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The Role of Neurokinin-1 Receptor in the Pathogenesis of Methamphetamine-induced Striatal Neurotoxicity in the Murine Brain

by

Jing Yu

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Biology

at

THE CITY UNIVERSITY OF NEW YORK

2002

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ABSTRACT

The Role of Neurokinin-1 Receptor in the Pathogenesis of Methamphetamine-induced Striatal Neurotoxicity in the Murine Brain

by

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Advisor: Professor Jesus A. Angulo

Methamphetamine (METH) is an addictive substance that also causes extensive neural degeneration in the central nervous system. Because METH augments striatal substance P levels, we hypothesized that signaling through the neurokinin-1 (NK-1) receptor by substance P initiates the neurotoxic cascade. To determine the role of NK-1 receptor in METH-induced neurotoxicity, we tested the effects of the selective NK-1 receptor antagonists WIN-51,708 and L-733,060 on several markers of dopaminergic terminal toxicity in the mouse striatum (namely, dopamine transporters, dopamine content, tyrosine hydroxylase, and glial fibrillary acidic protein). Male mice received four intraperitoneal injections of METH (5 or 10 mg/kg of body weight) at 2-hour intervals and were sacrificed 3-28 days after the treatment. Exposure to METH resulted in severe depletion of dopamine transporters, tissue dopamine content and tyrosine hydroxylase, as well as prominent expression of glial fibrillary acidic protein in the striatum. Administration of either NK-1 receptor antagonist 30 min prior to the 1st and 4th injection of METH prevented the loss of dopamine transporters assessed by

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autoradiography and Western blotting, the depletion of tissue dopamine assessed by High Pressure Liquid Chromatography, the reduction of tyrosine hydroxylase protein levels as well as the induction of glial fibrillary acidic protein determined by Western blotting. The immunohistological studies also demonstrated that the pretreatment of NK-1 receptor antagonist partially protected against METH-induced loss of tyrosine hydroxylase immunoreactive fibers and induction of astrogliosis. Meanwhile, we have provided the direct histological evidence, using Fluoro-Jade B staining, that METH also caused postsynaptic neuronal degeneration, which could be blocked by the pretreatment of NK-1 receptor antagonist. Pretreatment with NK-1 receptor antagonist had no effect on METH-induced hyperthermia. Exposure of mice to either of the NK-1 receptor antagonists alone was without effect on all of these markers. These results provide the first pharmacological evidence that tachykinins, particularly substance P acting through NK-1 receptors, play a crucial role in the pathogenesis of striatal neurotoxicity induced by METH. This finding could lead to novel therapeutic strategies to offset drug addictions as well as in the treatment of a number of disorders including Parkinson's and Huntington's diseases.

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List of Abbreviations

Ach	Acetylcholine
CNS	Central Nervous System
CPu	Caudate Putamen
DA	Dopamine
DAT	Dopamine Transporter
DOPAC	3,4-Dihydroxyphenylacetic Acid
FJB	Fluoro-Jade B
GFAP	Glial Fibrillary Acidic Protein
Glu	Glutamate
HPLC	High Pressure Liquid Chromatography
HVA	Homovanillic Acid
IEG	Immediate Early Gene
METH	Methamphetamine
NAc	Nucleus Accumbens
NK-1	Neurokinin-1
NKA	Neurokinin A
NMDA	N-methyl-D-aspartate
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
PKC	Protein Kinase C
PPT	Preprotachykinin
SN	Substantia Nigra
SP	Substance P
TH	Tyrosine Hydroxylase

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Chapter 1

Introduction

1.1 Methamphetamine-induced Neurotoxicity

1.1.1 Overview of Methamphetamine Abuse

Methamphetamine (METH; *N*-methyl-*O*-phenylisopropylamine) is a derivative of amphetamine with potent psychostimulatory action on the sympathetic and central nervous systems. Commonly known as "speed", "crystal", "ice", or "glass", METH is highly addictive, cheaper and longer lasting than cocaine. According to a report from the Executive Office of the President, the rapidly escalating abuse of METH use is spreading throughout the United States, especially West and Midwest areas, undergoing a major resurgence among adolescents and young people in their twenties (http://www.whitehousedrugpolicy.gov). METH comes in many forms and can be orally ingested, snorted, smoked, or injected. Similar to the other psychostimulants, METH is most often used in a "binge and crash" pattern. An intense sensation of rush occurs minutes after smoking or injecting the drug, while oral ingestion or snorting produces a long-lasting high which can continue for as long as half a day. The $t_{1/2}$ of METH in human is 8-12 h ranging up to 34 h (Anggard et al., 1973), but the pleasurable effects disappear even before the drug concentration in the blood falls significantly, therefore,

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the abusers try to maintain the high by binging on the drug. Like many other drugs of abuse, the highly addictive properties of METH originate from its ability to induce excessive dopamine (DA) overflow in the reward centers of the brain such as the mesolimbic and mesocortical pathways (Leshner and Koob, 1999). In addition to augmented DA transmission, METH also increases the release of other monoamines such as norepinephrine and serotonin (Kuczenski et al., 1995). High doses of METH can cause seizures, hypertensive crisis, cardiovascular or cerebrovascular accident, as well as elevate body temperature to dangerous, or sometimes lethal, levels. In addition to drug addiction and psychotic features, long-term METH abuse can result in long lasting neurotoxic damage in the central nervous system.

1.1.2 Methamphetamine-induced Neurotoxicity in the Neostriatum

Similar to cocaine, METH acts as an indirect DA agonist by inducing excessive DA overflow; however, in contrast to cocaine, METH is also neurotoxic in animal species and humans. Ever since early 80's, it has been widely documented that repeated exposure to recreational doses of METH produces persistent neurotoxicity to presynaptic axon terminals in rodents, nonhuman primates and humans (Woolverton et al., 1989; Brunswick et al., 1992; McCann et al., 1998; Villemagne et al., 1998). The long-term toxic damage is evidenced by the degeneration of DA fibers in the neostriatum (Ricaurte et al., 1984), characterized by the decrease of dopamine transporter (DAT) level, by the depletion of DA content and its metabolites, by the reduction of tyrosine hydroxylase (TH) levels and by the reactive astrocytosis (Ricaurte

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et al., 1984; Hirata et al., 1996; Chapman et al., 2001). Several studies have also demonstrated morphological alterations indicative of nerve terminal degeneration in the neostriatum (Ricaurte et al., 1984; Pu and Vorhees, 1995).

However, evidence of DA neuronal loss in the substantia nigra (SN) or ventral tegmental area (VTA) remains to be confirmed. Some groups recently reported loss of dopaminergic cells in SN after METH treatment (Sonsalla et al., 1996; Hirata and Cadet, 1997; Harvey et al., 2000), while the others have generally failed to observe significant changes of cell bodies in this area (Ricaurte et al., 1982; O'Callaghan and Miller, 1994; Bowyer et al., 1998). Despite the well-documented presynaptic DA terminal degeneration, there are very few studies on the postsynaptic neuronal change in striatum after METH exposure. Cadet et al. have indirectly demonstrated the loss of DA D_1 receptors (Cadet et al., 1998) as well as shown the first evidence of TUNEL-positive neurons in the striatum after the administration of toxic doses of METH (Deng et al., 1999). Neuronal cell death has also occurred in the cortex and hippocampus of METH treated animals (Ryan et al., 1990; Schmued and Bowyer, 1997; Deng et al., 1999; Deng et al., 2001). Nevertheless, these changes are not as profound as the DA terminal loss in the neostriatum.

Neostriatum (Fig. 1-1) consists of the striatum, also named caudate putamen (CPu), and its ventral extension, the nucleus accumbens (NAc). The neostriatum is the major recipient of inputs to the basal ganglia, a group of brain nuclei that control a wide variety of psychomotor behaviors. The vast majority of catecholamine terminals in the neostriatum are dopaminergic (Gerfen, 1992). Essentially all the functions ascribed to

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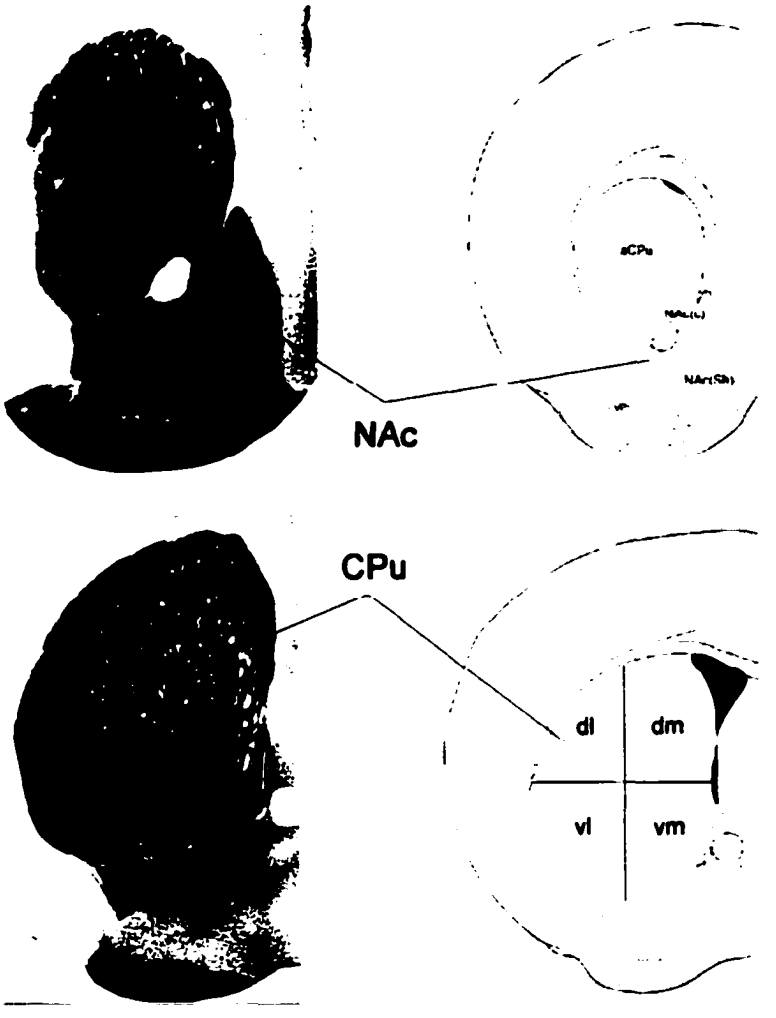

Figure 1-1. Schematic representation of neostriatum in coronal section. Adapted from *The Mouse Brain in Stereotaxic Coordinates* (Franklin and Paxinos, 1997). NAc, nucleus accumbens; aCPu, anterior caudate putamen; CPu, caudate putamen; dm, dorsal medial caudate putamen; dl, dorsal lateral caudate putamen; vm, ventral medial caudate putamen; vl, ventral lateral caudate putamen.

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the CPu and NAc are intimately linked to their rich dopaminergic innervation, which originates in two midbrain nuclei, the substantia nigra (SN) and the ventral tegmental area (VTA), respectively.

Recent reports have indicated that METH may cause long-term or permanent deficits in striatal dopaminergic indices in human chronic abusers (Wilson et al., 1996; McCann et al., 1998), implicating potentially sever health and social problems as these individuals may later develop serious movement disorders resembling Parkinson's disease. On the other hand, long-term neuronal cell death in the CPu of abstinent METH abusers (Ernst et al., 2000) may increase the likelihood of producing symptoms similar to Huntington's chorea. It is, therefore, of great importance to clarify the underlying mechanisms and, in turn, provide efficient protective interventions against the damaging effects induced by METH.

1.1.3 Mechanisms of Methamphetamine-induced Neurotoxicity

Methamphetamine, a cationic lipophilic molecule, enters the striatal dopaminergic terminal and is sequestered in the acidic vesicles where it acts as a weak base to reduce the vesicle pH gradient required for catecholamine accumulation, resulting in the diffusion of DA from vesicles into cytoplasm following its concentration gradient. The accumulated cytoplasmic DA elevates the substrate concentration gradient across the plasma membrane, promoting the release of DA through reverse transport at the dopamine transporter (DAT), while simultaneously inhibiting DA uptake by DAT, generating high concentration of extracellular DA (Sulzer et al., 1993). The aberrant

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accumulation of DA in either extracellular or intracellular compartment can induce oxidative stress (Cubells et al., 1994; Cadet and Brannock, 1998). Meanwhile, due to its lipophilicity, METH can diffuse through cell membranes including intracellular organelles, such as mitochondria, where it disrupts the electrochemical gradient and trigger a mitochondrial-dependent induction of necrotic as well as apoptotic cell death pathways (Davidson et al., 2001).

Dopamine Oxidation Diverse mechanisms have been proposed to underlie the neurochemical and behavioral alterations associated with neurotoxicity to METH (Davidson et al., 2001). In particular, the role of DA as a predominant mediator of the toxicity produced by METH has been widely promulgated (Schmidt et al., 1985; O'Dell et al., 1991; Cubells et al., 1994; Sulzer et al., 1995; Giros et al., 1996), as evidenced by the prevention of the toxicity by DA depleting pretreatments such as α-methyl-*p*-tyrosine (Ricaurte et al., 1984) or DA receptor antagonists (O'Dell et al., 1993), and by the correlation between the magnitude of METH-induced DA overflow and the degree of striatal DA dysfunction (O'Dell et al., 1991; Weihmuller et al., 1992; Marshall et al., 1993; Abekawa et al., 1994). The excessive DA transmission in the mesostriatal pathway induced by METH leads to the accumulation of DA in either cytoplasmic and/or extracellular compartments in the neostriatum (O'Dell et al., 1991; Sulzer et al., 1995). The accumulated DA is oxidized by monoamine oxidase (MAO) and by auto-oxidation, giving rise to oxygen-based free radical superoxide (O_2^-), which is metabolized into highly reactive hydroxyl radical (• OH) or reacts with nitric oxide (NO) to form nitrogen-based free radical peroxynitrite ($ONOO^-$) (Cadet and Brannock,

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1998). Emerging evidence suggests that METH-induced neurotoxicity is dependent upon the production of these reactive oxygen and nitrogen species that are potent oxidants causing necrotic or apoptotic cell death (Abekawa et al., 1996; Di Monte et al., 1996; Hirata et al., 1996; Itzhak and Ali, 1996; Imam et al., 1999). For example, METH-induced striatal DA terminal injury was attenuated in CuZn-superoxide dismutase (SOD) transgenic mice (Hirata et al., 1996) or by the peroxynitrite decomposition catalyst (Imam et al., 1999).

Glutamate-induced Excitotoxicity In addition to DA oxidation, METH exposure also increases extracellular glutamate (Glu) concentration (Nash and Yamamoto, 1992; Abekawa et al., 1994; Stephans and Yamamoto, 1994). The consequent activation of N-methyl-D-aspartate (NMDA) receptor may not only trigger the intracellular signaling pathways and regulate the expression of various gene products (Konradi et al., 1996), but also exacerbate DA release (Weihmuller et al., 1992; Marshall et al., 1993; Abekawa et al., 1994) and subsequently, via the synergistic actions of DA and Glu, may increase the formation of reactive oxygen and nitrogen species, in particular through the production of NO by the nitric oxide synthase (NOS) (Chen and Huang, 1992) and the successive formation of peroxynitrite. This notion is supported by the observations that NMDA receptor antagonists protect against METH-induced striatal DA terminal injury (Sonsalla et al., 1989; Sonsalla et al., 1991; Marshall et al., 1993; Pu and Vorhees, 1995). However, these effects of NMDA receptor antagonists were confounded by their influence on the regulation of core body temperature (Albers and Sonsalla, 1995).

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Interestingly, a toxic dose of METH caused increase of DA release in both CPu and NAc to the same magnitude, however, a gradual increase of extracellular concentration of glutamate occurred in CPu, but not in NAc, an area that is more resistant to METH-induced damage, suggesting that a rapid increase of DA overflow and a delayed increase of glutamate release work synergistically to cause METH-induced neurotoxicity in the striatum (Abekawa et al., 1994). Moreover, METH caused massive overexpression of neuronal NOS (nNOS) in the mouse striatum (Deng and Cadet, 1999). Several groups reported that high doses of NOS inhibitors attenuated METH-induced dopaminergic deficits in the striatum of mice (Abekawa et al., 1996; Di Monte et al., 1996; Itzhak and Ali, 1996), and that nNOS-deficient mice were resistant to METH-induced neurotoxicity (Itzhak et al., 1998). Therefore, it appears that METH-induced toxicity is a result of the combination of reactive oxygen and nitrogen species; both DA and Glu are involved.

Mitochondria Disruption and Apoptosis In addition to the direct diffusion into the mitochondria resulting in disruption of the electrochemical gradient established by the electron transport chain, METH increases the formation of DA-quinones (LaVoie and Hastings, 1999), which open the permeability transition pore, leading to the activation of caspases, the cell death cysteine proteases (Murphy et al., 1999). Moreover, the increased release of Glu induced by METH may activate NMDA receptors, facilitating the production of NO that inhibits complex IV within the electron transport chain and consequently initiates the apoptotic cascade (Heales et al., 1999). The accumulated evidence has suggested the involvement of apoptosis in METH-induced brain damage

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(Hirata and Cadet, 1997; Deng et al., 1999; Ladenheim et al., 2000; Deng et al., 2001) since the first report by Cadet et al in 1997 (Cadet et al., 1997). Several apoptosis-regulatory genes, including *Bax* (Cadet et al., 1997; Jayanthi et al., 2001) and *myc* (Thiriet et al., 2001), were activated by METH exposure. Further work is warranted to dissect the precise molecular mechanism and the apoptotic pathways associated with METH neurotoxicity.

Methamphetamine-induced Hyperthermia It is also important to note that several reports have suggested the direct correlation between METH-induced hyperthermia and the severity of dopaminergic neurotoxicity (Bowyer et al., 1994; LaVoie and Hastings, 1999; Xie et al., 2000). Interestingly, various pharmacological agents, including some DA receptor and NMDA receptor antagonists, appear to protect against METH-induced neurotoxicity by attenuating METH-induced hyperthermia (Albers and Sonsalla, 1995), while other protective agents, including some peptide receptor agonists, can protect without affecting the hyperthermic response to METH (Tsao et al., 1998; El Daly et al., 2000; Itzhak et al., 2000). Moreover, vesicular DA-depleting agent reserpine can lower body temperature but does not prevent METH-induced neurotoxicity (Albers and Sonsalla, 1995). Therefore, hyperthermia may not be solely responsible for the damaging effects elicited by METH, but it has to be taken into account while studying numerous pharmacological or genetic interventions and interpreting the implicated mechanisms.

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1.2 Substance P and Neurokinin-1 Receptor

1.2.1 Structure, Location and Function

Substance P (SP), an undecapeptide discovered in 1931 by von Euler and Gaddum, was the first identified neuroactive peptide and the first proposed neurotransmitter (Raffa, 1998). Comparing with the other two members, neurokinin A (NKA) and neurokinin B (NKB), SP is the most abundant of a family of mammalian tachykinin peptides that exert rapid action on smooth muscle and share a common C-terminal amino acid sequence, -Phe-X-Gly-Leu-Met-NH_2 (Raffa, 1998). When expressed in suitable host cells, they show a rank order of potency as: SP > NKA > NKB (Quartara and Maggi, 1997). SP and NKA are both encoded by the preprotachykinin (PPT) -A gene and formed after alternate RNA splicing and posttranslational processing of this gene. PPT-B gene gives rise to NKB only. SP immunoreactivity was detected in spinal cord, neostriatum, substantia nigra, globus pallidus and other regions in the central nervous system (CNS) (Raffa, 1998).

Three categories of neurokinin receptors have been characterized. All of them are G-protein-coupled receptors with seven transmembrane domains. They are designated according to the endogenous ligand that binds to it with the highest affinity: neuronkinin-1 receptor (for SP), neurokinin-2 receptor (for NKA), and neurokinin-3 receptor (for NKB) (Quartara and Maggi, 1997). The binding of SP to neurokinin-1 (NK-1) receptor induces a clathrin-dependent internalization of the SP/NK-1 complex into endosomes within minutes, which is followed by rapid degradation of the neuropeptide and recycling of the NK-1 receptor to the cell membranes (Mantyh et al.,

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1995). The stimulation of NK-1 receptor activates several second messenger systems which, in turn, trigger a wide range of downstream mechanisms, including cAMP accumulation, arachidonic acid mobilization, phosphatidylinositol turnover and elevation of intracellular calcium concentration. Meanwhile, some effects of NK-1 receptor activation can be mediated by G-proteins without the involvement of second messenger systems, or by a non G-protein-coupled mechanism, including the modification of some membrane channels (Quartara and Maggi, 1997).

However, all mammalian tachykinins have limited selectivity for a particular neurokinin receptor. There is an extensive "cross-talk" between different tachykinins at different receptors (Saria, 1999). Among various central nervous system areas, the basal ganglia, including neostriatum and substantia nigra, is especially enriched in both tachykinins and tachykinin receptors (Raffa, 1998). There is, however, a mismatch between the distribution of tachykinins and tachykinin receptors. The substantia nigra contains high density of neurokinin-3 (NK-3) receptors and, to a much less extent, neurokinin-1 (NK-1) receptors although SP has been detected in substantia nigra (Bannon and Whitty, 1995; Whitty et al., 1995). The NK-2 receptors, which are found most widely located in peripheral tissues, have less, if any, signaling capacity in the neostriatum where NKA precursor PPT-A mRNA is found (Raffa, 1998). The neostriatum contains a high concentration of both NK-1 and NK-3 receptors. Several lines of data have suggested that SP may act through NK-1/NK-3 receptors, while NKA may act preferentially through NK-3 receptors (Bannon and Whitty, 1995; Whitty et al.,

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1995). These data highlighted the remarkable complexity and plasticity of neuromodulation exerted by tachykinins.

1.2.2 Possible Roles in Central Nervous System

SP is widely distributed in CNS and has a variety of functions under both physiological and pathological circumstances. NK-1 receptor antagonists have been pursued as a pharmaceutical target for various conditions. Here is just to name a few.

Nociception For half a century, SP had been considered as a primary pain transmitter. In the last twenty years, the understanding of mechanisms involved in pain transmission has undergone a remarkable evolution. Data from the studies using NK-1 receptor antagonists (Quartara and Maggi, 1998) and PPT-A gene or NK-1 receptor knockout mice (Cao et al., 1998; De Felipe et al., 1998) have confirmed the essential role of tachykinins in nociception. Moreover, excitatory amino acids and tachykinins have been identified as co-transmitters in spinal cord. A large amount of evidence indicates that Glu, via NMDA receptor, provides the fast component of nociception; while tachykinins, via NK-1 receptor, produce the slowly developing and prolonged excitation (Quartara and Maggi, 1998). This process involves the interactions between excitatory amino acids and tachykinins at the postsynaptic level, including the stimulation of NK-1 receptor and subsequent protein kinase C (PKC)-dependent facilitation of NMDA transmission, as well as the formation of NO by Ca^{2+}-dependent NOS reaction (Woolf and Doubell, 1994).

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Excitotoxicity and Neuronal Survival After seven decades, the understanding of SP has evolved beyond the original concept as a pain transmitter of spinal cord. As a matter of fact, the interactions between excitatory amino acids and tachykinins have also been proposed in epileptic seizure and focal cerebral ischemia. NK-1 receptor antagonist reduced excitotoxin-induced seizures (Zachrisson et al., 1998) and PPT-A gene knockout mice were resistant to kainate-induced seizures as well as neuronal cell death (Liu et al., 1999b), presumably in part via attenuating Glu release. Focal cerebral ischemia induced remarkable expression of SP and NK-1 in glutamatergic neurons in cortex (Stumm et al., 2001) and NK-1 receptor antagonist reduced the infarct size (Yu et al., 1997).

Inflammation It was documented that SP is capable of modifying the response of various inflammatory cells, including macrophages, lymphocytes, granulocytes and mast cells (Saria, 1999). Reactive astrocytes express NK-1 receptors after CNS injury, causing elevation of intracellular calcium concentration and production of various cytokines (Quartara and Maggi, 1997), therefore participating in the immunological and inflammatory processes. NK-1 receptor antagonist has been shown to reduce the severity of the inflammatory CNS disease, such as meningoencephalitis (Kennedy et al., 1997), in which the reactive astrocytes could be pathogenic.

Behavior Blockade of NK-1 receptor does not have marked effects on gross behavior of experimental animals (Quartara and Maggi, 1998). However, distinct changes on more refined behavior have been reported, such as the involvements of SP/NK-1 in depression, anxiety and stress (Kramer et al., 1998; Rupniak et al., 2000). It

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is worthy to note that the rewarding effects of opiates were absent in NK-1 receptor knockout mice and the physical response of opiate withdrawal was also attenuated (Murtra et al., 2000). Remarkably, a new NK-1 receptor antagonist, MK-869, has been shown to be effective in treating major depressive disorder in a recent clinical study in humans (Kramer et al., 1998), representing the first promising clinical trial with NK-1 receptor antagonist on a large scale. It is conceivable that NK-1 receptor antagonist will be used for clinical treatment of CNS diseases as our understanding of its functions in various brain regions upon numerous insults continues to grow.

1.2.3 The Dynamic Modulator of Dopaminergic System

Tachykinins and tachykinin receptors are integral components of the mesencephalic DA system (Angulo and McEwen, 1994). As mentioned previously, the basal ganglia is particularly rich in both SP and NK-1 receptors (Saria, 1999).

Anatomical Circuits The principal neurons of both the striatum and the NAc are GABAergic medium spiny neurons, which in rodents constitute 90-95% of all striatal neurons (Chang et al., 1982). Two groups of medium spiny neurons have been identified based on anatomical and biochemical observations (Gerfen, 1992; Kawaguchi, 1997). One group, which expresses high levels of D_1 receptor and the tachykinin peptide SP as well as opioid peptide dynorphin, projects to the substantia nigra and the internal segment of the globus pallidus (GPi; the entopeduncular nucleus in the rodents); whereas the other group, which expresses high levels of D_2 receptor and the opioid peptide enkephalin, projects to the external segment of the globus pallidus (GPe; the

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globus pallidus in rodents). In addition to medium spiny neurons, the neostriatum contains three groups of interneurons: cholinergic interneurons, somatostatin/nitric oxide-expressing interneurons, and parvalbumin-containing interneurons. Each group comprises 1-3% of all striatal neurons (Kawaguchi, 1997).

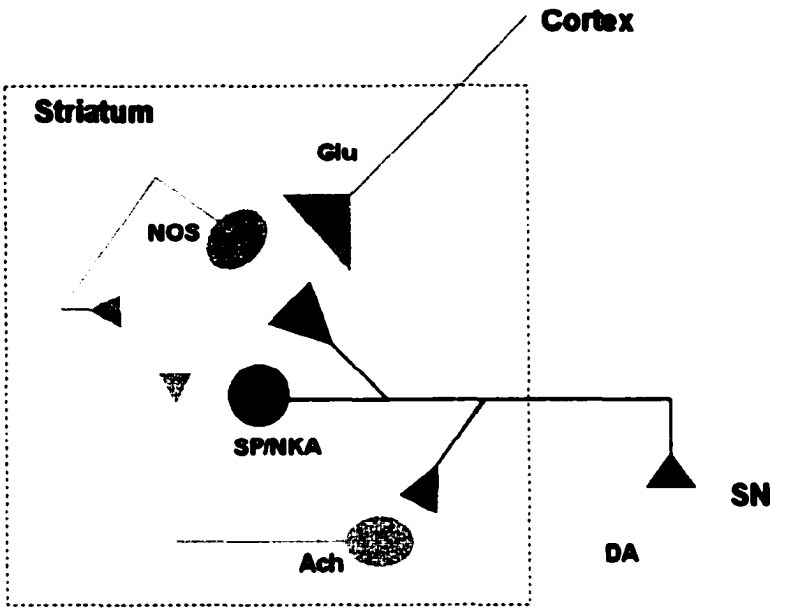

Figure 1-2. Schematic illustration of neurocircuits in the striatum.

The SP/NKA-containing neurons are postsynaptic to the dopaminergic terminals in the neostriatum (Fig. 1-2). From there, they can project to the substantia nigra (SN) and the ventral tegmental area, where they make direct (excitatory) contact with dopaminergic neurons that reciprocally send axons back to the neostriatum (Angulo, 1992). They can also relay collaterals within the neostriatum to make local synaptic

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contacts with the interneurons (Lee et al., 1997b) as well as the glutamatergic nerve terminals from the cortex or thalamus (Bolam et al., 1983). The immunocytochemical study has revealed the NK-1 receptors on cholinergic neurons and somatostatin/NOS neurons in the neostriatum (Kaneko et al., 1993; Chen et al., 2001). Therefore, the tachykinin peptides can modulate neostriatum function either directly by the action on DA neurons in the substantia nigra and ventral tegmental area, or indirectly via local interneurons and axon terminals.

Functional Modulation SP/NK-1 is not only anatomically intrinsic to basal ganglia circuitry, but also functionally modulates the activity of mesencephalic DA system. Pharmacological studies have demonstrated that SP locally stimulated spontaneous release of DA from rat striatal slices in a tetrodotoxin-sensitive fashion, which could be prevented by the SP antagonist (D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11}) SP (Petit and Glowinski, 1986) or by the NK-1 receptor antagonist WIN-51,708 (Khan et al., 1995). Microinjections of SP or NKA into the substantia nigra caused increased release of DA (Reid et al., 1990b; Reid et al., 1991) as well as Glu (Reid et al., 1990a) in the striatum. Furthermore, the firing of SP-containing neurons in striatum increased the firing probability of somatostatin/NOS and cholinergic interneurons by the receptor-mediated opening of tetrodotoxin-insensitive cation channels (Aosaki and Kawaguchi, 1996). Local perfusion of SP in the striatum elevated extracellular Ach concentration via NK-1 receptor-mediated mechanism (Anderson et al., 1993). Behaviorally, the direct application of SP into the ventral mesencephalon caused a marked increase in locomotion (Kelley et al., 1985). Moreover, NK-1 receptor has been associated with extrapyramidal side effects from chronic treatment with antipsychotic agents (Humpel et

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al., 1990; Liminga and Gunne, 1993). These data strongly suggest that SP/NK-1 is intimately involved in the neostriatum function and the regulation of neurotransmission in mesencephalic DA system either directly or indirectly.

1.3 Central Hypothesis and Research Design

1.3.1 Rationale for the Central Hypothesis

Given the indispensable and dynamic roles of tachykinins and neurokinin receptors in the neural activity of basal ganglia, it would not be surprising that the exposure of METH causes the alterations in the tachykinin system. Previous work has demonstrated that multiple administrations of METH elevated the levels of PPT-A mRNA, SP and NKA within striatonigral neurons (Sonsalla et al., 1986; Bannon et al., 1987; Zhang et al., 1997). It is believed that METH-induced augmentation of DA transmission causes the increase of tachykinin biosynthesis (Sonsalla et al., 1986; Young et al., 1986; Bannon et al., 1987), presumably via the postsynaptic D_1 receptor that is predominantly expressed by PPT mRNA-expressing medium spiny neurons (Gerfen, 1992; Zhang et al., 1997). However, it is not clear whether tachykinin system is involved in METH-induced neurotoxicity and, if so, whether it triggers the toxic cascades or protective mechanisms. Several lines of data have led us to speculate that the tachykinin system is part of the machinery responsible for the neurotoxic effects induced by METH.

First, both *in vitro* and *in vivo* studies demonstrated the excitatory effects of SP/NK-1 on DA transmission, as reviewed in the previous section. We have found that PPT-A mRNA level progressively elevated with the chronic treatment of METH and decayed

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when the drug was withdrawn, suggesting that the neurokinin system may play a role in the augmentation of locomotor activity elicited by METH (Zhang et al., 1997). Preliminary work in our lab has shown that administration of NK-1 antagonists significantly reduced DA release in striatum and the hyperlocomotion resulting from acute cocaine exposure (Kraft et al., 2001; Noailles and Angulo, 2002). Further evidence implicating tachykinins in the actions of psychostimulants is provided by the observation that NK1-/- mice showed no increase in the opiate-induced locomotor effects linked to the rewarding properties of the drug (Murtra et al., 2000). Therefore, it is reasonable to postulate that tachykinins may exert the modulatory effects, through NK-1 receptors, on METH-induced deleterious changes by augmenting DA overflow.

Second, local application of SP could also increase Glu release (Reid et al., 1990a) and the link between NK-1 receptor and NMDA receptor has been suggested in several pathological conditions involving excitatory amino acids (Woolf and Doubell, 1994; Liu et al., 1999b; Stumm et al., 2001). The augmentation of locomotor activity after chronic METH treatment, which is accompanied by the elevation of PPT-A mRNA levels as described above, was attenuated by the concurrent administration of NMDA receptor antagonist, indicating the interactions among SP, DA and Glu during METH exposure (Zhang et al., 1997). Moreover, NK-1 receptor has been located on somatostatin/NOS-containing interneurons (Kaneko et al., 1993; Chen et al., 2001), and most recent evidence has confirmed the direct projections from SP-containing neurons to NOS-containing neurons in striatum (Li et al., 2002) as well as the colocalization of nNOS and NK-1 receptors in the striatal interneurons (Li et al., 2001). Accordingly, we

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speculate that the activation of NK-1 receptors may exacerbate METH-induced damage by facilitating the NMDA receptor responses or increasing the formation of NO.

Third, the tachykinin family is not alone on its effects on mesencephalic DA system, other peptides including enkephalin have also been shown to modulate dopaminergic physiology (Angulo and McEwen, 1994). Pharmacological and deafferentation studies have established that mesencephalic dopaminergic transmission tonically inhibiting neostriatal enkephalinergic expression, while stimulating tachykinin system at both peptide and mRNA levels (Gerfen et al., 1991; Angulo, 1992). DADLE, a metabolically stable analog of the endogenous *delta* (δ) opioid peptide enkephalin, has been shown to block the METH-induced decrease in DAT binding sites and TH protein levels in the striatum (Tsao et al., 1998; Tsao et al., 2000). It is tempting to propose that tachykinins represent a family of peptides that might regulate neurotoxic pathways under conditions of excessive DA transmission in the striatum.

On the basis of the foregoing observations, we hypothesize that tachykinins, via the activation of NK-1 receptors, may serve as key participants in the neurodegenerative cascades induced by METH.

1.3.2 Research Design

As the first step to test the role of NK-1 receptor in METH-induced neurodegeneration, we conducted pharmacological, neurochemical and histological studies to assess the effects of concurrent administration of METH and selective

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nonpeptide NK-1 receptor antagonists on the markers of METH-induced neurotoxicity. The research design is summarized as follows.

- Specific Aim I (see Chapter 3)

Question — Does METH-induced neural cell damage affect both pre- and post-synaptic sites in the neostriatum?

Objective — Evaluate METH-induced neural cell damage at the pre- and post-synaptic sites in the neostriatum.

Experimental Design

a) Characterize the effects of METH on the pre-synaptic markers

b) Evaluate METH-induced post-synaptic neuronal degeneration

c) Establish a dose-toxicity response relationship

- Specific Aim II (see Chapter 4)

Question — Is NK-1 receptor involved in METH-induced neurotoxicity?

Objective — Examine the effect of NK-1 receptor antagonists on pre-synaptic marker of METH-induced neurotoxicity in the neostriatum (CPu and NAc).

Experimental Design

a) Establish the dose-response effect of NK-1 receptor antagonists on METH-induced loss of DAT sites

b) Verify the specificity at NK-1 receptor

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c) Establish the time-course of NK-1 receptor blockade on METH-induced damage

d) Examine the effect of NK-1 receptor antagonist on METH-induced hyperthermia

• Specific Aim III (see Chapter 5)

Question — To what extent does the blockade of NK-1 receptor protect against METH-induced neurotoxicity?

Objective — Evaluate the magnitude of protection afforded by NK-1 receptor blockade on METH-induced pre- and post-synaptic neurodegeneration.

Experimental Design

a) Neurochemical assessment of neuroprotection by NK-1 receptor antagonist

DAT protein levels

Tissue DA and its metabolites content

TH protein levels

GFAP protein levels

b) Histological assessment of neuroprotection by NK-1 receptor antagonist

TH immunohistochemistry

GFAP immunofluorescence

Fluoro-Jade B staining of neuronal degeneration

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Chapter 2

Materials and Methods

2.1 Animals and Drug Administrations

Ten weeks old male ICR/CD-1 mice weighing 36-44mg (Taconic, Germantown, NY) were single housed on a 12-h light/dark cycle with food and water available *ad libitum*, and were habituated for two weeks before commencement of drug treatments. For all the experiments in the project, except for the time course study, animals were sacrificed by decapitation on day 3 post-treatment. This time point, when the drugs in tissue have been washed out, was used in an attempt to assess toxic rather than pharmacological drug effects. For time course study, animals were sacrificed on 3, 7, 14 or 28 d after the last treatment. All animal use procedures were according to the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* and were approved by the animal care committee at Hunter College of the City University of New York.

Methamphetamine (Sigma, St. Louis, MO) was dissolved in saline and four injections (5 or 10 mg/kg) at 2-hr intervals were administered intraperitoneally for one day. Acute toxic dosing model with 5 or 10 mg/kg given four times at 2-hour intervals (i.p. or s.c.) in naïve animals is currently the most frequently used model in rodents.

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Given the differences in elimination half-life between rodent and human ($t_{1/2}$: rodent – 70 min; human – 12 h), this regimen best represents the "maintenance" abuse patterns in human (Cho et al., 2001) and can reliably produce striatal dopaminergic neurotoxicity (O'Dell et al., 1991).

WIN-51,708 is a heterosteroid-based nonpeptide NK-1 receptor antagonist (IC_{50} = 50 nM in rat forebrain membrane) (Quartara and Maggi, 1997) which displays remarkably high (> 400-fold) selectivity for the rat as opposed to the human NK-1 receptor (Pradier, 1995). It was dissolved in 45% 2-hydroxypropyl-β-cyclodextrin (RBI/Sigma, Natick, MA). L-733,060 is a benzylether piperidine-based nonpeptide NK-1 receptor antagonist (IC_{50} = 1.0 nM at the human NK-1 receptor expressed in CHO cells) with high affinity for human NK-1 receptor (Quartara and Maggi, 1997). L-733,060 and its less active enantiomer L-733,061 were dissolved in dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO). All three NK1 receptor antagonists were purchased from RBI/Sigma. Vehicle, WIN-51,708 (2.5, 5, 10, 20 or 30 mg/kg), L-733,060 (0.5, 1 or 2 mg/kg) or L-733,061 (1 or 2 mg/kg) were given 30 min prior to the 1st and 4th injections of methamphetamine or saline. We have found that this paradigm is most effective in preventing the neurotoxic changes induced by multiple administrations of METH (data not shown).

2.2 Autoradiographic Analysis of DAT

The autoradiographic analysis of DAT was done using [^{125}I]RTI-121 (3β-(4-[^{125}I]iodophenyl)tropane-2β-carboxylic acid isopropyl ester, 2200 Ci/mmol; New

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England Nuclear, Boston, MA). [^{125}I]RTI-121 has been shown to selectively label striatal DA uptake sites, but not serotonin uptake sites, with specific binding greater than 90% of total binding (Boja et al., 1995; Tsao et al., 1998). The autoradiographic assays were performed as previously described (Hirata et al., 1996; Tsao et al., 1998) with minor modifications. Briefly, after decapitation the brains were rapidly removed and frozen on dry ice. Twenty-micrometer sections of the brain regions encompassing the nucleus accumbens and caudate putamen were cut on a cryostat. Typically four slides with 6-8 sections from every mouse brain were used. Sections were vacuum-dried and incubated with 0.073nM [^{125}I]RTI-121 in assay buffer (137 mM NaCl, 2.7 mM KCl, 10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 10 mM NaCl) at room temperature for 60 min. Nonspecific binding was determined by 10μM GBR-12909. After incubation, wash the slides in ice-cold assay buffer twice for 20 min. The slides were then quickly dipped into ice-cold distilled water for several times, immediately dried under a fan and continue to air-dry overnight. Appose the slides to Hyperfilm MP (Amersham Pharmacia Biotech, Piscataway, NJ) for 51-53 hr together with [^{125}I]microscale. The binding of [^{125}I]RTI-121 to the brain was quantified by densitometry using a computer-based NIH image analysis system.

2.3 HPLC-EC Detection of DA and Its Metabolites

Brains were rapidly removed from decapitated mice and dissected on an ice-chilled mouse brain matrix (Harvard Apparatus, Holliston, MA). One-millimeter coronal sections were obtained and quickly frozen on dry ice. Tissues from nucleus accumbens,

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medial striatum and lateral striatum were punched out using a 500μm diameter cannula, expelled into 60 μl ice-cold 0.1 M perchloric acid containing 10 ng/ml 3,4-dihydroxybenzylamine (DHBA) as an internal standard, then frozen and thawed twice. Samples were then centrifuged at 10,000 x g for 10 min. The pellets were redissolved in 100 μl 0.1 M NaOH overnight before measuring the protein content by the Bradford method. The supernatants were used for the measurement of DA and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) content using high-performance liquid chromatography with electrochemical (HPLC – EC) detection. Ten microliters of sample were injected into a microbore reversed phase column (MD 150 (RP - C18), 4μm, 3x150 mm; ESA Inc., Bedford, MA) and separated by using ESA MD-TM mobile phase at a flow rate of 0.55ml/min. Peaks were detected by a Coulochem II detector (5200, ESA Inc., Bedford, MA) with two analytical electrodes (E1, -260mV; E2, 320 mV). To adjust for variability in the amount of the tissue punches, all the values were expressed as per milligram of protein.

2.4 Western Blot Analysis of DAT, TH and GFAP

Protein samples were extracted from the striatal area as previously described (Deng et al., 1999) with minor modifications. Briefly, dissected striata were homogenized in a lysis buffer (320 mM sucrose, 5 mM HEPES, 5 nM EDTA, 2 nM aprotinin, 10 nM leupeptin). For DAT Western blotting, the homogenates were centrifuged at 2,000 x g for 5 min at 4°C and the supernatants were further centrifuged at 28,000 x g for 30 min at 4°C. The pellets were resuspended in lysis buffer and used for analysis. For TH and

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glial fibrillary acidic protein (GFAP) Western blotting, the homogenates were centrifuged at 10,000 x g for 10 min at 4°C and the supernatants were used for analysis. The protein content was assayed by the Bradford method. Ten micrograms of protein were subjected to 12% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (BioRad, Hercules, CA). After the blocking of nonspecific binding, membranes were probed with polyclonal anti-DAT (1:500, Chemicon, Temecula, CA), monoclonal anti-TH (1:1000, Chemicon, Temecula, CA) or monoclonal anti-GFAP (1:5000, Sigma, St. Louis, MO) antibodies in the presence of 0.5% (for DAT) or 5% (for TH and GFAP) nonfat dry milk in TBS/T buffer at 4°C overnight. The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies for 1 h at room temperature, and the proteins were detected using the enhanced chemiluminescence (ECL) Western blotting detection system kit (Amersham Pharmacia Biotech, Piscataway, NJ). Used as an internal standard, Western blotting for α-tubulin was performed with a monoclonal anti-α-tubulin antibody (1:5000, Sigma, St. Louis, MO). Densitometry was performed by a NIH image system and the relative density of each band obtained for DAT, TH and GFAP was normalized against that of α-tubulin.

2.5 TH Immunohistochemistry

The animals were decapitated and brains were quickly removed and frozen on dry ice. The fresh frozen brains were cut into 20 μm sections on a cryostat, and thaw-mounted on Fisher Superfrost Plus slides, stored at –80°C until use. Thawed sections

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were completely dried and post-fixed in absolute ethanol at –20°C for 10 min. After several washes in 0.1 M PBS, sections were incubated with 0.3% hydrogen peroxide for 30 min, followed by incubation with 10 % blocking buffer containing 0.3 % hydrogen peroxide for 1 h at room temperature. Then the sections were incubated with polyclonal rabbit anti-TH antibody (1:100, Chemicon, Temecula, CA) diluted in 2 % blocking buffer and 0.3 % Triton X-100 in PBS at room temperature for 2 h and then at 4C for 36 h. Subsequent processing with biotinylated secondary antibody and ABC complex was performed using Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Sections were then visualized using DAB substrate kit for peroxidase (Vector Laboratories, Burlingame, CA). After washing in distilled water, sections were dehydrated and coverslipped. For the assessment of nonspecific staining, alternating sections were incubated without the primary antibody.

Densitometric measurements of optical density were performed using NIH Image software and with the experimenter blinded to the treatment groups. Images were acquired from each photomicrograph using Adobe Photoshop and converted to gray scale before the staining intensity was analyzed. Background was defined as the optical density over an area where no TH-positive fibers were stained. The optical density was averaged from four sections per animal (five to seven animals per group).

2.6 Double Labeling of Fluoro-Jade B and GFAP

To test the effects of METH and WIN-51,708 on postsynaptic striatal neurons, we performed double labeling of Fluoro-Jade B dye and immunofluorescence staining of

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GFAP. Slide-mounted sections were completely dried and post-fixed in absolute ethanol at –20°C for 10 min. After several washes in 0.01 M PBS, sections were incubated with the protein diluent from Vector Mouse on Mouse (M.O.M.) kit (Vector Laboratories, Burlingame, CA) for 5 min, followed by incubation with monoclonal anti-GFAP antibody (1:30, Sigma, St. Louis, MO) diluted in 0.3 % Triton X-100 and M.O.M. protein diluent in PBS at 4°C overnight. Sections were then incubated with biotinylated secondary antibody at room temperature for 30 min, followed by incubation with rhodamine avidin DCS (cell sorting grade; Vector Laboratories, Burlingame, CA) for 5 min. After washed for 5 min in PBS and 5 min in distilled water, sections were then placed in 0.0001 % Fluoro-Jade B staining solution (Histo-Chem Inc., Jefferson, AR) with 0.1 % acetic acid at ℃ for 1 h. After three washes in distilled water for 1 min each, sections were dried, immersed in xylene and coverslipped with DPX. The tissue was examined using an epifluorescent microscope with blue (460 – 500 nm) and green (532 – 587 nm) excitation light. Fluoro-Jade B and GFAP positive cells were counted from four sections per animal (five to seven animals per group).

2.7 Temperature Measurements

Rectal body temperatures were determined by a BAT-12 thermometer coupled to a RET-3 mouse rectal probe (Physitemp Instruments, Clifton, NJ), measured right before and 1 h after each single injection of METH, as well as 2 h after the last injection.

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2.8 Statistic Analysis

All data were presented as means ± SEM. The significance of difference between means was analyzed by Student's *t* test (single comparisons) or by ANOVA and post hoc analyses using the Fisher's protected least significant difference test, with the significance level set at $P < 0.05$. The degree of neuronal degeneration was assigned a rank score depending on the average number of Fluoro-Jade B (FJB)-positive cells in the striatum per section by counting four coronal sections per animal: 0 (–), < 1 cell / section; 1 (+): 1 - 50 cells / section; 2 (++): 51 - 100 cells / section; 3 (+++): 101 - 150 cells / section; 4 (++++): > 150 cells / section.

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Chapter 3

Standardization of METH Neurotoxicity

In order to assess the involvement of NK-1 receptor in METH-induced neurotoxicity, we need to first standardize the toxic effects from acute multiple METH administrations. It is well known that METH causes presynaptic dopaminergic terminal degeneration, manifested by several neurochemical and morphological markers. Some reports also suggested the damage to postsynaptic neurons (Cadet et al., 1998). Most importantly, Cadet et al. have demonstrated that METH could also induce apoptosis (Cadet et al., 1997) and have shown TUNEL-positive non-dopaminergic neurons in the striatum after the exposure of METH (Deng et al., 1999). Therefore, the first aim of this project is to evaluate METH-induced neural cell damage at both pre- and post- synaptic sites, and establish a dose-toxicity relationship so that we can employ an appropriate dose of METH for the pilot work on the role of NK-1 receptor in METH neurotoxicity.

3.1 Presynaptic Neurochemical Markers

As the first step, we determined the dose of METH that produces approximately 50% depletion of DAT sites in the neostriatum. DAT is a neurochemical marker widely recognized as an indicator of neurotoxicity in this part of the brain. It is more sensitive

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than other markers to both METH-induced toxic actions (Harvey et al., 2000) and the protecting effects of defensive agents (Tsao et al., 1998). In addition, the spatial resolution afforded by autoradiography is ideal for detection of the regionally specific changes in DAT binding sites. Using separate groups of mice (n=6 per group), we tested various doses of METH (2.5-10 mg/kg) to establish a toxicity spectrum. DAT sites were assessed in coronal sections of brain by autoradiography using [^{125}I]RTI-121. ICR (equivalent to CD-1) mice were used in the study and sacrificed on day 3 post-treatment. Administration of METH resulted in dose-dependent reductions of [^{125}I]RTI-121 binding to DAT sites in all three brain areas examined (lCPu, lateral caudate-putamen; mCPu, medial caudate-putamen; NAc, nucleus accumbens) compared to saline-treated controls (Fig. 3-1a, 3-1b). The 2.5 mg/kg dose had minimal effects on DAT loss, whereas 10 mg/kg caused severe decrements in DAT sites (~73%, 69% and 39% decrease in lCPu, mCPu and NAc, respectively). The 5 mg/kg of METH caused about 51%, 47% and 28% loss of DAT sites in lCPu, mCPu and NAc, respectively. This effect was further confirmed by Western blotting of DAT protein in striatum (Fig. 3-1c), for which we observed the reductions of about 50% and 70% at 5 and 10 mg/kg, respectively. Tissue DA content is another neurochemical marker for DA terminal function. In order to verify the dose-toxicity relationship of METH on presynaptic terminal, we quantified the tissue DA content by HPLC. Striatal tissue DA content was also depleted upon METH treatment by about 60% and 78% at 5 and 10 mg/kg (Fig. 3-1d).

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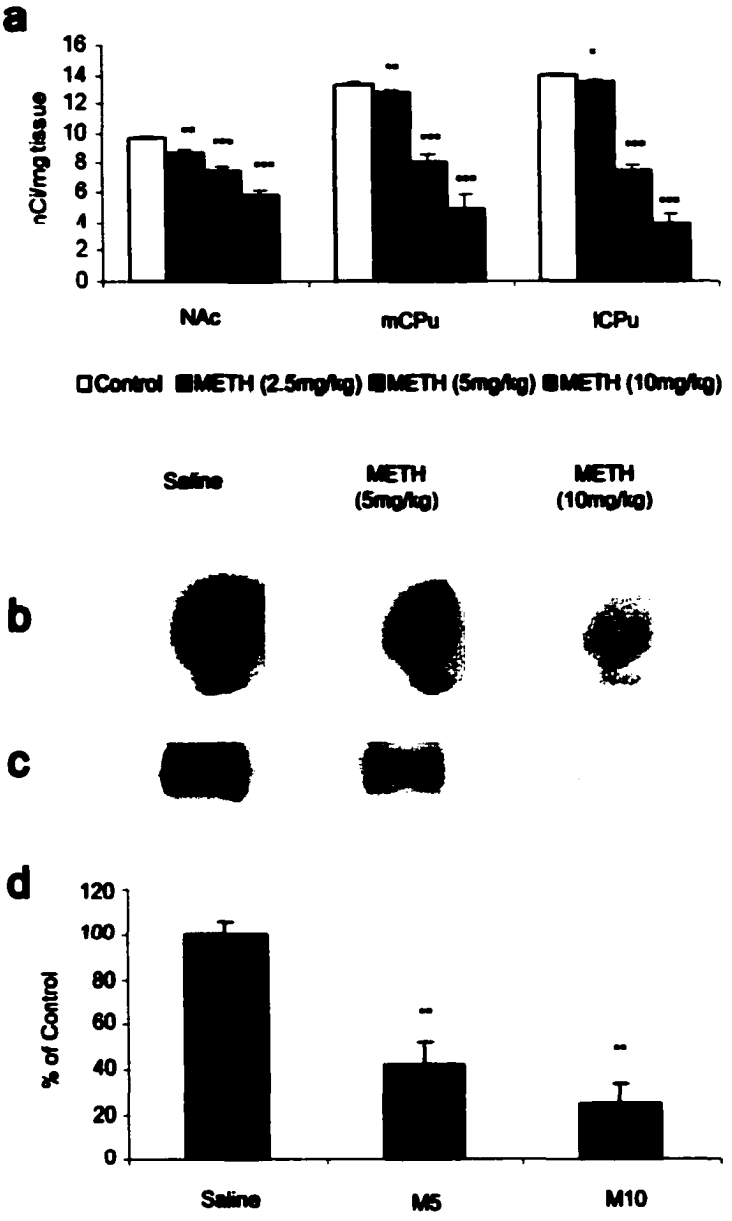

Figure 3-1. Effect of METH dose on neostriatal DAT sites and tissue DA content. Mice received four injections of METH at two-hour intervals. On day 3 post treatment, METH (n = 6) dose-dependently caused **(a)** loss of DAT [^{125}I]RTI-121 binding sites in nucleus accumbens (NAc), medial caudate putamen (mCPu) and lateral caudate putamen (lCPu), as demonstrated by **(b)** autoradiographic images of [^{125}I]RTI-121 binding in CPu, as well as **(c)** the reduction of DAT protein level on Western blots and **(d)** the depletion of tissue DA content in HPLC. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ as compared to saline control.

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3.2 Postsynaptic Neuronal Degeneration

Although it has been well documented for two decades that METH exposure can cause selective presynaptic DA terminal destruction, there was very few evidence suggesting that high doses of METH could also cause damage to postsynaptic striatal neurons (Cadet et al., 1998; Deng et al., 1999). In order to verify the hypothesis that METH administration can lead to degeneration in intrinsic striatal cell bodies, we used a newly developed technique, Fluoro-Jade B staining, to directly visualize the postsynaptic neuronal degeneration. Fluoro-Jade B is a newly developed anionic fluorochrome that selectively stains degenerating neurons after a variety of neurotoxic insults, including kainic acid, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 3-nitropropionic acid (3-NPA). Fluoro-Jade B staining is more sensitive, definitive and reliable than almost all the other conventional histochemical techniques in localizing neuronal degeneration (Schmued et al., 1997; Schmued and Hopkins, 2000). No positive Fluoro-Jade B staining was observed in any saline treated animals (n = 5), neither were the Fluoro-Jade B positive neurons evident in 5 mg/kg METH treatment group (n = 5); however, 10 mg/kg METH resulted in prominent staining of Fluoro-Jade B positive striatal neurons in three out of five treated animals (Fig. 3-2). In the three animals with positive Fluoro-Jade B staining, one of them showed Fluoro-Jade B positive cells throughout the striatum, the other two had less prominent staining which was relatively concentrated at the ventral lateral region of striatum. These results provide the direct demonstration of METH-induced neuronal degeneration in the

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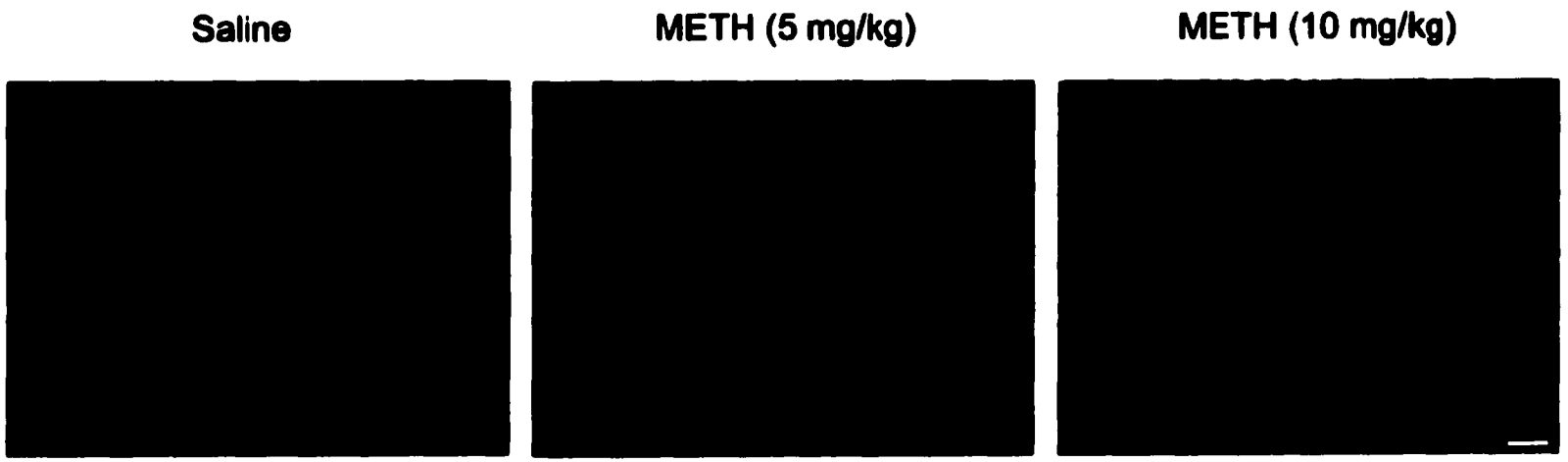

Figure 3-2. Effect of METH dose on FJB staining of striatal neuronal degeneration. No FJB positive neuron was seen in either saline treated control (n = 5) or animals treated with four injections of 5 mg/kg METH (n = 5). Administration of 10 mg/kg METH four times at 2 h intervals (n = 5) caused apparent FJB staining.

intrinsic striatal cell bodies after multiple high doses of METH administration, confirming the idea that METH can cause toxic effects beyond the monoamine system.

In the light of these results, we selected a dose of 5 mg/kg of METH for the next stage of the present project, because this dose caused approximately half of the maximum toxic effect so that it is ideal to test the role of NK-1 receptor in METH-induced neurotoxicity.

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Chapter 4

Involvement of NK-1 Receptor

The knowledge about the functional roles of neurotransmitters is obtained, to large extent, by pharmacological studies using specific high-affinity receptor antagonists. It was not until early 90's, with the appearance of small molecule nonpeptide antagonists for neuropeptide receptors, did the research on physiological and pathological function of neuropeptide advanced rapidly. In order to evaluate the involvement of NK-1 receptor on METH-induced neurotoxicity, we used the highly selective nonpeptide NK-1 receptor antagonists to evaluate the effects on DAT sites in the neostriatum, a reliable neurochemical marker sensitive to both METH insult and protective intervention.

4.1 Dose-Response Effect

We first tested the effect of NK-1 receptor antagonist WIN-51,708, which displays high affinity for the rodent NK-1 receptor (>400 fold compared to human NK-1 receptor), on the loss of DAT autoradiographic binding sites induced by METH. WIN-51,708 (n = 6 per group) was administered intraperitoneally at 2.5, 5, 10, 20 and 30 mg/kg of body weight at 30 min before the 1st and 4th injections of METH. Preliminary work has found that this schedule was as effective as four injections of the antagonist.

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All doses of WIN-51,708 afforded protection against METH-induced loss of DAT binding sites in mCPu and lCPu, with 20 mg/kg displaying almost complete protection (~90% and ~85% relative to vehicle-injected controls in mCPu and lCPu, respectively). However, no protective effect was apparent in NAc at the doses used in this study (Fig. 4-1a, 4-2).

4.2 Specificity at NK-1 Receptor

We then employed a second nonpeptide NK-1 receptor antagonist, L-733,060, in order to ascertain the specificity of the NK-1 receptor in the neurotoxic actions of METH. The L-733,060 compound has lower affinity for rodent as compared to human NK-1 receptor, but has a less active enantiomer (L-733,061) available as a control for nonspecific pharmacological activity. When tested at 0.5, 1 and 2 mg/kg, L-733,060 (n = 6 per group) was found to prevent the loss of DAT sites in CPu in a dose-dependent fashion, with 2 mg/kg providing the protection of ~80% and ~72% of DAT sites in mCPu and lCPu, respectively, compared to controls. No protection of DAT sites was observed in NAc. The less active enantiomer L-733, 061 (n = 6 per group) did not display protection at the same dose. Neither WIN-51,708 nor L-733,060 alone elicited changes on DAT sites in saline-treated controls (Fig. 4-1b, 4-2). Because WIN-51,708 has a higher affinity than L-733,060 for the rodent NK-1 receptor, and displays nearly complete protection of DAT sites from METH, it was chosen for subsequent experiments.

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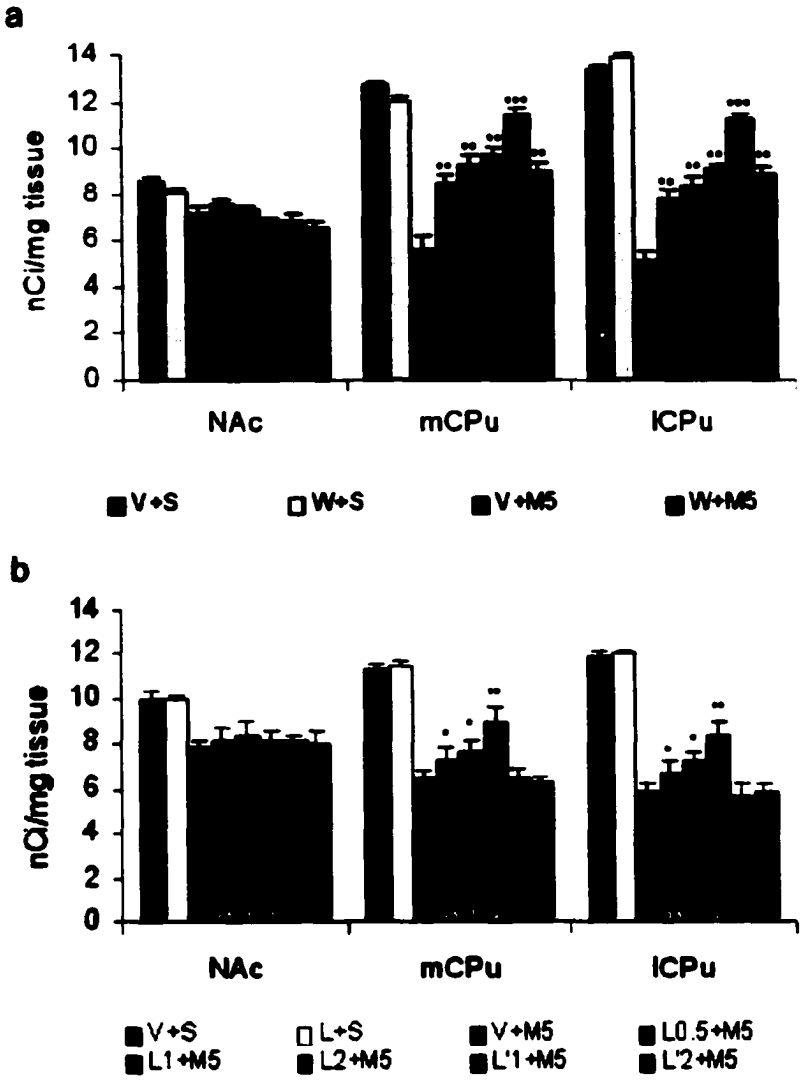

Figure 4-1. NK1 receptor antagonists blocked the loss of DAT [^{125}I]RTI-121 binding sites in the striatum induced by METH treatment. **(a)** WIN-51,708 (with different doses indicated at the bottom of each corresponding bar, n = 6) protected against DAT loss in medial (mCPu) and lateral (lCPu) caudate putamen, with 20mg/kg causing almost complete protection, whereas no effect in nucleus accumbens (NAc) was observed. **(b)** L-733,060 (using the same treatment paradigm as WIN-51,708), within 0.5-2mg/kg dose range (n = 6), also provided partial protection in the striatum, while it had no effect in NAc. The inactive enantiomer L-733,061 had no effect at the same dose. $^{*}p < 0.05$, $^{**}p < 0.01$ as compared to animals treated with vehicle + METH.

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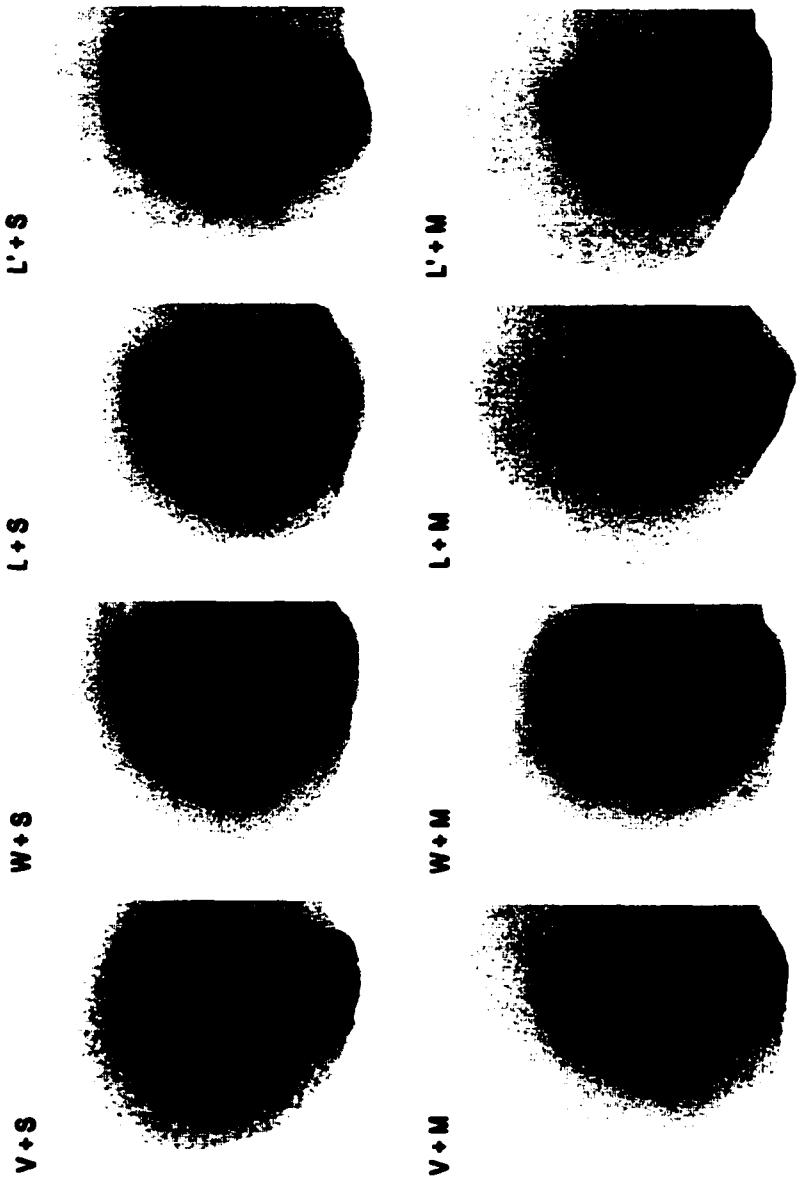

Figure 4-2. Autoradiographic images of DAT [^{125}I]RTI-121 binding sites in striata. *V+S*, vehicle + saline; *W+S*, WIN-51,708 + saline; *L+S*, L-733,606 + saline; *V+M*, vehicle + METH; *W+M*, WIN-51,708 + METH; *L+M*, L-733,060 + METH; *L'+S*, L-733,061 + saline; *L'+M*, L-733,061 + METH.

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4.3 Time Course

To establish the temporal pattern of NK-1 receptor blockade on METH-induced damage and to ascertain if the neuroprotection afforded by WIN-51,708 on the loss of [^{125}I]RTI-121 binding to DAT sites in the neostriatum was persistent, the time course was evaluated at days 3, 7, 14, and 28 post drug treatment (n = 6). In both mCPu and lCPu, WIN-51,708 consistently protected against the decrements of DAT sites at all time points. Rather unexpectedly, the NK-1 antagonist also protected against METH-induced decrease of DAT sites at days 14 and 28 post-treatment in NAc (Fig. 4-3).

4.4 Hyperthermia

Several laboratories have demonstrated that the treatment with METH produces hyperthermia and have postulated that the neurotoxic effects of METH may be due in large part to the elevation in body temperature (LaVoie and Hastings, 1999; Xie et al., 2000). We measured rectal temperature right before and 1 h after each single injection of METH, as well as 2 h after the last injection (n = 7). Administration of METH caused significant elevation of core body temperature. However, concurrent administration of METH and the NK-1 receptor antagonist WIN-51,708 failed to prevent hyperthermia. Administration of WIN-51,708 alone did not affect core body temperature (Fig. 4-4).

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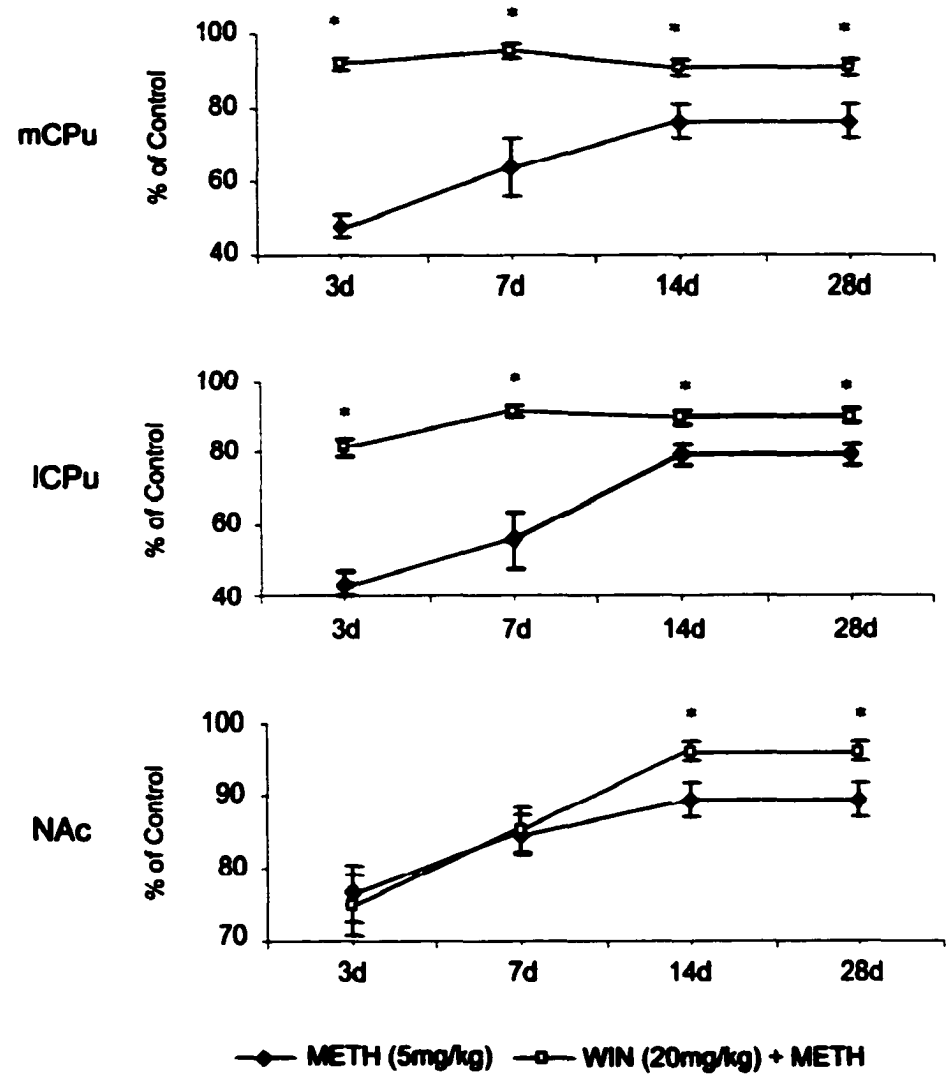

Figure 4-3. Time course of WIN-51,708 effect on METH-induced loss of DAT binding sites. METH (n = 6) exposure resulted in persistent loss of DAT [^{125}I]RTI-121 binding sites in mCPu, lCPu and NAc on day 3, 7, 14 and 28 post-treatment (n = 6). The treatment of WIN-51,708 prevented the loss in mCPu and lCPu at all time points; although it had no effect on METH-induced DAT loss in NAc on day 3 and 7 post-treatment, it prevented the loss in NAc on day 14 and 28 after the treatment. The results are expressed as mean ± S.E.M. percentage of the control (vehicle + saline) group (n = 6). *p < 0.05 compared to METH-treated animals (vehicle + METH).

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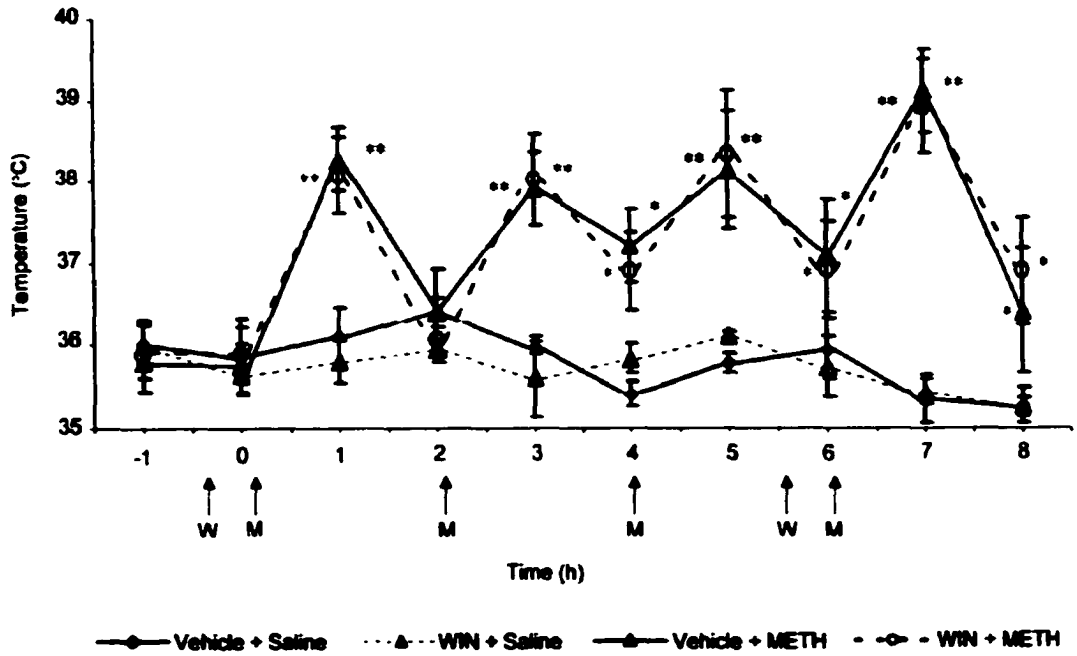

Figure 4-4. WIN-51,708 had no effect on METH-induced hyperthermia. Rectal temperature was recorded every 1 h, starting from 1 h before the 1st injection of METH to 2 h after the 4th injection of METH (n = 7). There was no statistic difference in METH-induced elevation of body temperature between animals treated with METH alone and concurrently treated with WIN-51, 708. The ambient temperature was 20 – 22.2 °C. The results are expressed as mean ± S.E.M. of seven animals. $^{*}p < 0.01$, $^{**}p < 0.005$ as compared to vehicle + saline control.

4.5 Discussion

The presented results demonstrate that the pharmacological blockade of NK-1 receptors is protective against METH-induced DA terminal deficit in the striatum, as evaluated by the characteristic marker, DAT autoradiographic binding sites. The

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consistent protection provided by two structurally and chemically distinctive NK-1 receptor antagonists confirmed the specific effects through NK-1 receptors.

The data also reveal the temporal and spatial patterns of the protective effects provided by NK-1 receptor antagonists. WIN-51,708 persistently protected against the loss of DAT binding sites at all time points, confirming that treatment with the NK-1 receptor antagonist prevented rather than delayed the neurotoxic effects of METH. Surprisingly, the NK-1 receptor blockade did not rescue NAc from METH-induced loss of DAT sites on the 3rd or 7th day post treatment, but provided protection on the 14th and 28th day, suggesting the presence of both immediate and delayed neurotoxic reactions that contribute to long-term toxic effects induced by METH exposure. It is well known that NAc is more resistant to METH toxicity (Eisch et al., 1992; Kokoshka et al., 1998), but the mechanism underlying the regional heterogeneity of METH has not yet been elucidated. DAT is believed to be an essential component of METH toxic machinery (Fumagalli et al., 1998). Supporting this notion, our autoradiographic study show that the basal level of DAT binding sites is higher in CPu than in NAc (Fig. 3-1a). Additionally, it was reported that a toxic dose of METH caused the delayed increase of Glu release only in the CPu, but not in the NAc (Abekawa et al., 1994). Up to date, we are not aware of any literature documenting the difference in the distribution of NK-1 receptor between CPu and NAc. However, a single injection of amphetamine or METH increased mRNA levels of neuropeptides SP, along with enkephalin and dynorphin, in the neostriatum with far greater elevation in CPu than in NAc (Hurd and Herkenham, 1992; Wang and McGinty, 1996), suggesting the differential actions of peptide systems

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to METH insult in these two areas. Interestingly, the opioid receptor antagonist naltrexone also failed to reverse the protective effects of enkephalin analog DADLE on METH toxicity in NAc, implicating a non-opioid receptor mechanism in this region (Tsao et al., 1998). Observed regional differences between the CPu and the NAc with respect to stabilization of DAT sites by NK-1 receptor antagonist treatment may reflect the existence of different mechanisms of METH-induced toxicity operating in these brain regions.

Hyperthermia has been widely considered as a critical component of METH-induced neurotoxicity (LaVoie and Hastings, 1999; Xie et al., 2000). Since SP, acting through NK-1 receptor, has been shown to enhance the secretion of cytokines (Martin et al., 1992), it is reasonable to propose that the protective effects of NK-1 receptor antagonists originate from attenuation of the hyperthermic reaction. However, our results demonstrate that METH-induced elevation of core body temperature is not influenced by co-administration of NK-1 receptor antagonist WIN-51,708. Therefore, the protection afforded by NK-1 receptor antagonists for neurochemical markers observed in the present study is independent of METH-induced hyperthermia. Together with studies on the protective agent DADLE (Tsao et al., 1998) and nNOS inhibitors (Itzhak et al., 2000), with knockout mice protected against METH-induced toxicity (Ladenheim et al., 2000), and with models wherein METH toxicity was exacerbated without temperature effects (Fumagalli et al., 1998; Deng et al., 1999), our observations reinforce the view that hyperthermia alone is not sufficient to produce the neurotoxic damages induced by METH. Elevated body temperature may, however, be critical to

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initiate and maintain some key steps in the toxic pathways, such as the function of DAT (Xie et al., 2000), the temperature sensitive enzymatic oxidation of DA (LaVoie and Hastings, 1999), or increased metabolic demand (Burrows et al., 2000).

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Chapter 5

Magnitude of The Action

The evidence collected thus far indicates that NK-1 receptor is indeed involved in METH-induced deleterious effects on presynaptic DA terminals. Then the next question is, to what extent does the blockade of NK-1 receptor protect against METH-induced neurotoxicity? In order to evaluate the magnitude of protection, we examine the effects of NK-1 antagonist on METH-induced pre- and post- synaptic neurodegeneration using neurochemical as well as histological assessment.

5.1 Neurochemical Assessment

5.1.1 DAT Protein Levels

Binding of [^{125}I]RTI-121 to DAT can be affected by a variety of environmental factors as well as functional or conformational changes of the transporter molecule. Therefore, the DAT autoradiographic binding sites may not necessarily reflect the actual DAT protein level or the integrity of the terminal membrane. We then measured DAT protein levels by Western blotting (Fig. 5-1). Administration of METH produced a ~44% decrease in DAT protein level, and the concurrent treatment of WIN-51,708 almost completely prevented the decrease (to ~90% of control). WIN-51,708 itself did

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not affect DAT protein levels in the striatum. Thus, the reduction of DAT sites is due to the depressed levels of transporter protein in the terminals. It is unlikely that the protection resulted from the up-regulation of DAT protein synthesis since the half-life for the DAT protein is about 7 days (Kokoshka et al., 1998). Taken together, the DAT autoradiography and immunoblotting data demonstrate that antagonism of the NK-1 receptor spares this important marker of the presynaptic dopaminergic terminals from the ravaging neurotoxic reactions unleashed by METH.

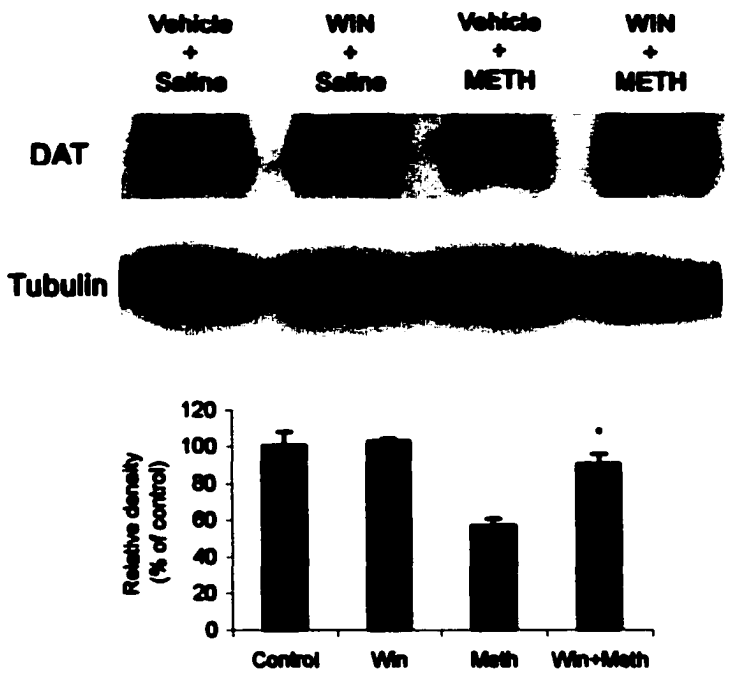

Figure 5-1. Western blots of DAT proteins. Administration of METH (n = 5) caused 43.4 ± 3.99% decrease in DAT protein expression as compared to saline treated control (n = 5). The concurrent treatment of NK-1 receptor antagonist WIN-51,708 (n = 5) protected the protein level to 89.9 ± 5.49% of control (* $p < 0.01$) as compared to animals treated with vehicle + METH). WIN-51,708 alone (n = 3) had no effect on DAT protein levels. The relative density of each band obtained for DAT was normalized against that of α-tubulin. The results are expressed as mean ± S.E.M. percentage of the control group.

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5.1.2 Tissue DA and Its Metabolites Content

Exposure to METH results in long-lasting reductions in tissue DA content in the striatum (Stephans and Yamamoto, 1994; Tsao et al., 1998). However, the study of enkephalin analog DADLE indicated that protection against DAT loss did not always result in protection against depletion of DA content and that the depletion of DA was not due to the breakdown or leakage of the terminal membrane (Tsao et al., 1998). Tissue DA content may be more related with the functional status of DAT or TH proteins (Tsao et al., 2000). In order to study the involvement of NK-1 receptor on METH-induced deficits of neostriatal DA, we used high-performance liquid chromatography with electrochemical detection (HPLC-EC) to quantify tissue DA content in groups of mice treated with METH alone or METH and the NK-1 receptor antagonist WIN-51,708. Exposure to METH reduced total tissue DA, DOPAC and HVA levels to ~50, ~62, & ~78 % of control in mCPu, respectively; ~33, ~24, & ~58 % relative to control in lCPu, respectively; and ~49, ~69, & ~81 % of control in NAc, respectively. Pre-treatment with WIN-51,708 afforded protection of DA, DOPAC and HVA levels to ~83, ~88, & ~93 % of control in mCPu, respectively; and ~70, ~71, & ~82 % of control in lCPu, respectively, without demonstrable protection in the NAc (Fig. 5-2).

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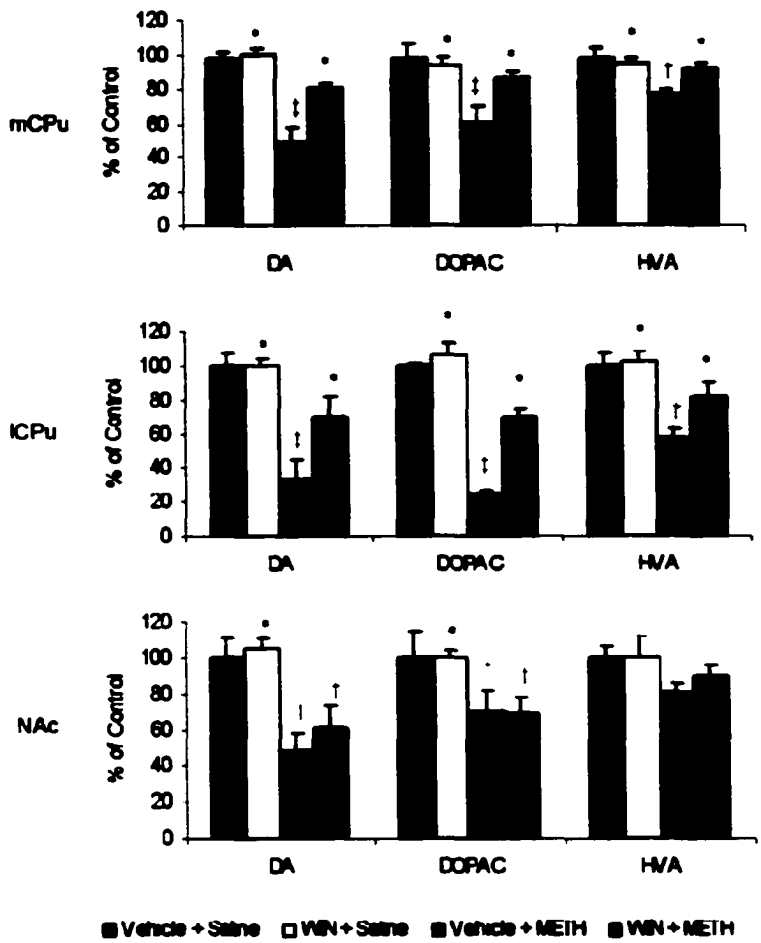

Figure 5-2. WIN-51,708 prevented the loss of striatal DA content induced by METH. Multiple METH injections (n = 10) caused significant depletion of tissue DA, DOPAC and HVA levels in the three brain regions examined on day 3 post treatment, except that HVA had no significant change in NAc at this dose range. The injections of WIN-51,708 (n = 11) partially blocked the depletion in mCPu and lCPu, but had no protective effect in NAc. WIN-51,708 given alone (n = 5) did not affect the tissue levels of DA and its metabolites. The results are expressed as mean ± S.E.M. percentage of the control (vehicle + saline) group (n = 7) values (ng/mg protein): mCPu: 100 ± 4.25 (DA), 100 ± 9.67 (DOPAC), 100 ± 6.72 (HVA); lCPu: 100 ± 6.73 (DA), 100 ± 1.24 (DOPAC), 100 ± 7.54 (HVA); NAc: 100 ± 10,833 (DA), 100 ± 14.58 (DOPAC), 100 ± 5.91 (HVA). † $p < 0.05$ or ‡ $p < 0.01$ compared to control animals (vehicle + saline), *$p < 0.05$ compared to METH-treated animals (vehicle + METH).

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5.1.3 TH Protein Levels

Tyrosine hydroxylase activity is rate-limiting for catecholamine biosynthesis and is present in the striatum in the presynaptic DA terminals. Previous studies have established this enzyme as a pivotal marker of neurotoxicity in the striatum. The protective effects of NK-1 receptor antagonists extend to this enzyme as measured by TH protein levels by Western analysis (Fig. 5-3a). Exposure to METH reduced TH protein levels to ~62% of saline-injected control values, whereas the administration of WIN-51,708 significantly blocked METH-evoked decrements of TH protein levels in the striatum (~81% of control). Administration of WIN-51,708 alone did not affect the basal levels of TH protein.

5.1.4 GFAP Protein Levels

We have demonstrated that three select neurochemical markers of the presynaptic terminal are spared from METH-induced damage as a result of blocking NK-1 signaling. We next decided to assess a marker of neurodegeneration extrinsic to the DA terminals in the neostriatum. GFAP is commonly considered as a marker for reactive astrogliosis activated by various neuronal insults including METH exposure (Pu and Vorhees, 1995; Deng et al., 1999; Ladenheim et al., 2000). Using Western blotting for GFAP protein, we found that there was no change of GFAP level in animals concurrently treated with WIN-51,708 and METH, as compared to vehicle-treated control, whereas the METH-treated animals showed a 150% increase in striatal GFAP

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protein level. WIN-51,708 was without effect on GFAP protein levels when administered alone (Fig. 5-3b).

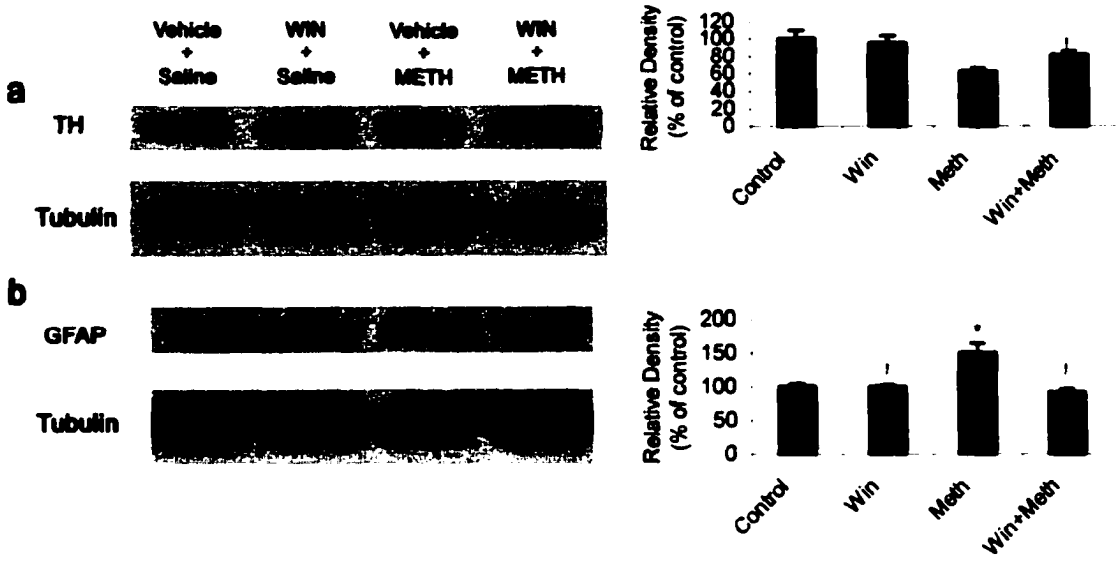

Figure 5-3. Protection TH and abrogation of GFAP induction by WIN-51,708 in the striatum of METH-treated mice. **(a)** Administration of METH (n = 7) produced a 38.1 ± 3.76% decrease in TH protein expression as compared to saline treated control (n = 7). The treatment of WIN-51,708 (n = 7) protected the protein level to 81.1 ± 4.88% of control. **(b)** METH exposure induced a 150.1 ± 14.67% increase of GFAP protein level (n = 6), whereas no change (91.5 ± 5.19% of control) was observed in animals with concurrent treatment of WIN-51, 708 (n = 6). WIN-51,708 alone (n = 3) had no effect on either TH or GFAP protein levels. The relative density of each band obtained for TH or GFAP was normalized against that of α-tubulin. The results are expressed as mean ± S.E.M. percentage of the control group. * $p < 0.01$ compared to control, ! $p < 0.01$ compared to METH treated group.

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5.2 Histological Assessment

It should be pointed out that, as shown in the time-course study, the loss of DAT binding sites induced by multiple 5 mg/kg METH paradigm has partially reversed in all three areas examined in the neostriatum. It may suggest that the compensatory changes have occurred in the surviving DA terminals. However, it is also possible that the neurochemical deficits induced by METH at this dose are due to the down-regulation of nigrostriatal DA system rather than DA terminal degeneration. At any rate, histological studies are warranted to assess the nature and magnitude of protection afforded by the blockade of NK-1 receptor. In order to maximize METH-induced damage, we employed 10 mg/kg METH in the histological studies, a dose that has been widely demonstrated to cause irreversible pre-synaptic terminal degeneration and has also elicited post-synaptic neuronal degeneration as shown on the Fluoro-Jade B staining (Fig. 3-2). In order to compare the pattern of presynaptic terminal loss with postsynaptic neuronal degeneration, the alternate sections from each animal were used for either TH immunohistochemistry or double labeling of Fluoro-Jade B and GFAP.

5.2.1 TH Immunohistochemistry

To confirm that NK-1 receptor antagonist could prevent METH-induced DA axonal degeneration rather than facilitate the compensatory expression of phenotypic markers by surviving cells, TH immunohistochemistry was used to evaluate the architecture of monoaminergic axons. TH immunohistochemistry has been shown to reliably represent

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the loss of DA terminals and well match with other methods, including silver staining, to demonstrate terminal degeneration (Rabinovic et al., 2000). The vehicle-saline treated control animals showed dense TH staining throughout striatum. The animals treated with WIN-51,708 alone demonstrated the patterns of TH staining indistinguishable from those in the control group. Intraperitoneal administration of 10 mg/kg METH four times at 2-h intervals for one day (n = 7) caused marked reduction in the intensity of TH immunoreactivity (TH-IR) 3 days after drug treatment (Fig. 5-4). Among them, five animals demonstrated extensive reduction of TH-IR over the entire striatum, while one showed relatively milder depletion mostly affecting the ventral lateral region of striatum, and the other one had only minor reduction in TH-IR. In comparison with the animals treated with METH alone, pretreatment of 10 mg/kg WIN-51,708 (n = 6) significantly prevented the decrease of TH-IR in four out of six treated animals ($p < 0.01$); whereas the other two showed substantial reduction of TH-IR, between them, one with the whole striatum affected and the other with the greatest decrease in the ventral lateral striatum. The average optical density of TH positive fibers was reduced to ~73% of control in METH-treated animals, but retained to ~85% of control in animals pretreated with WIN-51,708 ($p < 0.01$; data not shown).

5.2.2 Double Labeling of Fluoro-Jade B and GFAP Immunofluorescence

To assess whether the blockade of NK-1 receptor is also able to protect against METH-induced postsynaptic neuronal degeneration, Fluoro-Jade B staining was performed in slide-mounted frozen coronal sections in METH-treated animals with

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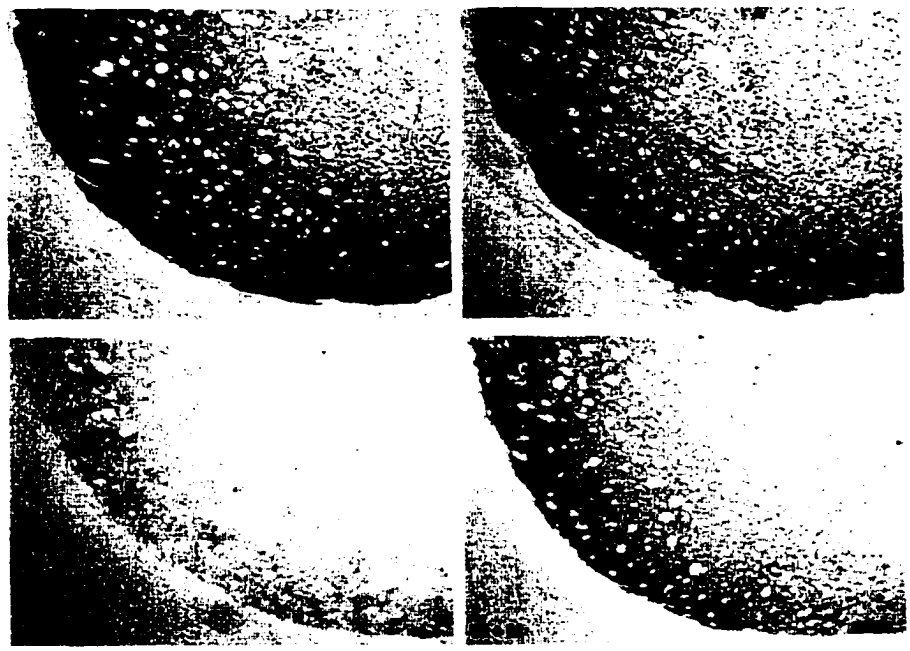

Figure 5-4. Effects on TH immunoreactivity. Administration of 10 mg/kg METH resulted in apparent decrease in the intensity of TH immunoreactivity in 5 out of 7 animals three days after treatment. Pretreatment of 10 mg/kg WIN-51,708 partially prevented the depletion in 4 out 6 animals. *V+S*, vehicle + saline (n = 5); *W+S*, WIN-51, 708 + saline (n = 5); *V+M*, vehicle + METH (n = 7); *W+M*, WIN-51,708 + METH (n = 6). Scale bar, 100 μm.

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or without pretreatment of WIN-51,708. These sections are the alternate sections from each animal with the ones used in TH immunohistochemical assessment. Reactive astrocytosis is widely accepted as a general marker responding to different kinds of neural insults. In the neurochemical study, we have demonstrated that pretreatment of WIN-51,708 prevented the increase of GFAP protein level induced by 5 mg/kg METH administration (a dose that did not cause postsynaptic neuronal degeneration). Therefore, reactive astrocytosis, assessed by immunofluorescence of GFAP double-labeled with Fluoro-Jade B, was taken as the main index of METH-induced neural damage (Fig. 5-5).

There was no Fluoro-Jade B positive neuron in either control or WIN-51,708 treated alone animals. METH administration resulted in positive Fluoro-Jade B staining in five out of seven treated animals. Among them, Fluoro-Jade B positive cells were seen throughout the striatum of two animals, relatively concentrated at the ventral lateral striatum of the other two animals, and at the lateral striatum in one animal. In the one animal with relatively moderate reduction of TH-IR in ventral lateral striatum, no Fluoro-Jade B positive labeling was apparent. In contrast, there was no Fluoro-Jade B positive neuron seen in four out of six animals pre-treated with WIN-51,708; while the other two showed Fluoro-Jade B staining either in the ventral lateral region or throughout the entire striatum, correlated with the pattern of reduction of TH-IR in the same animal. The reactive astrocytes are present in proximity to the degenerating neurons as well as in regions surrounding the degenerating area. No doubly labeled cells

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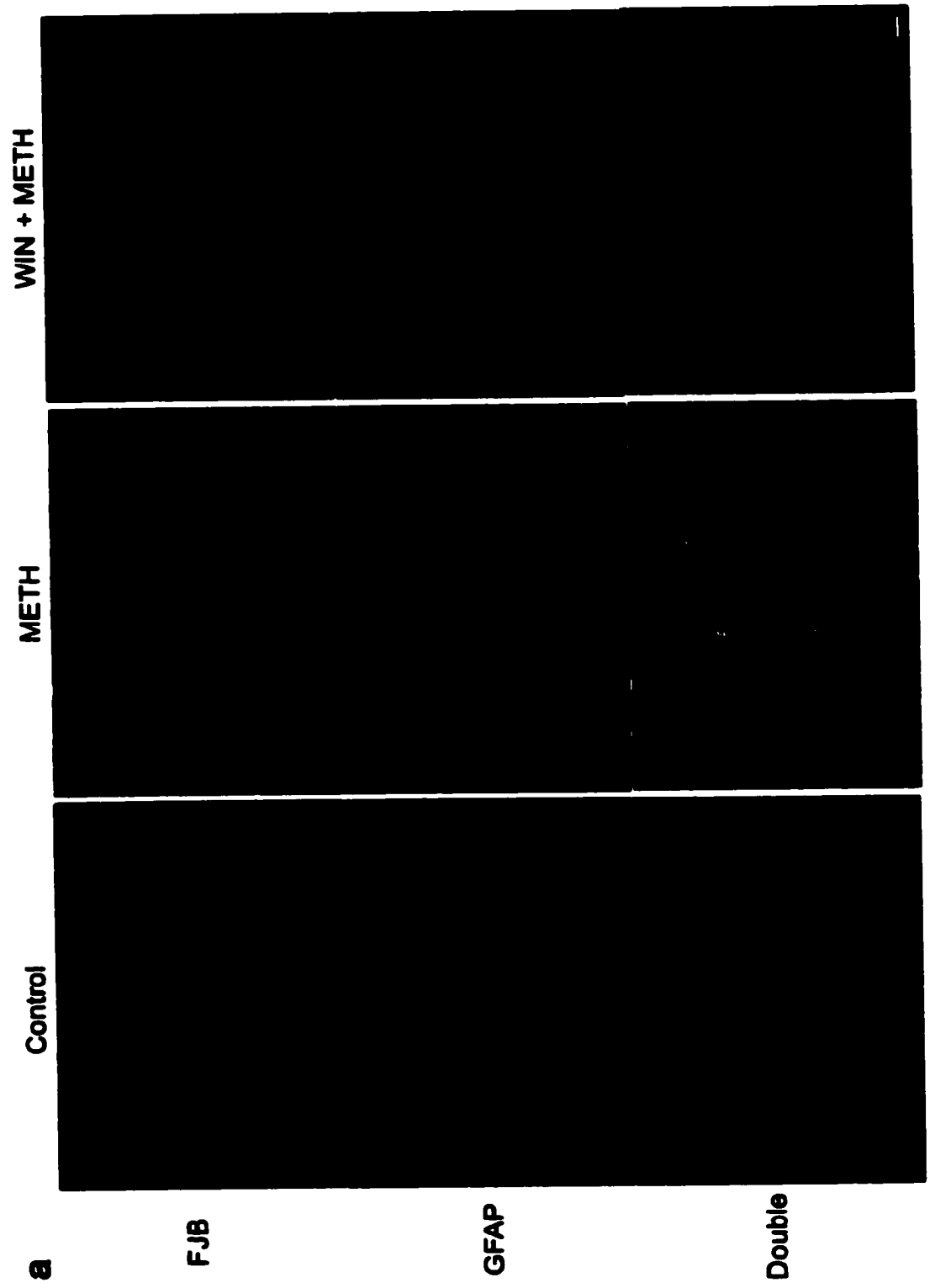

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b

Treatment Group	Number of Animals in Rank Score of FJB-positive cells				
	–	+	++	+++	++++
Control (n = 5)	5	0	0	0	0
WIN (n = 5)	5	0	0	0	0
METH (n = 7)	2	0	1	0	4
WIN+METH (n = 6)	4	1	0	1	0

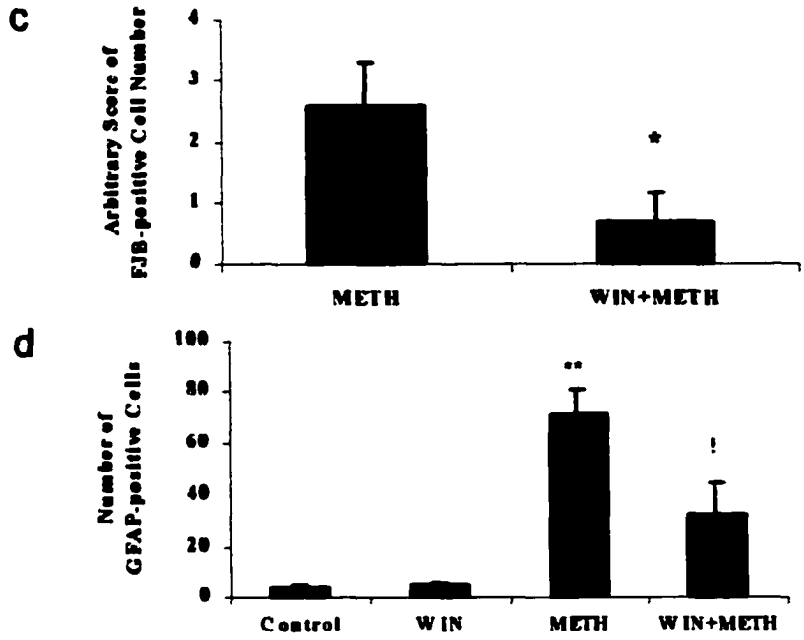

Figure 5-5. Effects on neuronal degeneration and astrocytosis. (a) Multiple exposure to METH (10 mg/kg) led to the staining of degenerating Fluoro-Jade B (FJB) positive neurons (*green*) in the striatum, and the reactive astrocytes (*red*; hypertrophic cell body and increased number) in adjacent to FJB positive neurons; pretreatment of WIN-51,708 (10 mg/kg) afforded protection, as suggested by (b) Number of animals showing FJB positive cells in each arbitrary rank score (0 [–], < 1 cell / section; 1 [+]: 1 - 50 cells / section; 2 [++]: 51 - 100 cells / section; 3 [+++]: 101 - 150 cells / section; 4 [++++]: > 150 cells / section) and (c) Average rank score in METH treated mice with or without WIN-51,708 pretreatment. (d) WIN-51,708 also caused quantitative decrease in the number of astrocytes. * $p < 0.05$ and ! $p < 0.01$ as compared to METH treated animals, ** $p < 0.001$ compared to control. Scale bar in (a), 20 μm.

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were observed, suggesting that the Fluoro-Jade B positive cells are mostly neuronal origin. METH exposure caused substantial increase in the number of astrocytes with larger cell bodies and longer processes. WIN-51,708 also prevented reactive astrocytosis in those pretreated animals with no significant TH-IR reduction or Fluoro-Jade B staining. As demonstrated in the METH-treated group, acute treatment of multiple high doses METH has greater impact on the presynaptic terminals than it does to the postsynaptic neurons, implying that presynaptic terminals are subjected to more damaging influences or they themselves are more vulnerable to toxic injury. On the other hand, the relative magnitude of loss of TH immunoreactivity correlated with the intensity of Fluoro-Jade B staining among the animals, suggesting that presynaptic terminals and postsynaptic neurons may be affected, at least in part, by some common mechanisms.

5.3 Discussion

In summary, the present study reveals that the pharmacological blockade of NK-1 receptors is protective against METH-induced presynaptic terminal and postsynaptic neuronal degeneration in the striatum, as assessed by several characteristic neurochemical markers including DAT autoradiographic binding sites and protein levels, DA and its metabolites contents, TH and GFAP protein levels, as well as by the histological evaluations including TH immunohistochemistry, Fluoro-Jade B staining and GFAP immunofluorescence.

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Administration of WIN-51,708 alone does not affect the levels of any neurochemical parameters assessed, it is therefore unlikely that the protection of either TH protein levels or tissue DA content occurs from the up-regulation of the nigrostriatal system to offset the neurotoxic effects produced by METH. In agreement with this view, the protective effects on DA as well as its metabolites DOPAC and HVA suggest that the rescue afforded by WIN-51,708 on METH-induced depletion of DA was likely due to the prevention of terminal damage rather than the compensatory increase of synthesis or inhibition of clearance. However, it is worth noting that although WIN-51,708 almost completely protected the loss of DAT induced by multiple 5 mg/kg METH administration, it blocked the reduction of either DA content or TH protein to a less extent. It appears that the neurochemical system might not be fully rescued even though the terminal membrane was preserved, implying that the destruction of terminal membrane might not be solely responsible for the depletion of DA content. A previous report demonstrated even more significant dissociation of the two closely related indices of METH toxicity by showing that the enkephalin analog DADLE completely blocked METH-induced loss of DAT without effects on the depletion of DA content (Tsao, 1998).

In line with the neurochemical data, TH immunohistochemistry and Fluoro-Jade B staining double-labeled with GFAP immunofluorescence demonstrated the protective effects of NK-1 antagonist on METH-induced neurodegeneration. This project has focused mainly on the well-established presynaptic DA terminal injuries, and also demonstrated that METH can cause toxic effects to the postsynaptic striatal neurons.

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The protective effects from NK-1 receptor antagonist on both presynaptic DA terminal destruction and postsynaptic neuronal degeneration suggest that NK-1 receptor may be involved in initiating the coordinated deleterious machinery leading to the long-term degeneration. These results also highlight the possibility that there are links between the mechanisms causing the DA terminal degeneration and giving rise to the intrinsic neuronal damage in the striatum. Both presynaptic and postsynaptic components are important to the neurotoxicity induced by METH. Supporting this suggestion, it has been shown that excitotoxic destruction of postsynaptic striatal neurons protect against METH-induced DA depletion (O'Dell et al., 1994), and the administration of toxic doses of METH is associated with increased p53-like immunoreactivity (Hirata and Cadet, 1997) as well as prolonged activation of AP-1 in striatal cell bodies (Sheng et al., 1996).

The results presented here strongly suggest an important role for NK-1 receptor in the manifestation of METH-induced neurotoxicity, pointing to a potentially new direction for elucidating the mechanisms of METH-induced neurotoxicity.

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Chapter 6

Implications and Limitations

The exact mechanism(s) by which NK-1 receptor mediates METH neurotoxicity is unclear. However, some implications can be gleaned based on the converging evidence from the anatomical, functional and molecular studies on tachykinin system and METH toxicity.

6.1 Regulations at Circuitry Level

In the layout of major circuits linked by the SP/NKA containing neurons, it appears that SP holds a strategic position in these circuits where it can modulate striatal function either directly or indirectly (Fig. 1-2). Therefore, NK-1 receptor may be involved, at the circuitry level, in METH-induced neurotoxicity. Intranigral injection of SP has been shown to increase striatal DA release that can be blocked by NK-1 receptor antagonists (Reid et al., 1990b; Reid et al., 1990a), and a parallel study in our lab showed that NK-1 receptor antagonist significantly reduced cocaine-evoked release of DA as well as locomotion (Kraft et al., 2001; Noailles and Angulo, 2002). Upon the massive DA overflow induced by METH, an amplifying feed-forward loop for DA release may be established, presumably in the striatonigral-nigrostriatal reciprocal connection. Although

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this speculation is against a conventional observation that a mismatch has been observed between the distribution of SP and NK-1 receptors in substantia nigra, where there is immunoreactivity of SP but not NK-1 receptor (Liu et al., 1994; Quartara and Maggi, 1998), a recent report using more refined reverse transcription-polymerase chain reaction (RT-PCR) technique has demonstrated the presence of NK-1 receptors in DA neurons of substantia nigra (Futami et al., 1998).

Nevertheless, SP may also regulate DA release indirectly via local circuits. It has been shown that SP locally stimulated DA release in the striatum via the SP receptors that are not located on DA nerve terminals (Petit and Glowinski, 1986). In the neostriatum, NK-1 receptors are mainly located on cholinergic neurons and somatostatin/NOS neurons (Kaneko et al., 1993; Chen et al., 2001). METH has been shown to increase Ach release in the striatum, possibly via a D1 and/or NMDA receptor mechanism (Taguchi et al., 1998). In neostriatum, D1 receptor has been found predominantly expressed by PPT mRNA-expressing medium spiny neurons (Gerfen, 1992). The D1 receptor-stimulated Ach release in striatum is mediated indirectly through local release of SP acting at NK1 receptors on striatal cholinergic interneurons (Anderson et al., 1994). Although Ach has dual "inhibitory" and "excitatory" actions, the endogenous Ach has been shown to stimulate striatal DA release (Petit and Glowinski, 1986; Dajas-Bailador et al., 1996) and potentiate NMDA response in striatal projection neurons (Calabresi et al., 1998). Therefore, it is reasonable to speculate that METH-induced DA release, indirectly via the action of SP and NK-1 receptor, lead to the stimulation of Ach release which may, in turn, further augment deleterious effects of

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METH (Fig. 1-2). Nevertheless, it is not possible at present stage to define the contribution of NK-1 receptor mediated increased release of Ach in METH toxicity.

Meanwhile, direct application of SP has also been shown to cause striatal Glu release (Reid et al., 1990a). Several studies utilizing *in vivo* microdialysis in rats demonstrated the immediate release of DA in response to the multiple exposures of METH and a delayed increase in Glu release in the striatum after the third injection of METH (Abekawa et al., 1994; Stephans and Yamamoto, 1994). Administration of NMDA receptor antagonist MK-801 prior to the last two injections of METH during a repeated four-injection paradigm afforded full protection against striatal DA terminal injury (Weihmuller et al., 1992). Although the mechanisms underlying the delayed increase in Glu release remain unclear, it is tempting to postulate that, beginning with the release of SP resulting from METH-induced DA overflow, signaling through NK-1 receptor might then, at the circuitry level, hypothetically lead to a neurotransmitter imbalance that ultimately triggers the deleterious overflow of excitatory amino acids in the striatum. Supporting this suggestion, the gradual increase of Glu release was only found in the striatum, but not in NAc, an area that is more resistant to METH insult (Abekawa et al., 1994), and our results indicated that NK-1 receptor antagonists failed to protect NAc from METH-induced damage in the early stage.

6.2 Postulated Model of Cellular Mechanisms

The contribution of tachykinins in excitotoxic neurotransmission has been proposed for various pathological conditions in the nervous system. In spinal cord, SP increases

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intracellular Ca^{2+} concentrations (Rusin et al., 1993), enhances responses to excitatory amino acids (Cumberbatch et al., 1995) and thus causes chronic sustained pain (Woolf and Doubell, 1994). In hippocampus, SP contributes to status epilepticus (Liu et al., 1999a), while NK-1 receptor antagonist reduces kainic acid-induced seizure (Zachrisson et al., 1998) and PPT-A gene knockout mice are resistant to excitotoxin-induced seizure and neuronal death (Liu et al., 1999b). In cortex, focal cerebral ischemia induces marked expression of PPT-A mRNA and NK-1 receptor on glutamatergic cells (Stumm et al., 2001) and NK-1 receptor antagonist reduces the infarct size after focal cerebral ischemia (Yu et al., 1997).

In view of the protective effects of NK-1 receptor antagonist in the present study, we postulate that similar cellular mechanisms are at work in METH-induced DA terminal degeneration in the neostriatum (Fig. 6). Electron microscopic study demonstrated that SP receptor immunoreactivity was localized on both presynaptic axon terminals and postsynaptic neurons in the neostriatum (Jakab and Goldman-Rakic, 1996). Under the supra-physiological condition induced by METH, the pronounced SP release resulting from massive DA overflow may lead to transient induction or upregulation of NK-1 receptor expression on glutamatergic terminals or NOS containing interneurons. The stimulation of NK-1 receptor, a G-protein coupled receptor, may enhance Glu and NO release as well as NMDA hypersensitivity via a Ca^{2+}-dependent mechanism. The stimulation of NK-1 receptor depolarizes the cell and increases intracellular Ca^{2+} concentration by the activation of a second messenger system. Consequently, the increased intracellular Ca^{2+} concentration may activate protein kinase

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C (PKC), leading to the potentiation of NMDA receptor response by reducing the Mg^{2+} blockade of ion channels (Chen and Huang, 1992). With more channels opened, more Ca^{2+} ions enter the cell and further increase PKC activity or Ca^{2+}-dependent vesicular Glu release. Thus, this positive feedback loop causes gradually enhanced and sustained NMDA receptor response and Glu sensitivity, as well as the phosphorylation of target proteins by PKC. The increased Glu flow may enhance DA release through NMDA receptors (Chowdhury and Fillenz, 1991; Krebs et al., 1991; Wang, 1991; Weihmuller et al., 1992; Gracy et al., 1999) or start another self-sustaining cycle via the interneurons. On the other hand, the elevated Ca^{2+} level following potentiation of NMDA receptors may activate NOS, a Ca^{2+}/calmodulin-dependent enzyme, increasing the synthesis of NO. Subsequently, the diffusible gas NO may interact with oxidizing DA metabolites to aggravate the oxidative stress.

Supporting this speculation, most recent immunohistochemical studies have demonstrated the colocalization of NOS and NK-1 receptor on striatal interneurons (Li et al., 2001) as well as the direct synaptic contacts between SP-containing axon terminals and NOS-containing interneurons in striatum (Li et al., 2002). The vast majority of NOS-positive neurons showed NK-1 receptor immunoreactivity, indicating that the NOS-containing interneurons were under direct control of SP-containing projection neurons in the striatum (Li et al., 2001).

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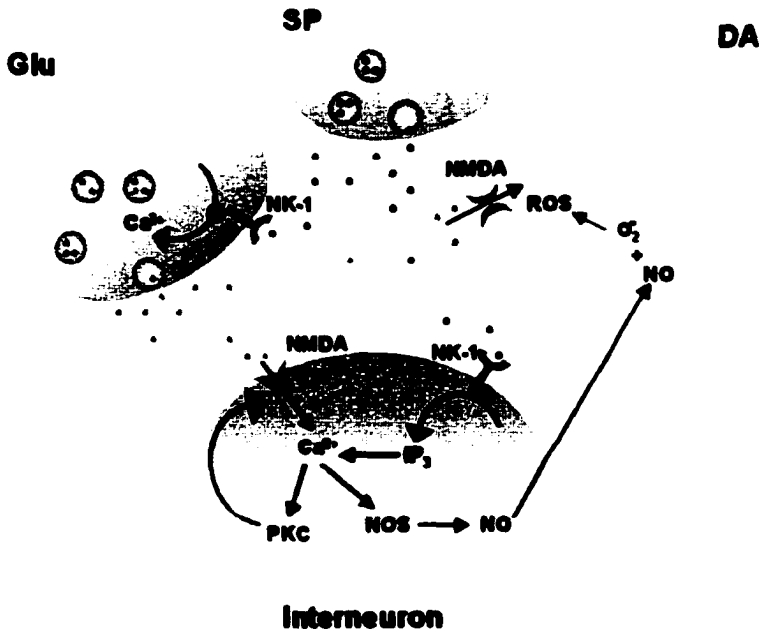

Figure 6. Schematic illustration of possible interactions between Glu and tachykinins in METH-induced alterations of neurotransmission in striatum. The pronounced SP release resulting from massive DA overflow may lead to transient induction or upregulation of NK-1 receptor expression on Glu terminals or NOS containing interneurons. The stimulation of NK-1 receptor, a G-protein coupled receptor, increases intracellular Ca^{2+} concentration by the activation of the second messenger systems, which may augment Glu release and NMDA receptor responses, as well as facilitate the formation of NO. Increased Glu flow may enhance DA release through the NMDA receptor or start another self-sustaining cycle via the local interneuron. NO may interact with oxidizing DA metabolites to aggravate the oxidative stress.

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6.3 Involvement of Molecular Events

Several lines of evidence have suggested that METH might cause acute and chronic effects through different mechanisms. For instance, the acute reduction of [^{3}H]dopamine uptake induced by multiple METH administration was partially recovered by 24 h after the fourth injection and again decreased six days later, suggesting a rapid, reversible phase of functional change and a latter, persistent phase of degeneration in multiple exposure of METH (Kokoshka et al., 1998). Moreover, a reversal of DAT levels occurred in p53 knockout mice on the 14th day after METH treatment, indicating that the secondary change has taken place upon METH exposure and it affects the molecular pathways that involve the accumulation of p53 protein in intrinsic striatal neurons (Hirata and Cadet, 1997). Our results also revealed that NK-1 receptor antagonist only afforded protection in NAc on day 14 and 28 post treatment, implicating that signaling through NK-1 receptor may play a role in the long-term damage, or in the prevention of recovery, of METH-induced insults. Because several immediate early genes (IEGs) induced by METH encode nuclear proteins that can regulate the expression of other genes, the induction of IEGs might be involved in the long-term alterations resulting from METH treatment. It has been demonstrated that DA/Glu interactions occur not only at the circuit level but also at the level of intracellular signaling within individual striatal neurons, which requires functional NMDA receptor and Ca^{2+}, to regulate the expression of IEGs (Konradi et al., 1996). Therefore, in addition to the synergistic actions of DA and Glu on the formation of reactive oxygen and nitrogen species causing

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acute damage, the molecular events involving the activation or inhibition of cell death-related genes may also be responsible for METH-induced long-term degeneration.

As the trigger of a signaling pathway involving G-protein and second messenger system, the binding of SP to NK-1 receptor may stimulate diverse down-stream effects. In vitro study has shown that SP stimulates target cells through the activation of activator protein 1 (AP-1) and cAMP-responsive binding (CREB) sites (Christian et al., 1994). In vivo studies also demonstrated the colocalization of SP and Fos-related antigen in striatal neurons (Zhang et al., 1992), as well as the induction of c-fos in the brain in response to SP (Yip and Chahl, 2000; Spitznagel et al., 2001) that could be inhibited by NK-1 receptor antagonist (Yip and Chahl, 2000). Because METH has been shown to activate the genes related to cell death (Cadet et al., 1997; Hirata and Cadet, 1997) and cause apoptosis in various brain regions (Deng et al., 1999), further work is warranted to investigate the involvement of NK-1 receptor in METH-induced molecular evens by using similar pharmacological manipulations.

6.4 The Possible Role of Astrocytes

Ladenheim et al. have recently demonstrated that METH-induced terminal degeneration and apoptotic cell death were attenuated in interlukin-6 knockout mice (Ladenheim et al., 2000), suggesting the involvement of cytokines in the pathogenic process of METH neurotoxicity. Converging evidence reveals that neuroglial cells, including astrocytes and microglia, contain NOS; and that cytokines can mediate NO production as well as activation of apoptotic pathways in various neurodegenerative

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diseases including Parkinson's disease (Hirsch et al., 1998; Xiao and Link, 1998; Choi et al., 1999). One possibility is that the reactive gliosis, as a phenomenon secondary to the primary insults, may cause necrosis or apoptosis to the neighboring areas via the increased production of NO or cytokines (Eliasson et al., 1999; Le et al., 2001; Wu et al., 2002).

It is worth noting that reactive astrocytes and microglia express SP receptors (Torrens et al., 1986; Mantyh et al., 1989; Luber-Narod et al., 1994; Lin, 1995) and SP has been shown to stimulate the secretion of various cytokines, such as interlukin-1, interlukin-6, tumor necrosis factor-α, from neuroglial cells in a number of CNS disorders (Martin et al., 1992; Gitter et al., 1994; Luber-Narod et al., 1994; Palma and Manzini, 1998). Furthermore, non-peptide NK-1 receptor antagonist selectively blocked SP-induced production of interlukin-6 in astrocytoma cell lines (Derocq et al., 1996). However, the potential of neuroglia to express NK-1 receptor may depend on the type of lesion (Stumm et al., 2001). What types of cytokines are secreted, if any, by what subtype of reactive neuroglia, and whether NK-1 receptor is involved in the production of specific cytokines after METH treatment remain to be open questions.

On the other hand, it is evident that neuroglial cells and cytokines have complex multi-functions in both pathogenic and regenerative processes (Xiao and Link, 1998). Of particular interest, the c-fos mutant mice, which are more susceptible to METH neurotoxicity, showed mush less reactive astrogliosis, implicating the involvement of c-fos expression in METH-induced astrogliosis and suggesting that astrocytes may be engaged in protective mechanism by the neurotrophic or anti-oxidative features (Deng et

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al., 1999). Hence, it cannot be certain from the present study whether the reduction of astrogliosis by NK-1 receptor blockade was directly via the interaction between astrocytes and NK-1 receptor antagonist to reduce the production of NO and cytokines, or indirectly as a consequence of the protection against METH-induced neurodegeneration through other mechanisms. It is important for future studies to examine these questions in greater detail.

6.5 Neuropeptides – The Modulators

It is known that SP and opioid peptide dynorphin (Dyn) are co-expressed in striatonigral neurons, whereas the other opioid peptide enkephalin (Enk) is primarily expressed in striatopallidal neurons (Lee et al., 1997a). Interestingly, although immunoreactivities for all these peptides are intense in the target regions of their mRNA-expressing neurons, the binding activities of all their receptors are intense mainly in the neostriatum (Mansour et al., 1988; Kaneko et al., 1993). It suggests that the tachykinin and opioid peptides produced in neostriatal projection neurons might be used primarily in the neurotransmission in neostriatum and they might interact to respond to the same stimulus. In fact, the "direct" striatonigral pathway (with high levels of SP and D1 receptor) and the "indirect" striatopallidal pathway (with high levels of Enk and D2 receptor) are synaptically linked in the neostriatum (Yung et al., 1996).

In addition to the augmented PPT-A mRNA and SP level (Sonsalla et al., 1986; Zhang et al., 1997), administration of METH also caused acute increase in preprodynorphin mRNA and Dyn peptide levels in the striatum (Hanson et al., 1988;

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Smith and McGinty, 1994). In contrast, far less increase of either preproenkephalin mRNA (Smith and McGinty, 1994; Wang and McGinty, 1996; Zhang et al., 1997) or Enk (Alburges et al., 2001) level was detected after acute METH exposure. The elevation in preproenkephalin mRNA and Enk levels was unlikely due to the increased DA overflow, because DA tonically inhibit Enk system (Angulo and McEwen, 1994). Conceivably, it might be mediated by the yet undefined intrinsic striatal mechanisms involving the interaction of this peptide with other neurotransmitters. It was reported that Enk analog DADLE decreased presynaptic DA release via opioid receptor-mediated mechanism in striatal slices (Schlosser et al., 1995). The observation that Enk analog DADLE attenuated METH-induced striatal DA deficits in an opioid receptor-dependent manner declared the modulatory role of Enk in METH neurotoxicity (Tsao et al., 1998).

The other opioid peptide Dyn, which is selective for kappa (κ)-opioid receptor, co-localizes with SP in the striatonigral pathway (Lee et al., 1997a). However, it is functionally quite different from SP. Similar to Enk, Dyn acts to negatively modulate cellular responses to dopaminergic stimulation (Spanagel et al., 1990; Steiner and Gerfen, 1993; Heidbreder and Shippenberg, 1994). Microdialysis studies have shown that the kappa (κ)-opioid receptor agonists decreased DA and Glu release in the neostriatum (Spanagel et al., 1990; Hill and Brotchie, 1995; Rawls and McGinty, 1998) as well as cocaine-evoked increase in DA overflow in NAc (Heidbreder and Shippenberg, 1994). Thus, it is not surprising that the κ-opioid receptor agonist U69593 attenuated METH-induced alterations on presynaptic dopaminergic function (El Daly et

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al., 2000). The two opioid peptides, Enk and Dyn, may represent the compensatory inhibitory responses to excessive DA neurotransmission induced by METH.

Given their dynamic modulatory effects on dopaminergic system, considering the converging evidence showing their involvement in METH neurotoxicity, the neuropeptides may not only anatomically but also functionally linked to participate in the active neural reactions induced by METH. It has become clear that the mechanisms by which METH causes neurodegeneration are far more complex than it first appears and certainly beyond the classical neurotransmitter system.

6.6 Limitations

In addition to the common drawbacks in pure pharmacological study, such as the possible pharmacology masking effects and the variations among animals, there are some limitations inherent in this study. First, the antagonists need to compete with endogenous neuropeptides whose actions could be modulatory under different circumstances such as the concentrations or subtypes of active receptors. The selective antagonists interfere with the actions of peptide on a certain receptor may activate other subtypes of receptor (Betancur et al., 1997), or unexpectedly interact with ion channel binding sites (Quartara and Maggi, 1997). Second, although WIN-51,708 has remarkably high affinity for rodent NK-1 receptor, it does not have a commercially available inactive enantiomer to control the non-specific pharmacological activity at high dose. Moreover, as a pharmacological compound, it has some toxic side effects, such as loss of muscle tone and dyspnea (Quartara and Maggi, 1997), especially when

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administered systemically at high dose. In fact, while increasing METH dose to 10 mg/kg, the mortality rate of WIN 20mg/kg group (~ 66 %) was slightly higher than that of METH treated alone (~ 50 %), so that we used 10 mg/kg dosage of WIN-51,708 in the histological study. In this regard, the gene knockout mouse will be an ideal and important genetic approach to verify the hypothesis.

However, as reviewed in Chapter 1, there is a "cross-talk" between tachykinins and their receptors. Given that neostriatum and substantia nigra are particularly enriched in both SP and NK-3 receptors (Bannon and Whitty, 1995; Whitty et al., 1995; Quartara and Maggi, 1997), the genetic ablation of NK-1 receptor may lead to compensatory alterations during development, such that SP may signal through NK-3 receptor. This is especially a concern in METH neurotoxicity since the neostriatum and substantia nigra are two major areas involved in the selective dopaminergic degeneration. Should that be the case, the employment of NK-3 receptor antagonist in NK-1 receptor knockout mice could be a desirable solution.

On the other hand, our results do not exclude the possible involvement of neurokinin A (NKA) in the active pathological process of METH toxicity. NKA and SP are synthesized from the same precursor, preprotachykinin-A (PPT-A) gene, and co-exist in the neurons of nigrostriatal dopaminergic pathway (Angulo and McEwen, 1994). Although SP is the natural ligand with highest affinity for NK-1 receptor, the accumulating evidence suggests that the affinity of NKA for NK-1 receptor may be comparable with that of SP (Hastrup and Schwartz, 1996; Sagan et al., 1996). Furthermore, SP and NKA appear to have similar effects on most aspects of NK-1

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receptor signaling in spinal cord and CHO cells (Sagan et al., 1996; Trafton et al., 2001). As a matter of fact, it was observed that multiple high doses of METH identically increased nigral and striatal concentrations of both SP and NKA (Hanson et al., 1986). Therefore, the PPT-A gene knockout mice completely lack of SP and NKA, a situation that can never be achieved with the pharmacological antagonists, will be highly valuable in assessing the functional role of tachykinins in METH neurotoxicity.

6.7 Significance of The Study

Due to the similarity of degenerative changes in the striatal dopaminergic system, and supported by the recent accumulating data showing the loss of cell bodies in substantia nigra (Sonsalla et al., 1996; Hirata and Cadet, 1997), METH-induced neurotoxicity has been suggested as one of the potential models of Parkinson's disease. Likewise, Huntington's disease is a hereditary neurodegenerative disease characterized by progressive premature cell death most predominantly affecting the striatum. It was reported that the striatal SP level was reduced in rigid Huntington's disease patients, but was slightly elevated in less advanced cases (Storey and Beal, 1993). Although the underlying mechanisms are still elusive, oxidative stress, excitotoxicity and mitochondrial dysfunction are believed to play essential roles in the neostriatal degeneration occurred in both disorders (Beal et al., 1986; Palfi et al., 1996; Przedborski and Jackson-Lewis, 1998; Alexi et al., 2000; Jakel and Maragos, 2000). Additionally, some in vitro studies have shown that levodopa, a traditional symptomatic antiparkinsonian drug that augments DA activity, induced the formation of free radicals

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(Pardo et al., 1993) and apoptosis in cultured neuronal cells (Ziv et al., 1997). It raises the concern about the potential neurotoxicity from long-term levodopa treatment in Parkinson's disease (Simuni and Stern, 1999), emphasizing the significance of exploring neuroprotective agents. Although many questions remain unanswered, the present study identified that NK-1 receptor could contribute to neostriatal vulnerability in the neurodegenerative process involving oxidative stress, excitatory neurotransmission or apoptotic cell death. This finding will not only contribute to the understanding of neuroplasticity in the neostriatal dopaminergic system, but also may lead to a new approach in the therapeutic interventions for drug addictions as well as for a number of diseases, including Parkinson's disease and Huntington's disease.

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Chapter 7

Conclusions and Future Directions

In this thesis, we have studied the role of neurokinin-1 receptor in the pathogenesis of methamphetamine-induced striatal neurotoxicity. Our hypothesis was based on the potential mechanisms underlying METH neurotoxicity and the dynamic modulatory effects of neuropeptides, especially substance P, on the basal ganglia function. The present study has made the following contributions to the research on METH-induced neurotoxicity, functional roles of NK-1 receptor and its ligand in dopaminergic system, as well as neuroplasticity in the neostriatum. We

- Standardized the toxic effects of METH-induced deficits, with spatial resolution, on several classic markers of presynaptic DA terminal including DAT sites and DA content;
- Provided the direct histological evidence of METH-induced postsynaptic neuronal degeneration in the striatum;
- Demonstrated that pharmacological blockade of NK-1 receptor afforded protection against METH-induced
    - Loss of DAT binding sites and proteins
    - Depletion of DA and its metabolites content
    - Reduction of TH protein levels and immunoreactivity

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- Elevation of GFAP protein levels and immunoreactivity
- Intrinsic neuronal degeneration in the striatum

– Verified that the protective effects were specifically mediated by the blockade of signaling through NK-1 receptor;

– Confirmed the long-lasting effect of NK-1 receptor;

– Excluded hyperthermia as a confounding factor in the interpretation of results.

Our results provide the first pharmacological evidence that tachykinins, particularly SP, work through NK-1 receptors in the pathogenesis of striatal neurodegeneration produced by multiple exposures to METH. For the future, it will be essential to characterize the underlying mechanisms by which NK-1 receptor mediates neurotoxicity to METH. As discussed in the previous chapter, several mechanistic topics merit further investigation:

– The exact locations of functional NK-1 receptor upon METH exposure;

– The involvement of NK-1 receptor in METH-induced molecular events such as activation of apoptotic pathways and induction of immediate early genes;

– The role of NK-1 receptor in METH-induced formation of reactive oxygen and nitrogen species;

– The effects of NK-1 receptor signaling on the activities of essential components in the excitotoxic cascade, such as NMDA receptor, NOS and PKC;

– The actions of NK-1 receptor in METH-induced activation of neuroglial cells, including astrocytes and microglia, and their roles in facilitating neurotoxicity.

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In addition to the pharmacological methods, genetic manipulation could be a powerful complementary approach in the functional and mechanistic study. Given the close connections between the members of tachykinin peptide family, we might possibly advance the understanding of the cascades leading to METH neurotoxicity by

- Combining pharmacological blockade of NK-3 receptor in NK-1 receptor gene knockout mice;
- Evaluating the effects of METH administration on the mice lacking PPT-A gene.

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