al., 2006; Wang and Angulo, 2011). Besides, pharmacological inhibition of nNOS in the striatum also attenuated METH-induced striatal apoptosis (Wang and Angulo, 2011). All these findings indicate the toxic role of NO in neurodegeneration induced by METH. Initial studies on NO-mediated signaling indicated that this molecule interacts with soluble guanylyl cyclase (sGC) by stimulating its activity (Murad, 2006). The consequent product of cyclic GMP is commonly used as a biomarker of NO, which can mediate several pathways inside neurons and glial cells (Murad, 2006; Garthwaite, 1995; Gudi, 1999). In the present study, we observed that NTR1 agonist attenuates METH-induced cGMP accumulation in the striatum to control levels. This result also suggests that the NTR1 agonist attenuates METH-induced NO accumulation in the striatum. Since the NTR1 agonist also attenuated METH-induced glial cell activation in the striatum, it is rational to propose that NTR1 attenuates NO overproduction by inhibiting the activation of glial cells, inactivating iNOS. Whether the NTR1 agonist modulates NO production by nNOS in the striatal interneurons requires further investigation. For example, by detecting the levels of nNOS in the striatum after intrastriatal infusion of NTR1 agonist and antagonist, we could assess if neurotensin exerts effects on NO production in the striatal interneurons.

Higher concentrations of NO are clearly neurotoxic, so inhibition of NO overproduction by NTR1 agonist results in lower neurotoxicity levels (Calabrese et al., 2007). NO can induce
neurotoxicity through react with superoxide anions \( (O^2- \text{, produced by inducible nitric oxide synthase under inflammatory conditions or neuronal nitric oxide synthase as in the case of excitotoxicity}) \) to form peroxynitrite (Beckman et al., 1990), which is a strong oxidant that can affect multiple cell components such as GAPDH, Parkin and matrix metalloproteinase 9 (Pacher et al., 2007; Beckman and Koppenol, 1996). Peroxynitrite can also induce protein nitration resulting in damage to cellular components (Radi, 2004). Furthermore, NO has been shown to activate both the constitutive and inducible isoforms of cyclooxygenase, which are upregulated in brain cells under pro-inflammatory conditions (Mollace et al., 2005; Mancuso et al., 2007). During the catalytic cycle of cyclooxygenase, the release of free radicals and formation of prostaglandins occur, which are both closely related to neuroinflammation that may lead to neurotoxicity (Mancuso et al., 2007).

In addition to NO neurotoxicity, DARPP32 (Dopamine- and cAMP-regulated phosphoprotein) was also considered in the context of METH-induced neurotoxicity. Highly concentrated in the striatal spiny medium-sized neurons, DARPP32 interprets information arriving at dopaminergic neurons in multiple brain regions, including the striatum, via neurotransmitters including dopamine, glutamate, neotensin, NO, GABA and serotonin (Svenningsson et al., 2004; Svenningsson et al., 2002; Greengard, 2001). These neurotransmitters transduce their signals through their respective receptors, or, in the case of NO, diffuse into striatal projection neurons. In addition to these neurotransmitters, psychostimulant drugs of abuse are also found to convey their signals via the DARPP32 pathway indirectly by enhancing dopamine and glutamate neurotransmission in the striatum (Greengard, 2001). All these signals projecting into the striatum projection neurons regulate the phosphorylation of DARPP32 at Thr34, leading
DARPP32 to become an inhibitor of protein phosphatase 1 (PP1) which controls numerous physiologically important substrates; they include neurotransmitter receptors, ion pumps and transcription factors to regulate cell cycle and even programmed cell death (Greengard, 2001). The physiological significance of the DARPP32-PP1 cascade has been well demonstrated in DARPP32 knockout mice (Fienberg et al., 1998), in which all behavioral, physiological, biochemical, pharmacological, and toxicological responses to the psychostimulant drugs of abuse are either diminished or dramatically attenuated compared to the wild type. These results demonstrate that DARPP32 participates in psychostimulant-modulated neurotoxicity. Chen and Chen (2005) also observed METH induced phosphorylation of DARPP32 in the striatum, while not affecting DARPP32 levels from one hour to four hours after drug administration.

Examining the phosphorylation of DARPP32 at Thr34 modulated by METH and NTR1 activation was a critical aspect of our study. We hypothesized that such phosphorylation may be affected by NTR1 through regulation of dopamine and glutamate neurotransmission. To that end, we administrated both NTR1 agonist and NTR1 antagonist followed by METH i.p. injection and subsequent detection of DARPP32 and p34-DARPP32 in the striatum using western blots. We found that METH induces phosphorylation of DARPP32 at Thr34 without affecting the level of DARPP32 in the striatum at one hour after drug administration, which is consistent with Chen and Chen’s findings. Western blot analysis also indicated that the NTR1 agonist attenuates such phosphorylation, while the NTR1 antagonist exerts the opposite effect to augment such phosphorylation. Interestingly, the NTR1 agonist alone also decreases the phosphorylation of DARPP32 at Thr34, while the NTR1 antagonist itself promotes such phosphorylation. These data support our hypothesis that NTR1 modulates the DARPP32 phosphorylation pathway.
potentially through dopamine and glutamate neurotransmission. Because inhibition of PP1 by p34-DARPP-32 may ultimately induce apoptosis, our results suggest that neurotensin’s protection against apoptosis may also occur by inhibiting this mechanism.

Our laboratory's previous studies demonstrated that striatal neuropeptides play a dynamic homeostatic role under conditions of excessive and prolonged dopamine accumulation. We have also shown that several neuropeptides, with the exception of substance P, attenuate the impact of METH. (Yarosh and Angulo, 2012; Zhu et al., 2006; Afanador et al., 2013; Zhu et al., 2006; Wang and Angulo, 2011) For example, exposure to METH increases the levels of preproNPY mRNA in the striatum, suggesting an increased use of this neuropeptide (Yarosh and Angulo, 2012), and this neuropeptide Y (NPY), synthesized by striatal interneurons, which encompass less than 1% of all striatal neurons (Figueroedo-Cardenas et al., 1996; Kawaguchi, 1997; Kubota et al., 1993). In addition, genetic and pharmacological studies demonstrate that NPY counters the damaging effects of METH, protecting striatal neurons from this psychostimulant (Wilson et al., 1996; Zhu et al., 2006). Another neuropeptide, somatostatin, is also synthesized by the same population of striatal interneurons producing NPY (Figueroedo-Cardenas et al., 1996). Our laboratory recently reported that somatostatin also counters the damaging effects of METH, protecting striatal neurons from METH-induced apoptosis (Afanador et al., 2013). Furthermore, substance P, a neuropeptide synthesized by striatal spiny projection neurons, augments production of NO, which further augments METH-induced striatal apoptosis through NK1R receptor in Somatostatin/nNOS/NPY interneurons (Zhu et al., 2006; Wang and Angulo, 2011). Thus, neuropeptides, the endogenous agents expressed and utilized in the healthy striatum, modulate dopaminergic responses under physiological homeostatic conditions as well as in
aberrant states involving a surfeit of dopamine, as induced by METH. These studies demonstrated that striatal neuropeptides are strategically positioned to modulate striatal dopaminergic responses. Although dopamine plays a critical role, there are currently no available drugs that target neurotoxicity induced by METH. Therefore, it is essential to identify compounds that modulate dopamine neurotransmission such as neuropeptide neurotensin. In the present study, we demonstrated that neurotensin modulates METH-induced apoptosis in the striatum through diverse pathways.

In summary, our data indicate that neurotensin can modulate METH-induced striatal neuron apoptosis independent of, and in addition to, body temperature regulation. In particular, through systemic delivery, the NTR1 agonist, PD149163, prevented METH-induced elevation of core body temperature. Through intrastriatal infusion, however, PD149163 maintained its effect of attenuating METH-induced striatal apoptosis without affecting body temperature. We also found that the NTR1 agonist attenuates METH-induced striatal apoptosis through diverse mechanisms including glial cell activation, DARPP32 phosphorylation, and NO accumulation in the striatum. Each of these factors may interact with each other to exert the overall protective effect against METH-induced neurodegeneration. For example, glial cell activation induces the production of NO, which promotes the DARPP32 phosphorylation through NO-cGMP-PKG pathway. NO accumulation and glial cell activation may also induce striatal apoptosis through reacting with reactive oxygen species and toxic proinflammatory factors in addition to the DARPP32 phosphorylation pathway. The potential mechanism model of neurotensin modulating METH-induced neurotoxicity in the striatum is summarized in figure 16. Based upon these findings,
PD149163 may serve as a suitable candidate for further investigation as a neuroprotective agent in the brain.
Figure 16. Mechanism model of neurotensin attenuating METH-induced striatal neurodegeneration. Neurotensin is bio-synthesized by the striatal medium spiny neurons (MSNs) and secreted inside the striatum, where neurotensin receptor 1 is the principal neurotensin receptor located in both dopamine terminal and corticostriatal neurons. Neurotensin release is up-regulated by METH administration inside the striatum as shown above. Once activated, NTR1 can enhance the neurotransmission of dopamine and glutamate through reaction with D1/D2 dopamine receptor and NMDA receptor both localized on the membrane of striatal MSNs. Activation of D1 receptor will promote phosphorylation of DARPP32 while NMDA receptor and D2 receptor function to dephosphorylate DARPP32. In addition to dopamine and glutamate neurotransmission modulation, the NTR1 agonist also attenuates METH-induced NO and cGMP accumulation in the striatum. Effect of NTR1 on dopamine, glutamate and NO neurotransmission all potentially modulate DARPP32 phosphorylation as evidenced by the fact that NTR1 agonist attenuates phosphorylation of DARPP32 while NTR1 antagonist reverses such effect. Besides, NTR1 activation also attenuates METH-induced glial cell activation and hyperthermia. NTR1 agonist can attenuate METH-induced striatal apoptosis independent of body temperature regulation.
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