A ROLE FOR NICOTINE IN NEUROPROTECTION AGAINST DISORDERS ASSOCIATED WITH MONOAMINERGIC AND COGNITIVE DYSFUNCTION

by

Paula de Lima Vieira Brock

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STATEMENT OF DISSERTATION APPROVAL

The dissertation of **Paula de Lima Vieira Brock**

has been approved by the following supervisory committee members:

Annette E. Fleckenstein	, Chair	**01/30/2015** Date Approved
Glen R. Hanson	, Co-Chair	**01/30/2015** Date Approved
Diana G. Wilkins	, Member	**01/30/2015** Date Approved
J. Michael McIntosh	, Member	**01/30/2015** Date Approved
Misty D. Smith	, Member	**01/30/2015** Date Approved

and by **Karen S. Wilcox** , Chair/Dean of

the Department/College/School of **Pharmacology and Toxicology**

and by David B. Kieda, Dean of The Graduate School.

ABSTRACT

The aim of this dissertation was to investigate potential mechanisms whereby nicotine (NIC) is neuroprotective to the dopamine (DA), serotonin (5-HT), and memory systems. As early as 1939, clinical studies have indicated that tremors, or Parkinson's disease (PD), are less likely to occur among tobacco smokers. More recent epidemiological studies have found an inverse correlation between tobacco smoking and PD risk. PD is the second most common neurodegenerative disorder characterized by death of DA and 5-HT neurons in the nigrostriatal pathway and is associated with motor and memory dysfunction. Extensive preclinical studies have since demonstrated that NIC is neuroprotective in models of PD. The mechanism by which NIC is neuroprotective is of particular interest in the field of neurodegeneration to understand disease risk and to develop better prevention and treatment strategies. Noteworthy, the abuse of methamphetamine (METH), a potent psychostimulant, causes long-term neurotoxic effects resembling some aspects of PD, including deficits to the DA, 5-HT and memory systems. The data presented in this dissertation indicate that long-term (56 d) oral NIC administration to rats starting in adolescence attenuates both the dopaminergic and memory deficits, but not the serotonergic deficits, caused by a high-dose METH regimen. The dopamine transporter (DAT) function and density in the

striatum and nucleus accumbens core were used as markers of dopaminergic integrity, and the novel object recognition (NOR) test was used as marker of memory function. NIC is also neuroprotective when given short-term (21 d) beginning in adolescence, but not when it is initiated during adulthood. However, neuroprotection occurs when the duration of NIC administration is extended to 39 d beginning in adulthood. NIC pretreatment alone is sufficient for neuroprotection against METH-induced DAT deficits as well as NOR deficits. Furthermore, NIC ameliorates the NOR deficits caused by METH when given as posttreatment, suggesting that NIC has cognitive protection and cognitive enhancement properties. Lastly, the densities of α4β2 and α6β2 nicotinic acetylcholine receptors are upregulated and downregulated, respectively, after administration of NIC in combination with METH. These data suggest an involvement of these receptors in neuroprotection against METH-induced dopaminergic and memory deficits.

To my husband, Stewart Brock, from whom I received the love, support, and encouragement necessary to complete this work.

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CHAPTER 1

INTRODUCTION

Overview

Nicotine (NIC) has shown to be neuroprotective in preclinical models of neurodegenerative disorders such as Parkinson's disease (PD) (Quik et al., 2012) and Alzheimer's disease (Gould et al., 2013) as well as psychiatric conditions such as depression (Tizabi et al., 2009) and schizophrenia (Jubelt et al., 2008). Several mechanisms underlying this neuroprotection have been proposed including increases in neurotrophic factors (Maggio et al., 1998; Takarada et al., 2012), antioxidant activities (Newman et al., 2002; Xie et al., 2005) and alterations in the proteasome system (Kane et al., 2004), with one common feature being the involvement of nicotinic acetylcholine receptors (nAChRs). NIC affects many systems in the body including cardiovascular, respiratory, digestive, skeletal muscles, and the brain via these receptors. One scientific area of interest is the understanding of nAChRs in health and disease, as these studies might lead to new therapeutics as well as new preventive strategies against neurological and psychiatric disorders. NIC is naturally present in many common vegetables, particularly bell peppers, eggplant, and tomatoes (Domino et al., 1993; Siegmund et al., 1999). Diets rich in these vegetables have shown to be protective against PD (Nielsen et al., 2013). This dissertation will

specifically assess the potential neuroprotective effect of NIC in the monoamine and memory systems of the striatal and hippocampal brain regions that are affected by the abuse of methamphetamine (METH), with particular focus in investigating the role of nAChRs in potential neuroprotection. Noteworthy, present studies investigated the ability of NIC to modify neuronal function and prevent damage; thus, the implications of these studies lie under preventive measures in contrast to treatment of neurodegenerative disorders.

Striatum and hippocampus

Striatum and related regions. In rodents, the striatum is the main input nucleus of the basal ganglia. The striatum is divided into two anatomical regions, the dorsal and the ventral striatum. The dorsal striatum or neostriatum (referred herein as “striatum”), which reflects the caudate and putamen in humans, is the forebrain region that regulates motor function as well as learning and behavioral reinforcements. Approximately 90–95% of the striatum is composed of mini spiny projection neurons consisted of inhibitory γ-aminobutyric acid (GABA) neurotransmitter, and they provide the striatal output network. The remaining neuronal population is comprised of GABAergic and cholinergic interneurons. The striatum is densely innervated by dopaminergic neuronal input from the substantia nigra pars compacta (referred herein to “substantia nigra”) and to a lesser extent from the ventral tegmental area. The striatum also receives glutamatergic input projections from cortical regions (Zhou et al., 2002).

The ventral striatum, otherwise known as and referred to herein as “nucleus accumbens,” modulates motor and motivation action behavior and

participates in reward learning and addiction. It receives dopaminergic innervations mainly from the ventral tegmental area and to a lesser extent from the substantia nigra. The nucleus accumbens also receives extensive glutamatergic input from the hippocampus, prefrontal cortex, and amygdala. Additionally, the nucleus accumbens sends output GABAergic projections to basal forebrain cholinergic neurons. The nucleus accumbens is subdivided into two anatomical regions, the “core” and the “shell.” The nucleus accumbens core maintains a similar circuitry to the striatum by sending inhibitory projections to the substantia nigra and subthalamic nucleus. In contrast, the shell of the nucleus accumbens has no connectivity with the substantia nigra and subthalamic nucleus. These anatomical differences between core and shell strongly impact their distinct functions in goal/behavior selection, being the core involved in general selection and the shell in outcome-specific selection (Mannella et al., 2013). This dissertation focuses on the study of the nucleus accumbens core as opposed to the shell because of the involvement of the core, along with the striatum, in the dopaminergic disorders described herein.

Hippocampus and perirhinal cortex (PRh). The hippocampal formation consists of three main subregions, the dentate gyrus formed by granule cells, the CA1 and CA3 fields formed by pyramidal cells, and the subiculum. The main afferents to the hippocampal formation arise in the entorhinal cortex. These efferents from entorhinal cortex are projected to the dentate gyrus, which is the entry of the hippocampal formation, forming the perforant pathway. The dentate gyrus then sends its efferents to the CA3 and CA1 fields. The efferents from

dentate gyrus to the CA3 region form the mossy fiber pathway. The CA3 region sends efferents to the CA1 region forming the Schaffer collateral pathway. The CA1 field then sends its efferents to the subiculum, the exit of the hippocampal formation, which project back to the entorhinal cortex closing a loop pathway. The main innervations of the entorhinal cortex come from the PRh and parahippocampal gyrus (Milner et al., 1998).

The hippocampal formation and the PRh are the regions that intimately regulate explicit memory, particularly episodic memory (Vargha-Khadem, 1997). Learning and memory can be divided by explicit or implicit memory. Implicit memory, also known as nondeclarative memory, relates to habitual and procedural tasks and is mediated by functions of the basal ganglia, cerebellum, and parts of the cerebral cortex. Explicit memory, also known as declarative memory, is dependent upon functions of the medial temporal lobe including the hippocampal formations and PRh. Explicit memory is further divided into three types: episodic memory, which concerns memories of events or episodes; semantic memory, which concerns memories of general knowledge; and autobiographic memory, which combines both episodic and semantic memories (Milner et al., 1998). This dissertation deals with the study of episodic memory only because relapse, an important social, economical, and health consequence of METH abuse, is particularly affected by deficits in episodic memory, but not other types of memory also disrupted among METH abusers (Simon et al., 2004).

Recent studies have identified the PRh and hippocampal formations as

the main regions responsible for episodic memory (Kinnavane, 2014). At least initially, episodic memory activity is encoded in the hippocampus. Once processed in hippocampus, episodic memories are consolidated and stored in the PRh. Because the functions of the striatum or nucleus accumbens are not associated with episodic memory, this dissertation consists in the evaluation of two distinct systems. In other words, this dissertation investigates the possibility that nicotine (NIC) is neuroprotective to the hippocampal/PRh and to the striatal/nucleus accumbens deficits caused by METH.

Parkinson's disease (PD)

PD is the second most common neurodegenerative disease after Alzheimer's disease, affecting over 6 million people worldwide >60 years old. PD consists of a progressive and typically slow degeneration of neurons starting at the brain stem (Goetz, 2011). At this early stage, patients will present with symptoms that are not exclusive to PD and thus are rarely diagnosed (Becker et al., 2002). When the degeneration process reaches the midbrain, particularly substantia nigra and striatum, dopamine (DA) neurons are damaged causing motor complications such as tremors, rigidity, and bradykinesia. Unfortunately, by the time patients present to their physicians with motor deficits, 60–70% of neurons in the substantia nigra have already degenerated, as shown by reductions in DA transporter (DAT) binding, and 80% of DA content in the striatum is reduced, making it difficult to treat (Broussolle et al., 1999; Becker et al., 2002). During more advanced stages, when degeneration reaches the cortex, patients might develop cognitive deficits (Dickson, 2012). Therefore, PD patients

form a heterogeneous population with a range of disease onset and symptoms making it difficult to diagnose, treat, and to understand its etiology.

Etiology of PD. The etiology of PD is complex, and many genes plus environmental factors have been associated with its risk (Venderova and Park, 2012). Until a few years ago, PD etiology was heavily attributed to environmental factors such as exposure to pesticides and older age, and it is classified as a sporadic disease, which comprise 80–85% of the cases (Baltazar et al., 2014). Recent studies have demonstrated that PD etiology is also associated with specific genetic traits that might interact with environmental exposure (Edwards et al., 2010; Venderova and Park, 2012). These studies consistently indicate that dysfunction of the proteasome system and mitochondria and aggregation of cell structure proteins might contribute to the extensive dopaminergic damage occurred in this disease in a multiple gene-environment interaction manner.

PD genetics. Analyses of brain samples from postmortem PD patients have historically revealed overexpression and aggregation of alpha-synuclein protein in Lewy bodies (Spillantini et al., 1997). Lewy body formation has thus been the gold standard of PD pathology and known to cause neuronal death. However, Lewy bodies are not always present, and alpha-synuclein gene mutations or copy number variations have only been studied in rare familial cases that tend to manifest at very early age (Singleton et al., 2003). In addition, although the association of alpha-synuclein and PD is well established in studies of rare familial cases (Polymeropoulos et al., 1997; Kruger et al., 1998), functional studies of alpha-synuclein have only occurred within the last 5 years.

Alpha-synuclein importantly regulates dendritic arborization, neurogenesis, and cell membrane curvature (Cronin et al., 2009; Winner et al., 2012; Westphal and Chandra, 2013). In the hippocampus, the brain region in which neurons are constantly renewed, mutant alpha-synuclein impaired neuronal formation and survival (Winner et al., 2012). Lack of cell structure due to reduced arborization and loss of neuronal survival are key features of PD. Genome wide association studies (GWAS) recently provided the tool for researchers to find mutations associated with the sporadic cases. The alpha-synuclein gene, SNCA, has been now associated with sporadic PD cases through GWAS and meta-analyses studies (Simon-Sanchez et al., 2009; Edwards et al., 2010; Nalls et al., 2011; Lill et al., 2012).

Environmental factors and PD susceptibility. Both genetic traits and environmental factors seem to explain PD susceptibility (Brighina et al., 2008; Bove and Perier, 2012; Chung et al., 2013). Particularly, exposure to pesticides is known to increase PD risk (Elbaz et al., 2009) and induces DA damage in the midbrain (Bove and Perier, 2012). Several studies have evaluated the potential for interaction between these and other environmental factors with candidate gene variants that could potentially explain a larger population of patients (Brighina et al., 2008; Venderova and Park, 2012). One of the studies showed that patients with PD were more likely than control subjects to have used pesticides (Brighina et al., 2008). Furthermore, in younger individuals, data indicated an association of pesticide exposures with PD and an association of SNCA REP1 genotype score with PD (Brighina et al., 2008). In addition, some

drugs of abuse, specifically amphetamines, known to disrupt DA function in the midbrain, have recently shown to increase PD risk (Callaghan et al., 2010; Callaghan et al., 2012). Preclinical studies evaluating amphetamines abuse and candidate PD genes demonstrated that METH causes an upregulation of the SNCA protein, alpha-synuclein, suggesting one mechanism by which this class of drugs can lead to increased PD risk (Liao et al., 2005). Overall, some environmental factors can interact with some gene variants to modify PD risk in specific groups of individuals such as young people. While genetic models try to recapitulate progressive degeneration, drug-induced models recapitulate the sporadic and most common cases of PD that affect the mitochondrial complex I and induce oxidative stress. These mimic the final molecular mechanism that is dopaminergic loss in the nigrostriatal pathway.

The main treatment for PD is levodopa, which is associated with significant side effects such as dyskinesia, and anticholinergic drugs, which are associated with memory deficits. Nicotine (NIC) has shown to ameliorate dyskinesia and memory function.

Methamphetamine (METH)

METH is a small lipophilic molecule structurally similar to the endogenous neurotransmitter DA first synthesized by Nagai Nagayoshi in 1893 and later crystalized by Akira Ogata in 1919. METH has been approved for human use by the United States Food and Drug Administration (FDA) since November 2000, under the brand name Desoxyn®, for the treatment of attention deficit hyperactivity disorder (ADHD) and exogenous obesity. Due to its high abuse

liability and potential for severe side effects, METH is also a schedule II substance as defined by the United States Controlled Substance Act under Title 21 Code of Federal Regulations (CFR) 1308.12.

During the 1990s, METH abuse was described as “an epidemic” in the U.S. with a significant increase in emergency room visits (54% across all ages and 88% for persons under 18) (Gaines, 2014) and continues to be a problem in the 2010s (SAMHSA, 2014). In 2005, the national economic burden caused by METH abuse was estimated to be over 23 billion dollars (Nicosia et al., 2009). Some examples of such high economic cost include the burden of addiction, premature deaths, aspects of lost productivity, health care costs, child endangerment issues, and involvement in the criminal justice system.

Despite the incidence of abuse having declined steadily since 2000 (Johnston et al., 2010), METH abuse is still a significant problem in our society with an estimated abuse of over 1% among adolescents and young adults (Johnston et al., 2014) and high emergency room visits (SAMHSA, 2014). Despite evidence indicating that METH neurotoxicity might be partially reversible with time, this recovery is likely dependent on the amount and route of administration of METH exposure (Friedman et al., 1998; Harvey et al., 2000) and is likely incomplete (Woolverton et al., 1989). Furthermore, abuse of METH/amphetamine has been associated with brain abnormalities, including cerebrovascular damage such as arteritis (Rumbaugh et al., 1971), vasculitis (Bostwick, 1981), and intracranial hemorrhage (Cahill et al., 1981) and increased prevalence of ischemic stroke, particularly in the subcortical white matter and

basal ganglia (Yen et al., 1994). Particularly, and specifically discussed in this dissertation, METH abuse increases the likelihood to develop PD later in life, likely due to METH-induced deficits in the basal ganglia. Combined, these data suggest that although the incidence of METH abuse has dropped, millions of past METH addicts from the “epidemic” period might develop PD in the near future.

METH abuse causes cognitive deficits. Extensive clinical literature has revealed that METH abusers display neurocognitive impairment as assessed by measures of attention, learning and memory, and/or executive functioning. These are associated with poor functional outcomes, such as relapse (Scott et al., 2007). Moreover, neurocognitive deficits caused by METH abuse are long lasting and persist for at least 6 months after abstinence (Hoffman et al., 2006). Of particular interest of this dissertation is that METH dependence is associated with episodic memory deficits (Scott et al., 2007; Kalechstein et al., 2009; Casaletto et al., 2014). This is of importance because deficits in episodic memory, but not other kinds of cognitive function, are associated with relapse (Simon et al., 2004). In laboratory animals, both contingent and noncontingent administration of METH leads to episodic memory deficits (Belcher et al., 2008; Herring et al., 2008; Reichel et al., 2012) assessed by a spontaneous novel object recognition (NOR) test discussed below.

METH and mechanisms of cognitive dysfunction. METH intake produces deficits in episodic memory as well as decreases in hippocampal volume that remain evident even after prolonged drug abstinence in humans and experimental animals (Orikabe et al., 2011; Akiyama et al., 2011; North 2012).

Several clinical studies have demonstrated that METH-induced neurocognitive deficits are correlated with hippocampal abnormalities. For instance, Thompson et al. (2004) showed that METH abusers have 7.8% smaller hippocampal volumes than subjects without a history of substance abuse, and these deficits correlated with memory performance as assessed by a word-recall test. Furthermore, Sekine et al. (2006) showed by positron emission tomography that serotonergic deficits as assessed by serotonin transporter (SERT) densities are significantly reduced in several brain regions of abstinent METH abusers. Although this former study showed that reductions in SERT densities were associated with aggressive behavior, it is unclear whether hippocampal and/or cortical serotonergic deficits are also associated with neurocognitive dysfunction.

In laboratory animals, contingent or noncontingent METH administrations also cause significant memory deficits as assessed by NOR (Belcher et al., 2008; Reichel et al., 2012). These same METH regimens cause serotonergic deficits in the hippocampus and PRh as assessed by SERT density, function, and immunoreactivity and serotonin (5-hydroxytryptamine; 5-HT) content as well as deficits in the dopaminergic system of the striatum and nucleus accumbens as assessed by DAT density, function and immunoreactivity, and DA content (Belcher et al., 2005; Belcher et al., 2008; McFadden, Hunt, et al., 2012; Reichel et al., 2012). As discussed above, NOR memory is a declarative type of memory mediated by functions of the hippocampus and cortex as oppose to nondeclarative (habitual) memory mediated by the basal ganglia (Kinnavane et al., 2014). Thus, the studies performed in this dissertation assessed whether

NOR deficits caused by METH are associated with deficits in serotonergic markers in the hippocampus and PRh.

Long-term potentiation (LTP) in hippocampal formations and PRh is a well-established synaptic plasticity mechanism by which learning and memory occurs (Bliss and Collingridge, 1993). Recently, *exvivo* studies showed that METH administration increases baseline synaptic transmission and reduces LTP in the CA1 region of the hippocampus (Hori et al., 2010; Swant et al., 2010). The CA1 hippocampal region is important for acquisition of episodic memory (Kinnavane et al., 2014). *In vivo* studies with mice showed that METH abstinence after chronic daily dosing reduced spatial memory and CA1 hippocampal LTP (North et al., 2012). Other preclinical studies demonstrated that chronic METH administration to young rats, as well as gestational exposure to METH, causes reduction in LTP in CA1 pyramidal neurons (Hori et al., 2010). These findings suggest that METH-induced deficits in episodic memory are caused by reductions in CA1 LTP. The effects of METH on LTP in the PRh are less studied.

METH and dopaminergic neurotoxicity. In addition to neurocognitive impairment and serotonergic deficits in associated neuronal regions, METH abuse also causes significant reductions in dopaminergic markers in the striatum and nucleus accumbens core, as demonstrated in human, nonhuman primate, and rodent studies (Hotchkiss and Gibb, 1980; Woolverton et al., 1989; Daberkow et al., 2005; Kousik et al., 2014). Such deficits persist for at least 8 months in rats and 3 years in humans (Bittner et al., 1981; McCann et al., 1998; McCann et al., 2008). Particularly, preclinical studies have demonstrated that

METH preferentially damages the dopaminergic terminals in the nucleus accumbens core, rather than in the shell (Broening et al., 1997). Furthermore, the striatum is the forebrain region that intimately regulates motor function, and clinical and preclinical studies have demonstrated that METH-induced striatal dysfunction is associated with psychomotor impairment and motor learning deficits (Volkow et al., 2001; Daberkow et al., 2005).

METH dosing. We chose to evaluate the binge model of METH because METH addicts undergo periods where they take large quantities of drug in a short period of time (0.7–3 g/d; approximately 12–50 mg/kg) (Simon et al., 2000; Semple et al., 2003; Hoffman et al., 2006). In the United States, arrest reports of drivers show METH blood levels can be above 300 μg/mL (Logan, 1996). To examine METH-induced neurotoxicity in animal models, various noncontingent, high doses of METH have been used. For example, 30 mg/kg/day (Cadet et al., 2011) have been used to study the neurotoxic consequence of METH exposure. In this dissertation, 30/mg/kg/day (i.e., 4 x 7.5 mg/kg, 2-h apart) was used. These dosing regimens cause significant DA deficits in the striatum and nucleus accumbens core resembling some aspects of PD.

METH and PD. An additional problem for METH abusers is the risk for developing PD later in life, as recent studies demonstrated that the risk for developing PD is 1.76-fold higher in abstinent METH abusers (Callaghan et al., 2010; Callaghan et al., 2012; Curtin et al., 2014). Clinical and preclinical studies have shown overlapping neuropathologies between METH abuse and PD (Gibb et al., 1987; Woolverton et al., 1989; Kousik et al., 2014). For example, as

discussed above, PD is characterized by progressive degeneration of DA neurons in the substantia nigra leading to deficits in DA content and DAT density in the striatum and motor function impairment. Similarly, detoxified METH addicts show psychomotor impairment and deficits in DAT density (Volkow et al., 2001). Motor function impairment is less likely to occur among METH addicts because of the lack of degeneration of DA neuron cell bodies in the substantia nigra. In comparison to patients with PD, abstinent METH addicts present smaller reductions in DAT density in the striatum suggesting that METH abuse emulates early-stage PD (McCann et al., 1998; McCann et al., 2008). Furthermore, recent preclinical studies have demonstrated that METH administration causes upregulation of alpha-synuclein protein in the substantia nigra and striatum (Fornai et al., 2005; Liao et al., 2005). Such phenomenon might contribute to the increased risk of METH abusers in developing PD.

METH mechanisms of neurotoxicity. The biological mechanisms by which METH induces dopaminergic neurotoxicity are complex, but have been extensively studied (Fleckenstein et al., 2007). Briefly, METH is taken up by the plasmalemmal DAT or diffuses through the cellular membrane of presynaptic dopaminergic terminals. Once inside, METH can diffuse through vesicular membranes and changes the internal pH of cytoplasmic vesicles (Cubells et al., 1994). The vesicular monoamine transporter-2 (VMAT-2) present on the membrane of these vesicles stops sequestering DA. The excess of cytoplasmic DA is released through DAT. METH is able to reverse the function of DAT from taking up synaptic DA to releasing DA. Particularly, DA released via either

reversal of transporters displays characteristics of tonic DA release. The excess cytoplasmic DA can also auto-oxidize, forming reactive oxygen species (ROS) that are believed to contribute to neuronal damage. The substantial release of DA could deplete DA stores and alter vesicle trafficking (Di Chiara and Imperato, 1988). Recent evidence has revealed that METH augments both tonic and phasic DA release, but phasic DA release is the predominant event that likely contributes to the long-term dopaminergic deficits (Howard et al., 2011; Howard et al., 2013). These studies demonstrated that during METH administrations both phasic and tonic DA release are elevated. However, 4–7 weeks later phasic DA release is significantly reduced, whereas tonic DA release is unaffected, indicating that terminals that mediate phasic DA release are more susceptible to the toxic effects of METH. It has been suggested that METH-induced phasic DA release occurs through readily releasable pool of vesicles (i.e., membrane-associated fraction) as opposed to tonic DA release that occurs from reversal of VMAT-2 from the reserve pool of vesicles leading to cytoplasmic DA (Cubells et al., 1994; Grace, 1995; Covey et al., 2013; Daberkow et al., 2013). In summary, DA levels, DAT function, and DAT and TH immunoreactivity are reduced in humans and laboratory animals that administered high doses of METH. Although in this dissertation VMAT-2 and phasic/tonic DA release are not directly assessed, some of the experiments were conducted based upon a hypothesis on VMAT-2 or phasic/tonic DA release mechanisms.

Tonic versus phasic DA signaling and METH neurotoxicity. Tonic DA release is measured in a timescale of minutes to hours and occurs in

spontaneously active dopaminergic neurons to enhance performance of motor behavior (Floresco, 2007; Redgrave et al., 2010). Tonic DA release is also associated with perseverance in achieving goals (Salamone et al., 2003). Phasic DA release is measured in a timescale of milliseconds and occurs from excitation (primarily glutamatergic) of dopaminergic neurons. Phasic DA release is important for learning processes within the basal ganglia (Schultz, 2002). METH neurotoxicity disrupts mainly phasic DA release, suggesting that dopaminergic terminals that mediate phasic DA release are more sensitive to METH effects (Howard et al., 2011; Howard et al., 2013). In agreement with these neurochemical studies, behavioral studies have shown that METH administrations to rats causes persistent deficits in motor learning and as mentioned above, learning process in the basal ganglia is associated with phasic DA signaling (Daberkow et al., 2005).

METH as a PD model. Clinical studies have shown that postmortem dopaminergic markers, including DAT expression, DA levels, and tyrosine-hydroxylase (TH) immunoreactivity are reduced in the caudate and putamen of former METH abusers. Serotonergic deficits in the striatum, hippocampus, and frontal cortical regions have also been reported in postmortem human brains from former METH abusers. A typical model of METH-induced dopaminergic and serotonergic deficits in rats consists of repeated administrations of a high-dose METH regimen (typically 4–6 injections of 5–10 mg/kg/injection at 2–6-h intervals). One to 14 days after this high-dose METH regimen, markers of DA neurons integrity reveal significant deficits in TH immunoreactivity in the

substantia nigra and striatum, DA content in the striatum, and DAT function and immunoreactivity in the striatum and nucleus accumbens core that are detected up to 8 months later (Koda and Gibb, 1973; Sonsalla et al., 1996; Hirata and Cadet, 1997; Krasnova and Cadet, 2009; Yamamoto et al., 2010; Ares-Santos et al., 2012). Hippocampal serotonergic markers such as SERT function and 5-HT content are also detected within several days of METH abstinence with this model. This model consistently mimics the physiological deficits in dopaminergic and serotonergic markers observed in human METH abusers and PD patients.

Nicotine (NIC)

NIC is an alkaloid present in the nightshade family of plants (Green et al., 2013). NIC is present in vegetables (Domino et al., 1993; Siegmund et al., 1999), however, at concentrations 500-fold less than second hand smoke (Henningfield, 1993). However, these low levels of NIC found in diet taken long-term can be sufficient to reduce PD risk (Nielsen et al., 2013). Many other compounds naturally found in plants (Green et al., 2013) and in small organisms such as snails (McIntosh et al., 1999) affect nAChRs and these compounds are used in basic research as agonists and antagonists for nAChRs.

NIC is known to be the addictive substance in cigarettes, and its chronic administration has a profound impact in brain chemistry (Benowitz, 2010). NIC activates all subtypes of nAChRs located throughout the body thereby affecting various molecular and cellular mechanisms (Benowitz, 2010; Colombo et al., 2013). Some of these mechanisms include desensitization and upregulation or downregulation of nAChRs function and expression (Perez et al., 2008; Perez et

al., 2009). These receptors are pentameric; i.e., they are formed by an assembly of five subunits each coded by a distinct gene. Seventeen genes have so far been identified in vertebrates (α1-10, β1-4, γ, δ, ε). These receptors mediate a variety of functions depending on their localization, particularly allowing influx of Ca^{+} and Na^{+} and inducing neurotransmitter release (Yu and Wecker, 1994; Grady et al., 2001; Azam and McIntosh, 2006; Perez et al., 2009).

NIC and nAChRs in the striatum. Three main subtypes of nAChRs are expressed in the striatum: the α4β2, α6β2, and α7 subtypes. The α4β2 and α6β2 subtypes of nAChRs are highly found in dopaminergic terminals in the striatum and mediate DA release. Of note, the α4β2 subtype is expressed on interneurons, GABA neurons, and dopaminergic terminals, whereas the α6β2 subtype is expressed predominantly on dopaminergic terminals (Quik and McIntosh, 2006; Nashmi et al., 2007; English et al., 2012; Luo et al., 2013). In contrast, the α7 subtype is localized on glutamatergic axons and mediates glutamate (Glu) release in the striatum (Zhou et al., 2002; Zoli et al., 2002; Quik et al., 2005). The effect of chronic NIC administration on the function and expression of these subtypes of nAChRs have been demonstrated in humans, nonhuman primates, and rodents (Perry et al., 1999; Perez et al., 2009). For example, chronic NIC administration to laboratory animals causes upregulation of α4β2, downregulation of α6β2, and upregulation of α7 nAChRs binding in the striatum (Slotkin et al., 2004; Doura et al., 2008; Perez et al., 2008).

NIC and nAChRs in the nucleus accumbens. Ligand binding studies have shown that nucleus accumbens contain α4β2, α6β2, and α7 nAChRs

(Doura et al., 2008). *In vivo* and *in vitro* studies have shown that NIC administration reduces DA release in the nucleus accumbens core and shell via α6β2 nAChRs (Perez et al., 2012; Schilaty et al., 2014). Of particular interest for this dissertation, functional studies have shown that α6β2 nAChRs primarily mediate DA release in the core, whereas α4β2 nAChRs mediate DA release in the shell (Schilaty et al., 2014). These studies utilized α-conotoxin-MII (αCtxMII) ligand, a selective antagonist for α6β2 nAChRs, in which its application to brain slices inhibited the NIC-induced DA release in the core, but not in the shell (Schilaty et al., 2014). The application of dihydro-β-erythroidine (DHβE), a selective antagonist for the α4β2 subtype, inhibited NIC-induced DA release in the shell, but not in the core (Schilaty et al., 2014). In summary, chronic NIC administration downregulates α6β2 nAChRs, thereby reducing DA release in the nucleus accumbens.

nAChRs regulation of tonic and phasic DA release. It has been demonstrated in striatal and nucleus accumbens core slices that α6β2 nAChRs mediate phasic DA release, whereas α4β2 nAChRs mediate tonic DA release (Meyer et al., 2008; Wickham et al., 2013). Additionally, the majority (~80%) of nAChR-modulated DA release in the striatum and nucleus accumbens is regulated by α6β2 nAChRs (Perez et al., 2008; Perez et al., 2009). As discussed above, METH neurotoxicity disrupts mostly phasic DA release, as opposed to tonic DA release. These findings suggest that (1) dopaminergic fibers that express α6β2 nAChRs fire DA in phasic patterns; (2) they are more susceptible to METH neurotoxicity; (3) reduction in phasic DA release during METH

administrations is neuroprotective. Thus, α6β2 nAChRs potentially serve as markers of dopaminergic terminals that are more susceptible to neurotoxicity. Furthermore, this suggests a mechanism by which persistent dopaminergic deficits could be attenuated. In other words, these data suggest that blockage of phasic DA release via inhibition of α6β2 nAChRs could potentially protect against METH-induced toxicity. Data presented in this dissertation indicate that α6β2 nAChRs-containing dopaminergic terminals are susceptible to METH neurotoxicity. However, whether inhibition of α6β2 nAChRs attenuates METH-induced dopaminergic deficits has yet to be tested.

As noted above, long-term exposure to NIC causes upregulation of α4β2 and downregulation of α6β2 nAChRs binding, indicating that phasic DA release is reduced and tonic DA release is augmented with chronic NIC administration. In preclinical studies, nAChR-stimulated striatal [^{3}H]DA release was reduced after 10 d NIC self-administration in mice, and data indicated that this reduction was mediated by α6β2 nAChRs. However, no increases in nAChR-stimulated [^{3}H]DA release via α4β2 nAChRs were observed (Marks et al., 2014). In electrically stimulated endogenous DA release studies, several weeks of NIC administration to rodents significantly reduced DA release in the striatum and nucleus accumbens, and these effects were mediated by α6β2 nAChRs (Exley et al., 2013). In contrast, others have shown that chronic NIC treatment to rodents increases electrically evoked endogenous DA release in the striatum likely via upregulation of α4β2 nAChRs (Perez et al., 2008). However, a single injection of NIC caused reductions in both tonic and phasic electrically stimulated DA

release, with a higher ratio of phasic bursts relative to tonic firing (Zhang et al., 2009). Thus, results suggest that chronic, but not acute, NIC administration reduces phasic-like DA signaling via downregulation of α6β2 nAChRs and increases tonic-like DA release via upregulation of α4β2 nAChRs.

NIC and nAChR in the hippocampus and PRh. Several subtypes of nAChRs have been found in the hippocampus and cortex (Sudweeks and Yakel, 2000). For example, functional studies have found the presence of nAChRs containing α7 (Alkondon and Albuquerque, 1995; Jones and Yakel, 1997; Frazier et al., 1998; McQuiston and Madison, 1999), α4β2 (McQuiston and Madison, 1999; Sudweeks and Yakel, 2000), α2 (McQuiston and Madison, 1999; Sudweeks and Yakel, 2000), or α3β4 subunits (Azam and McIntosh, 2006). The α3β4 nAChRs mediate norepinephrine release in the hippocampus (Azam and McIntosh, 2006). *In vivo* studies have demonstrated that norepinephrine does not mediate cognitive deficits induced by METH or by the simple process of aging (Schweizer et al., 2003; Rau et al., 2006; Reichel et al., 2012). On the other hand, extensive literature has pointed out that the α4β2 and α7 nAChRs impact memory formation in physiological and pathophysiological states (Felix and Levin, 1997; Levin and Simon, 1998; Nott and Levin, 2006). These two subtypes are expressed pre- and postsynaptically throughout the hippocampus and cortex modulating Ca^{2+} influx (Kenney and Gould, 2008). Both α4β2 and α7 subtypes modulate glutamatergic and GABAergic activities in the hippocampus and cortex (Alkondon and Albuquerque, 2004). Particularly, α4β2 subtypes are highly expressed in CA1 and the dentate gyrus, whereas α7 is mostly found in the

dentate gyrus. Presynaptically, α4β2 play a greater role in modulating GABA release than α7 subtypes (Freedman et al., 1993; Gray et al., 1996; Alkondon and Albuquerque, 2004; Yamazaki et al., 2005). Postsynaptically both subtypes modulate synaptic plasticity (Alkondon and Albuquerque, 2004; Yamazaki et al., 2005; Kenney and Gould, 2008). Particularly, α4β2 and α7 nAChRs seem to mediate LTP in the CA1 hippocampus and cortex, an important process that underlies learning and memory (Kenney and Gould, 2008). NIC induces 5-HT release in the hippocampus and cortex, suggesting the presence of nAChRs in serotonergic terminals in these regions (Seth et al., 2002). The presence of α7 nAChRs in serotonergic terminals in the hippocampus has been demonstrated (Aznar et al., 2005). The presence of α4β2 nAChRs has been shown in the nucleus raphe (which projects serotonergic innervations to the hippocampus) (Cucchiaro and Commons, 2003). It is unclear whether nAChRs are localized to a specific region of the hippocampus.

nAChRs regulation of LTP. Several preclinical studies have investigated the mechanism by which NIC augments LTP in the Schaffer collateral pathway (CA3-CA1) of the hippocampus (Yamazaki et al., 2005; Yamazaki et al., 2006). These studies have demonstrated that NIC increases LTP by reducing GABAergic inhibition of CA1 (Alkondon et al., 1997; Yamazaki et al., 2005). In these studies, NIC reduced the amplitudes of IPSCs evoked by low intensity electrical stimulation (<200 μA) via α4β2 and by high intensity electrical stimulation (>300 μA) via α7 nAChRs. These findings indicate that both α4β2 and α7 nAChRs are expressed in the hippocampus and particularly important for

mediating NIC-induced LTP in CA1. Furthermore, NIC did not affect glutamate release, but rather repressed GABA release in the CA1 cells via α4β2 nAChRs. These data demonstrate that NIC mainly augments LTP via α4β2 nAChRs reduction in GABA release (Alkondon et al., 1997; Yamazaki et al., 2005). Chronic NIC exposure also increases α4β2 nAChRs expression on glutamatergic axons of the perforant pathway of the hippocampus (cortex-DG). In these studies chronic NIC augmented LTP in the perforant pathway, indicating that the upregulated α4β2 nAChRs are functional (Nashmi et al., 2007).

NIC and dopaminergic neuroprotection

For over 50 years, it has been known that the incidence of diseases associated with motor dysfunction is less likely to occur among tobacco smokers. A clinical study from 1938 reported that although tobacco smokers were more likely to develop diseases of the circulatory system (heart disease), gastrointestinal system (ulcers), respiratory system (lung cancer and asthma) and nervous system (anxiety), they were less likely to present tremors (Short, 1938). Several epidemiological studies have since revealed an inverse correlation of tobacco smoking and the development of PD (Hernan et al., 2001; Chen et al., 2010).

In recent epidemiological studies, data were stratified by smoking behavior, which provides detailed information on the impact of duration and intensity of cigarette smoking and PD risk (Chen et al., 2010). Among current smokers, intensity of smoking (i.e., number of cigarettes smoked per day) did not affect the inverse association of smoking and PD risk. However, among past

smokers higher intensity (i.e., greater number of cigarettes smoked per day) was associated with greater protection against PD. These data indicate that the neuroprotective effect of tobacco smoking goes away with time and that intensity only matters when tobacco exposure has ceased. These data further suggest that neuroprotection is mediated by neurochemical changes occurring after long-term exposure to tobacco, rather than an immediate effect of the neuroprotective substance present in tobacco. Additional relevant information from these studies concerns the fact that approximately 68% of the participants started smoking before the age of 20. Although the age covariate was not taken into account in data analysis, this information permits speculation that age of smoking initiation might also play a role in neuroprotection.

NIC neuroprotection in clinical PD studies. Many investigators have studied whether NIC is the substance in cigarettes affording this neuroprotection. An observational study showed that consumption of NIC-containing food such as peppers and tomatoes is inversely associated with PD risk (Nielsen et al., 2013). Furthermore, clinical trials are already evaluating the potential use of NIC in PD therapy (Villafane et al., 2007; Itti et al., 2009). Villafane et al. (2007) showed that NIC treatment via NIC patch for 17 weeks ameliorates motor symptoms of PD in comparison to baseline as assessed by the Unified PD Rating Scale (UPDRS). In addition, NIC added to the first line PD therapy L-DOPA improved outcome as indicated by the reduction in the dose of the medication patients had to take overtime. Furthermore, positive effects of NIC only occurred after long-term treatment. Noteworthy, Itti et al. (2009) showed that not only motor symptoms

and efficacy improved over the long course of NIC treatment, but NIC also improved DA neurons integrity as assessed by neuronal imaging techniques. These findings suggest that NIC interacts with PD, affording neuroprotection to remaining terminals, and thus slowing disease progression.

NIC neuroprotection in preclinical studies. Several preclinical studies have demonstrated that NIC is neuroprotective to DA neurons in drug-induced PD models (Quik et al., 2012). NIC protected in MPTP, paraquat, and 6-hydroxydopamine lesion models in rodents and monkeys (Khwaja et al., 2007; Huang et al., 2009). For example, 7-week NIC administration via drinking water to rats starting at PND 30 protected against 6-hydroxydopamine lesions when assessed via ^{125}I-RTI-121 autoradiography and DA content in the striatum. Similarly, a similar NIC administration via drinking water to mice for 6 weeks starting at young adulthood attenuated paraquat-induced dopaminergic deficits. Fewer studies have reported NIC neuroprotection after acute NIC administration (Maggio et al., 1998; Ryan et al., 2001).

NIC neuroprotection and nAChRs signal pathway. NIC has shown to have antioxidant effects (Linert et al., 1999; Soto-Otero et al., 2002; Egea et al., 2007), to reduce alpha-synuclein fibrillation (Hong et al., 2009), to increase neurotrophic factors (Maggio et al., 1998; Belluardo et al., 2008; Takarada et al., 2012), to activate the function of the proteasome system (Chapman, 2009), and to enhance LTP in memory-related brain regions (Fujii et al., 1999; Kroker et al., 2011). But the subtype of nAChR that mediates these neuroprotective effects is unknown. Chronic NIC administration causes upregulation in the density of α7

nAChRs, suggesting that activation of these receptors would afford protection. However, pretreatment with a selective α7 antagonist protects against METH-induced neurotoxicity (Northrop et al., 2011). The α7 subtypes are located in glutamatergic terminals in the striatum and regulate glutamate release. Blockage of striatal glutamate release is neuroprotective. Thus, the potential NIC protection to dopaminergic neurons in the striatum is likely not mediated by α7 nAChRs. In contrast, studies have revealed a role for α4β2 and α6β2 subtypes of nAChRs in neuroprotection since they mediate DA function in the striatum (Perez and Quik, 2011). However, whether selective agents for these receptors are also protective is unknown.

Duration of NIC treatment, neuroprotection, and nAChRs. Length of NIC exposure is known to influence the expression and function of selective subtypes of nAChRs and correlate with neuroprotection. In rodents, 6–7 weeks of NIC administration afforded dopaminergic neuroprotection against 6-hydroxydopamine- or paraquat-induced damage (Khwaja et al., 2007; Huang et al., 2009). In these studies, NIC administration caused upregulation of α4β2 and downregulation of α6β2 nAChRs density when assessed via ^{125}I-epibatidine and ^{125}I-CtxMII autoradiography. Several others have shown that chronic oral NIC administration to rodents causes upregulation of α4β2 and downregulation of α6β2 using either ^{125}I-A-85380 (selective for α4β2 and α6β2) or ^{125}I-epibatidine (selective for α4β2, α6β2, and α3β4) in combination with ^{125}I-CtxMII (a selective ligand for α6β2). However, NIC administration not always induces upregulation of α4β2 and downregulation of α6β2 in the striatum. In Even et al. (2008) and

Nguyen et al. (2003), mice that received NIC via osmotic minipumps for 17–21 days showed upregulation of α4β2 but no change in α6β2 expression when assessed via autoradiography with ^{125}I-epibatidine in the presence or absence of cytosine or with α6-knockout mice. Pietila et al. (1998) showed that upregulation of α4β2 only occurs after at least 4 weeks of oral NIC administration when assessed via [^{3}H]NIC binding. Inconsistency with expression of nAChRs after chronic NIC administration has been explained by two factors: (1) the form of NIC administration (i.e., continuous via minipumps or intermittent via drinking water) as withdrawal from NIC also affects nAChRs expression and is only achieved with intermittent administration protocols and (2) techniques used to assess the expression of nAChRs since selective ligands are necessary to distinguish α4β2 to α6β2 subtypes (Govind et al., 2012).

Age of NIC administration and nAChRs. Age of NIC administration affects not only the expression of nAChRs, but also dopaminergic function in the striatum. Collins et al. (2004) demonstrated that 7 days of NIC administration via intraperitoneal injections increased striatal DAT expression in periadolescents (PND 30), but not in young adult (PND 60) rats, when assessed via ^{125}I-RTI autoradiography. Furthermore, the expression of α4β2 nAChRs was upregulated in young adults, but not in adolescents, when assessed via ^{125}I-epibatidine (Doura et al., 2008). NIC metabolism and elimination appears to be increased in adolescent rats in comparison to young adults, as demonstrated by hepatic CYP enzyme expression (Yun et al., 2010) and brain NIC/metabolites disposition (Vieira-Brock et al., 2013). Since NIC withdrawal leads to upregulation of α4β2

and downregulation of α6β2 nAChRs (Pietila et al., 1998), adolescents might present a different pattern of nicotinic receptor expression/function than adults due to increased elimination of NIC. Doura et al. (2008) demonstrated that NIC-induced changes in nAChRs expression differ between adolescent and adult rats with upregulation of α4β2 and downregulation of α6β2 in the striatum being more robust in adolescents. These data indicate that there are differences in the response to NIC between adolescents and young adult rats. However, whether these changes impact the potential neuroprotective effect of NIC in METH-treated rats is unknown.

α4β2 nAChRs and neuroprotection against METH-induced neurotoxicity. Upregulation of α4β2 nAChRs by chronic NIC exposure potentially mediates neuroprotection against METH-induced dopaminergic deficits. As noted above, these subtypes of nAChRs mediate tonic DA release in the striatum and nucleus accumbens. Part of the mechanism by which METH causes neurotoxicity is via reactive oxygen species (ROS) that are formed due to oxidized DA in the cytoplasm. Thus, reduction in cytoplasmic DA attenuates neurotoxicity. In fact, drugs that increase vesicular sequestration of DA by upregulating VMAT-2 function are protective. In similar ways, an increase in α4β2 nAChRs density might increase tonic DA release during METH administrations, consequently reducing cytoplasmic DA and ROS. These events would occur due to the fact that METH administrations induce acetylcholine release, which in turn bind to plasmalemmal α4β2 nAChRs, whereby tonic DA release would occur.

α6β2 nAChRs and neuroprotection against METH-induced neurotoxicity. Conversely, downregulation of α6β2 nAChRs by chronic NIC exposure potentially mediate protection by reducing phasic DA release. Studies with rodents and monkeys have shown that chronic NIC administration not only leads to decreases in α6β2 nAChRs density, but also a reduction in phasic DA release (Meyer et al., 2008; Perez et al., 2008; Exley et al., 2013). It has been also shown that METH-induced neurotoxicity is mainly mediated by exocytotic DA release and phasic DA signals (Daberkow et al., 2013). These events might cause neurotoxicity via downstream activation of D1/D2/D3 receptors by DA as indicated by findings that antagonists of D1/D2/D3 receptors protect against METH-induced neurotoxicity (Sonsalla et al., 1986; Broening et al., 2005; Gross et al., 2011). Thus, it can be speculated that reduction in phasic DA release during METH administrations would afford neuroprotection.

Interestingly, the nucleus accumbens core, as opposed to the shell, express α6β2 nAChRs and mediate phasic DA release. METH administrations mainly damage phasic DA signaling and the core of the nucleus accumbens, suggesting that α6β2-containing dopaminergic terminals are more vulnerable to METH neurotoxicity than terminals that mediate tonic DA signaling, which do not contain α6β2 nAChRs. In addition, these data suggest that in the absence of phasic DA release, or absence of α6β2 nAChRs, METH would have lower toxicity potential. Interestingly, chronic NIC administration leads to a significant reduction in α6β2 nAChRs and protects against dopaminergic neurotoxins. One hypothesis of this dissertation is that NIC neuroprotection occurs via

downregulation of α6β2 nAChRs.

NIC-induced exchange of α6 for α4 subunits. Previous studies have shown that NIC causes α4β2 nAChR upregulation by inducing higher assembly of β2 subunits with α4 subunits and consequently reducing assembly of β2 subunits with α6 subunits (Kuryatov et al., 2005; Sallette et al., 2005; Colombo et al., 2013). In fact, α6 subunits are highly degraded during chronic NIC treatment, supporting the idea that chronic NIC treatment leads to a switch in subunits assembly. Thus, because α4β2 upregulation occurs at the cost of α6β2 downregulation, chronic NIC exposure reduces α6β2-containing terminals and augments α4β2-containing terminals. In other words, terminals that would normally express α6β2 receptors after chronic NIC exposure express α4β2 instead. Thus, chronic NIC reduction in α6β2-containing terminals and augmentation of α4β2-containing terminals, either individually or combined, potentially attenuate the neurotoxic effects of METH. Of note, studies conducted in cells expressing nAChRs showed that the upregulated receptors retain function (Kuryatov et al., 2005; Sallette et al., 2005; Nashmi et al., 2007).

NIC and cognitive function

Several clinical (Freedman et al., 1994; Adler et al., 1998; Jubelt et al., 2008; Newhouse et al., 2012) and preclinical (Levin and Torry, 1996; Aleisa et al., 2011; Mizoguchi et al., 2011; Gould et al., 2013) studies have indicated that NIC and other agonists of nAChRs have cognitive-enhancing properties and/or cognitive neuroprotective effects. For example, treatment with transdermal NIC (or nAChR agonists) ameliorates different aspects of neurocognition in patients

with schizophrenia (Freedman et al., 1994; Levin et al., 1996; Adler et al., 1998; Olincy et al., 2006; Jubelt et al., 2008). In patients with dementia, long-term treatment with transdermal NIC improved attention, memory, and psychomotor speed (Newhouse et al., 2012). In rhesus monkeys, acute administration of varenicline, an α4β2 nAChR partial agonist, or PNU-282987, an α7 nAChR full agonist, significantly improved working memory in both cocaine-naive and cocaine-treated monkeys (Gould et al., 2013).

NIC mechanisms for cognitive protection. Chronic NIC administration to healthy individuals (Perry et al., 1999) or nonlesioned rodents (Abdulla et al., 1996; Melichercik et al., 2012; Kruk-Slomka et al., 2014) increases nAChRs binding, leading to increases in LTP, a widely accepted process of memory formation (Fujii et al., 1999; Fujii et al., 2000; Welsby et al., 2006, 2009) and improves various types of memory in normal rats (Abdulla et al., 1996; Melichercik et al., 2012; Kruk-Slomka et al., 2014). Additionally, these positive effects of NIC on memory seem to last several days after NIC removal (Abdulla et al., 1996; Levin and Torry, 1996) perhaps due to long-lasting increases in nAChRs and LTP (Abdulla et al., 1996; Yamazaki et al., 2006; Huang et al., 2008). In previous studies, *in vivo* NIC pretreatment prevented LTP deficits in area CA1 of the hippocampus in parallel with attenuation of memory deficits induced by cholinergic lesion, chronic stress, or beta-amyloid infusion (Yamazaki et al., 2002; Alkadhi, 2011; Srivareerat et al., 2011). Furthermore, α4β2 nAChRs have also been correlated with cognitive deficits associated with aging and Alzheimer's disease (Perry et al., 2000). Overall, these data suggest that

reductions in α4β2 nAChRs lead to cognitive deficits, and NIC administration either protects against memory deficits or improves memory by increasing α4β2 nAChRs and LTP.

NIC neuroprotection against METH-induced memory deficits. METH abuse is associated with episodic memory deficits (Scott et al., 2007; Casaletto et al., 2014). In these individuals that abuse METH, both acquisition (or learning) and retrieval (or recall) of memory are impaired. As discussed above, METH reduces LTP in the CA1 (Swant et al., 2010), a hippocampal region important for episodic memory acquisition (Kinnavane et al., 2014). Both α4β2 and α7 nAChR stimulation increase LTP in CA1 (Kroker et al., 2011). Specifically, α4β2 mediates excitatory postsynaptic current (EPSC) in the CA1 region of the hippocampus (Bell et al., 2011).

Some clinical studies have shown the potential benefit of nicotinic drugs in ameliorating memory deficits among METH abusers. Among METH-dependent participants, 5 d oral administration of varenicline (a nicotinic receptor agonist) improved working memory in comparison to placebo-treated participants. Of note, previous work has shown that short-term low-dose administration of varenicline and rivastigmine, two agonists of nAChRs, were not efficacious in improving episodic memory in METH addicts (Kalechstein et al., 2011; Kalechstein et al., 2014). Two possible explanations suggested by the authors were that the dose and/or duration of treatment might have to be increased. Another possible reason for the lack of efficacy might be that the function of nAChRs is reduced in METH addicts. Thus, dose and duration studies as well as

functional studies of nAChRs are needed in order to confirm the utility of nAChR agonists in treating cognitive deficits in METH abusers.

Assessment of episodic memory in rodents. Episodic memory is a type of explicit memory mediated by hippocampal and cortical functions. In rats, episodic memory is commonly assessed by NOR (Kinnavane et al., 2014). NOR is a simple, but powerful, test for assessment of episodic memory in rats and mice that has been used and studied extensively since its description by Ennaceur and Delacour (1988). NOR relies on the instinct of rodents to explore novel objects more than familiar objects. In other words, when rodents are given enough time to spend exploring an object, this same object becomes familiar to them when presented again sometime later. At this later time, it has been observed that rodents would spend more time exploring an object that they have not been presented before over that familiar object. Consequently, when episodic memory function is defective, rodents would not discriminate which object is familiar and which object is novel and hence would explore both objects at similar amounts of time.

Contribution of hippocampal formations and PRh to NOR. Both, the hippocampus and the PRh are important for NOR. Particularly, the PRh is important for every aspect of recognition memory, i.e., acquisition of memory during the familiarization phase to retrieval of memory during recognition testing. The subregions of the hippocampus play different roles in recognition memory. CA1 is important for acquisition of memory during familiarization, whereas CA3 and dentate gyrus seem to participate in the recognition phase when memory

has to be retrieved (Kinnavane et al., 2014). Furthermore, preclinical studies with systemic or local infusion of NIC indicated the important role of α4β2 and α7 subtypes of nAChRs and the hippocampus and PRh in NOR (Melichercik et al., 2012).

Research hypotheses

NIC has shown to be neuroprotective to the dopaminergic and episodic memory system. Several mechanisms have been proposed. NIC has shown to have antioxidant effects, to reduce alpha-synuclein fibrillation, to increase neurotrophic factors, to activate the function of the proteasome system and to enhance LTP in memory-related brain regions. However, the subtype of nAChR that mediates these neuroprotective effects is unknown. Revealing the mechanisms by which NIC protects, including the nAChR subtypes involved, is of clinical importance because selective preventive and/or treatment strategies are necessary considering that NIC *per se* is associated with multiple side effects.

As discussed above, chronic NIC administration leads to upregulation of α4β2 subtype of nAChRs in several brain regions including the striatum, nucleus accumbens core, hippocampus, and PRh. In parallel, chronic NIC administration leads to downregulation of α6β2 nAChRs in the striatum and nucleus accumbens core (this subtype is not expressed in the hippocampus and cortex). The α4β2 and α6β2 nAChRs subtypes mediate tonic and phasic DA release in the striatum and nucleus accumbens core, respectively. In the hippocampus and PRh, α4β2 nAChRs mediate LTP.

The neuroprotection strategies for METH-induced dopaminergic

neurotoxicity proposed and investigated in this dissertation are (1) reduction of cytoplasmic DA in order to decrease ROS formation and (2) reduction in exocytotic DA release whereby postsynaptic DA receptors are activated, which contribute to long-term neurotoxicity. Chronic NIC administration might fulfill these two strategies by (1) augmenting tonic DA release and thus causing reduction of cytoplasmic DA through upregulation of α4β2 nAChRs sites and (2) reducing phasic DA release, thus causing reduction in exocytotic DA release through downregulation of α6β2 nAChRs sites. Of note, DA release *per se* was not investigated in this dissertation work, but rather, the density of α4β2 and α6β2 nAChRs in the striatum and nucleus accumbens core and their association with dopaminergic function in rats treated with METH alone or in combination with NIC was explored. This proposed working hypothesis is presented as a diagram in Figure 1.1.

The neuroprotection strategy for METH-induced episodic memory deficits proposed and investigated in this dissertation is an augmentation of LTP in the hippocampus and PRh. NIC administration might fulfill this strategy by increasing LTP via upregulation α4β2 nAChRs sites. LTP *per se* was not investigated in this dissertation work, but rather the density of α4β2 nAChRs in the hippocampus and PRh and their association with memory function in rats treated with METH alone or in combination with NIC. This proposed working hypothesis is presented as a diagram in Figure 1.2.

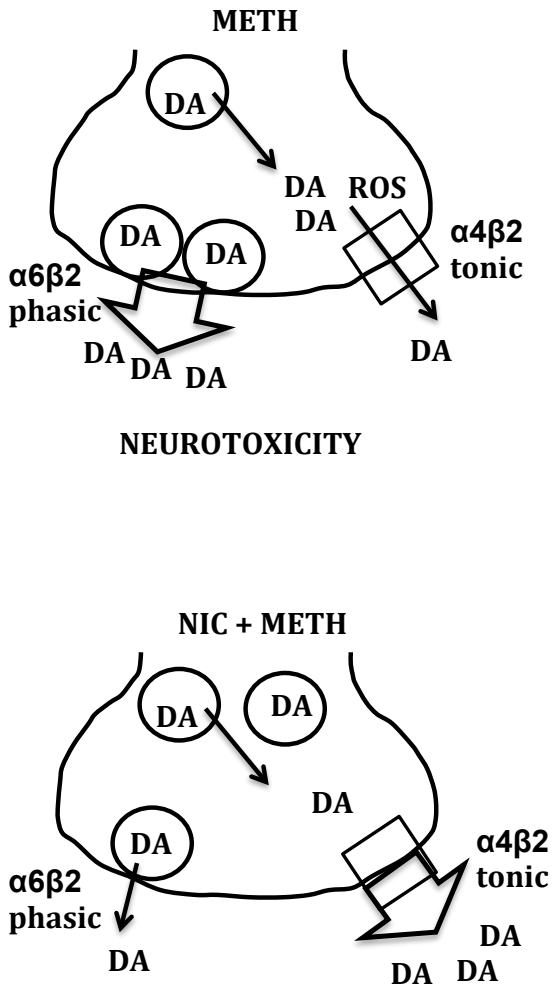


Figure 1.1 Working hypothesis for the mechanism of NIC dopaminergic neuroprotection in the striatum. METH reduces the reserve DA pool and increases the readily releasable DA pool leading to cytosolic DA accumulation and excitotoxicity, respectively. NIC-induced upregulation of α4β2 nicotinic receptors potentially decreases excess of cytosolic DA via augmentation of tonic DA release, and NIC-induced downregulation of α6β2 nicotinic receptors potentially reduces excitotoxicity via reduction of phasic DA release to offset METH effects and afford protection.

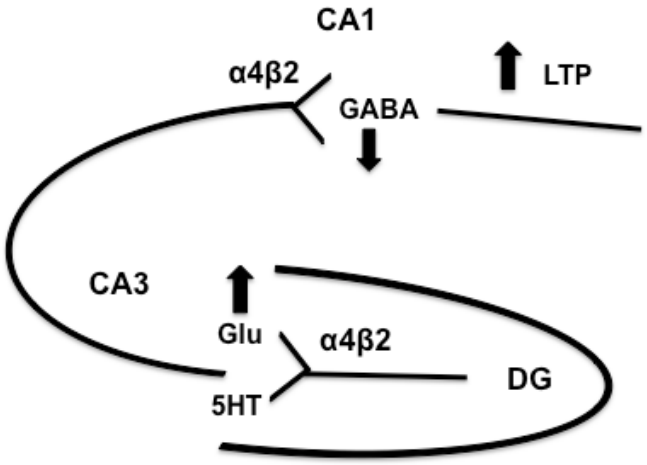


Figure 1.2 Working hypothesis for the mechanism of NIC memory neuroprotection in the hippocampus. NIC-induced upregulation of α4β2 nicotinic receptors in the hippocampus and PRh potentially increases LTP to protect against NOR deficits caused by METH.

CHAPTER 2

CHRONIC NICOTINE ADMINISTRATION ATTENUATES METHAMPHETAMINE-INDUCED DOPAMINERGIC DEFICITS: IMPACT OF AGE OF NICOTINE EXPOSURE

Introduction

Methamphetamine (METH) is a potent psychostimulant abused among adolescents and young adults (Grant et al., 2007; Johnston et al., 2014). Repeated METH administrations in humans (Sekine et al., 2001; Volkow et al., 2001; McCann et al., 2008) and rodents (McFadden, Hadlock, et al., 2012; Kousik et al., 2014) cause long-term impairment of striatal dopaminergic neuronal function resembling some aspects of Parkinson's disease (PD) (McCann et al., 1998; Lotharius and Brundin, 2002; Kish et al., 2008). Recent clinical studies from others and us have shown that abstinent amphetamine/METH abusers have an increased risk for developing PD later in life (Callaghan et al., 2010; Callaghan et al., 2012; Curtin et al., 2014) and although the majority of PD patients have never abused METH, overlapping neuropathologies appear to underlie the degenerative processes involving these two conditions (Granado et al., 2013, for review; Kousik et al., 2014). Preclinical studies indicate that aberrant dopamine (DA) sequestration and release leading

to oxidative stress might be one of the mechanisms that likely contribute to this dopaminergic damage (Fleckenstein et al., 1997; Lotharius and Brundin, 2002; Riddle et al., 2006, for review).

Clinical evidence suggests that PD is less likely to occur among cigarette smokers (Hernan et al., 2001; Hernan et al., 2002; Chen et al., 2010), and preclinical research has indicated that nicotine (NIC) is neuroprotective in the nigrostriatal dopaminergic system (Huang et al., 2009; Garcia-Montes et al., 2012; Quik et al., 2012, for review). Despite the fact that the majority of METH abusers smoke cigarettes (~80%; McCann et al., 2008), and thus self-administer NIC, only a few studies have specifically assessed the potential neuroprotective effect of NIC in the METH model of dopaminergic deficits (Maggio et al., 1998; Ryan et al., 2001). These studies have demonstrated that acute NIC injections are neuroprotective against METH-induced striatal dopaminergic deficits; however, the circumstances under which *in vivo* NIC is neuroprotective in preclinical models (i.e., duration of NIC exposure and age of NIC initiation) warrant further investigation.

In order to provide further insights into the mechanism whereby NIC is neuroprotective, it is important to note that the majority of human adults addicted to cigarettes started smoking during adolescence (Kandel and Logan, 1984; Chen and Kandel, 1995; Breslau and Peterson, 1996). Furthermore, adolescence is a vulnerable time period for drug abuse and associated with significant brain development; thus, NIC-induced neurochemical changes potentially influence several aspects of the adult brain functioning (Spear, 2000;

Barron et al., 2005). For example, epidemiological NIC neuroprotection studies indicated that 68% of the participants who demonstrated protection started smoking before the age of 20 (Chen et al., 2010). These clinical data suggest that cigarette smoking (and thus NIC exposure) starting at a young age may play a role in neuroprotection. However, whether age of NIC initiation is a factor in neuroprotection is unknown.

The present series of studies were aimed to investigate any potential age-related effect on NIC neuroprotection in the METH model of striatal dopaminergic dysfunction. Furthermore, in order to more closely mimic the intermittent and chronic nature of NIC exposure in smoking, NIC was given long-term via drinking water as opposed to acute injections. The data described herein demonstrate that chronic oral NIC administration attenuates the persistent striatal deficits caused by METH, as assessed by evaluating dopamine transporter (DAT) function and expression in rats. In addition, longer NIC administration is necessary for neuroprotection to occur in adults, suggesting that both age of onset and duration of exposure play a role in neuroprotection.

Methods

Animals. Male Sprague-Dawley rats (Charles River Breeding Laboratories, Raleigh, NC) initially weighing 125–150 g (corresponding to postnatal day (PND) 40) or 245–270 g (corresponding to PND 60) (Spear, 2000; Tirelli et al., 2003) were housed 2–3 rats per cage and maintained under a controlled light/dark cycle (14:10 h) and in an ambient environment of 20 °C (with the exception of the 6-h period during which METH or saline vehicle was

administered during which the ambient environment was maintained at 24 °C). Food and water were available *ad libitum*. During METH or saline administrations, core body (rectal) temperatures were measured using a digital thermometer (Physitemp Instruments, Clifton, NJ) every 1 h beginning 30 min before the first saline or METH administration and continuing until 30 min after the final saline or METH administration. Rats were placed on a cooler environment if their body temperature exceeded 40.5 °C and returned to their home cage once their body temperature dropped to 40 °C. All experiments were approved by the University of Utah Institutional Animal Care and Use Committee, in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals 8th Edition* (Institute of Laboratory Animal Resources, 2011).

Drug treatments. METH hydrochloride was provided by the National Institute on Drug Abuse (Research Triangle Institute; Research Triangle Park, NC) and administered at 4 x 7.5 mg/kg/s.c, 2-h intervals calculated as free-base. (-) NIC (1.010 g/ml; Sigma-Aldrich Co. LLC) was administered *ad libitum* p.o. at concentrations of 10, 20, 50, or 75 µg/ml via the water bottles as delineated in Figure 2.1. To increase palatability, 1% saccharin (Sweet & Low, Cumberland Packing Corp., NY) was added to the animals' drinking water only in experiments in which the initial NIC concentration was 75 µg/ml (i.e., experiments delineated in Figure 2.1B, 2.1C, and 2.1D), or during the highest escalating rate (i.e., experiment delineated in Figure 2.1E). In our current studies, NIC water consumption was ~30 ml/rat/day, tap water consumption was ~45 ml/rat/day, and

saccharin water consumption was ~60 ml/rat/day, similarly to previous reports (Bordia et al., 2008). These NIC doses in rats yield plasma concentrations similar to plasma NIC and cotinine concentrations typically found in human smokers (10–50 ng/ml for NIC and 300 ng/ml for cotinine) (Benowitz, 1994; Matta et al., 2007).

Tissue preparation. Rats were decapitated 7 d after METH treatment. Both striata were dissected out on ice, placed in cold sucrose buffer (0.32 M sucrose, 3.8 mM NaH_2PO_4, and 12.7 mM Na_2HPO_4) and used for [^{3}H]DA uptake and western blotting as described below. Brains of experiments PND 40–61 and PND 61–100 were hemisected and the right contralateral tissues were analyzed as described in Chapter 3. Hippocampal and perirhinal cortex tissues were also analyzed and data were reported in Chapter 5.

[^{3}H]DA uptake assay. Striatal synaptosomes were prepared as previously described (Hanson et al., 2009). Following decapitation, the striatum was quickly dissected out and homogenized in ice-cold sucrose buffer (0.32 M sucrose, 3.8 mM NaH_2PO_4, and 12.7 mM Na_2HPO_4). [^{3}H]DA uptake assays were conducted according to Hanson et al. (2009). For plasmalemmal uptake of [^{3}H]DA, striatal synaptosomes were prepared accordingly and resuspended in ice-cold Krebs' buffer (126 nM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 16 mM sodium phosphate, 1.4 mM $MgSO_4$, 11 mM dextrose, 1 mM ascorbic acid, pH 7.4). Assay tubes containing 1.5 mg striatal tissue and 1 μM pargyline were incubated for 3 min at 37 °C with [7,8-^{3}H]DA (0.5 nM final concentration, Perkin Elmer, Boston, MA). Nonspecific values were ascertained in the presence of 10 μM cocaine. Samples

were filtered using a filtering manifold (Brandel, Inc Gaithersburg, MD) through Whatman GF/B filters (Whatman International LTD, Maidstone, England) soaked previously in 0.05% polyethylenimine and washed three times with 3 ml of ice-cold 0.32 M sucrose. Protein concentration was used for normalization and determined by the Bradford Protein Assay.

DAT western blotting. Western blotting was conducted according to our previous method (Hadlock et al., 2009). Equal quantities of protein (8 µg) were loaded into each well of a 4 to 12% NuPAGE Novex Bis-Tris Midi gradient gel (Invitrogen, Carlsbad, CA) and electrophoresed by using a XCell4 Surelock Midi-Cell (Invitrogen). Membranes were blocked for 30 min with Starting Block Blocking Buffer (Thermo Fisher Scientific, Waltham, MA) and incubated for 1 h at room temperature with a DAT antibody (a generous gift from Dr. Roxanne Vaughan, University of North Dakota, Grand Forks, ND). The polyvinylidene difluoride membrane was then washed five times in Tris-buffered saline with Tween (250 mM NaCl, 50 mM Tris, pH 7.4, and 0.05% Tween 20). The membranes were then incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody (BioSource International, Camarillo, CA). After five washes in Tris-buffered saline with Tween, the bands were visualized by using Western Lightning Chemiluminescence Reagents Plus (PerkinElmer Life and Analytical Sciences, Waltham, MA) and quantified by densitometry using a FluorChem SP Imaging System (Alpha Innotech, San Leandro, CA). Protein concentrations were determined by using the Bradford Protein Assay.

Brain METH and amphetamine concentrations. Brain METH and its

metabolite, amphetamine, concentrations were measured by liquid chromatography-tandem mass spectrometry as described previously (Truong et al., 2005). The whole brains (except for the striatum) were weighed and homogenized separately in 10 ml of water. A VibraCell homogenizer (Sonics, Newton, CT) was used for the homogenization. A 0.5-ml volume of the homogenate was used for the analysis. An Agilent liquid chromatograph (Agilent Technologies, Santa Clara, CA) coupled to a ThermoQuest Finnigan TSQ 7000 tandem mass spectrometer (Thermo Fisher Scientific) was used for the analysis. Electrospray ionization was used. The lower limit of quantification (LOQ) was 1 ng/ml in the homogenates.

Data analyses. Statistical analyses were conducted using GraphPad Prism 5.01 software (La Jolla, CA). Mean concentrations $\pm$ standard error of the mean (SEM) were analyzed using one-way analysis of variance followed by Newman-Keuls post hoc test. Differences among groups were considered significant if the probability of error was less than 5% ($p < 0.05$). Sample sizes are indicated in figure legends.

Results

Figure 2.1 depicts the experimental design of the studies presented herein. In panel A, rats received NIC for a total of 56 d throughout the entire experiment from adolescence (PND 40) to adulthood (PND 96) and METH administration 7 d before the end of the experiment at PND 89. In order to investigate whether the effects of NIC on METH-induced dopaminergic deficits remain with shorter NIC administration, in panel B NIC administration was

shortened to 21 d beginning in adolescence. In panel C, rats also received NIC for 21 d beginning in adolescence, but METH administrations were given only after 21 d that NIC was stopped (i.e., during adulthood) to evaluate how long NIC effects last. Next, in panel D, rats received NIC for 21 d, but beginning in young adulthood (PND 61). Lastly, in panel E, we extended NIC administration to 39 d beginning at PND 61 to further investigated whether the effects of NIC on METH-induced dopaminergic deficits are solely dependent on the age of NIC initiation, or if they are also dependent the length of NIC administration.

Results presented in Figure 2.2 demonstrate that *ad libitum* exposure to an escalating-dose regimen of NIC (10–75 µg/ml; p.o.; see Methods and Figure 2.1, panel A for details) from PND 40–PND 96 attenuated the persistent (e.g., 7-d) METH-induced decrease in striatal DAT [^{3}H]DA uptake ($F_{3,30} = 39.61$, $p < 0.05$) and immunoreactivity ($F_{3,30} = 38.13$, $p < 0.05$) when METH (4 x 7.5 mg/kg/injection, s.c.) was administered on PND 89 (panels A and B). In other words, 56 d of NIC exposure via drinking water afforded protection when exposure was initiated on PND 40. This NIC regimen did not attenuate METH-induced hyperthermia (panel C).

Results presented in Figure 2.3 demonstrate that *ad libitum* exposure of NIC (75 µg/ml; p.o.; see Methods and Figure 2.1, panel B for details) from PND 40–PND 61 attenuated the persistent (e.g., 7-d) METH-induced decrease in [^{3}H]DA uptake when METH (4 x 7.5 mg/kg/injection, s.c.) was administered on PND 54 ($F_{3,31} = 18.81$, $p < 0.05$). In other words, 21 d of NIC exposure afforded protection when, as was accomplished for Figure 2.2, exposure was initiated on

PND 40. This NIC regimen did not attenuate METH-induced hyperthermia (data not shown).

It is noteworthy that the protection afforded by 21 d NIC exposure (i.e., Figure 2.3) does not persist when NIC exposure is initiated on PND 40, but terminated on PND 61 (Figure 2.4). In particular, this NIC exposure regimen (75 µg/ml; p.o.; see Methods and Figure 2.1 panel C for details) did not attenuate the METH (4 x 7.5 mg/kg/injection, s.c.)-induced decrease in [^{3}H]DA uptake when METH is subsequently administered on PND 82, and as assessed 7 d later on PND 89 ($F_{3,34}$ = 43.52, ns). This NIC regimen did not attenuate METH-induced hyperthermia (data not shown).

In contrast to Figure 2.3, 21 d of NIC exposure was not sufficient to attenuate the persistent (7-d) METH-induced decrease in striatal [^{3}H]DA uptake when NIC exposure was initiated on PND 61 (Figure 2.5). In particular, *ad libitum* exposure of NIC (75 µg/ml; p.o.; see Methods and Figure 2.1 panel D for details) from PND 61–PND 82 did not attenuate the persistent (e.g., 7-d) METH-induced decrease in [^{3}H]DA uptake when METH (4 x 7.5 mg/kg/injection, s.c.) was administered on PND 75 ($F_{3,21}$ = 25.79, ns). This NIC regimen did not attenuate METH-induced hyperthermia (data not shown).

Results presented in Figure 2.6 demonstrate that *ad libitum* exposure to an escalating-dose regimen of NIC (10–75 µg/ml; p.o.; see Methods and Figure 2.1 panel E for details) from PND 61–PND 100 attenuated the persistent (e.g., 7-d) METH-induced decrease in striatal [^{3}H]DA uptake ($F_{3,34}$ = 13.12, $p < 0.05$) when METH (4 x 7.5 mg/kg/injection, s.c.) was administered on PND 93. In other

words, 39 d of NIC exposure afforded protection when exposure was initiated on PND 61. This NIC regimen did not attenuate METH-induced hyperthermia (data not shown).

Finally, we assessed the concentration of METH and metabolite in the brain of METH-treated rats chronically pretreated with oral NIC in order to investigate whether NIC neuroprotection in the METH model occurs because of changes in the pharmacokinetics of METH. PND 40 rats received increasing concentrations of NIC via drinking water (10–75 μg/ml) for 49 d. METH or saline administrations occurred at PND 89 followed by a 1-h sacrifice. METH and the metabolite amphetamine concentrations were not statistically different between METH-treated rats pre-exposed to tap water or NIC water (9.7 $\pm$ 0.6 ng/mg and 8.4 $\pm$ 0.8 ng/mg, respectively, p = 0.23). METH and amphetamine were not detected in the saline-treated rats pre-exposed to tap water or NIC water (<LOQ).

Discussion

These current studies demonstrate that long-term (i.e., 56 d) oral NIC administration to rats, beginning during a time period corresponding to adolescence (Spear, 2000; Tirelli et al., 2003), attenuates METH-induced persistent striatal dopaminergic deficits when assessed during adulthood. NIC also affords striatal dopaminergic neuroprotection when short-term oral NIC (i.e., 21 d) is initiated during adolescence. In contrast, short-term oral NIC administration (i.e., 21 d) commenced in young adulthood does not attenuate METH-induced striatal dopaminergic deficits. However, NIC is neuroprotective when administered for 39 d during adulthood. These data indicate that both age

of NIC initiation and duration of NIC exposure play a role in the neuroprotective effect of NIC to striatal dopaminergic deficits caused by METH.

The current studies showed that 21 d, 39 d, or 56 d oral NIC administration *per se* did not induce changes in striatal DAT function and/or expression when assessed during adulthood. These data are consistent with previous findings demonstrating that chronic NIC administration via drinking water beginning in adolescence did not affect striatal DAT expression when assessed in adulthood (Huang et al., 2009). Similarly, 7 d of NIC via osmotic minipumps had no effect on striatal DAT function and expression in adult rats (Izenwasser and Cox, 1992; Collins et al., 2004).

Consistent with similar NIC neuroprotection studies utilizing dopaminergic neurotoxins other than METH, current data demonstrate that chronic oral NIC administration is neuroprotective against striatal dopaminergic deficits caused by a high-dose METH regimen. For example, previous studies have shown that NIC administration via drinking water at an escalating dose of 12.5 μg/ml to 50 μg/ml for 7 weeks to rats beginning in adolescence attenuates declines in striatal DAT densities caused by 6-hydroxy-DA lesion (Huang et al., 2009). In a different study, 6 weeks NIC administration via drinking water to mice attenuated paraquat-induced striatal DAT densities deficits when NIC was initiated during adulthood (Khwaja et al., 2007).

It is well established that attenuation of METH-induced hyperthermia protects the persistent dopaminergic deficits caused by the stimulant. For example, exposure of animals to a low ambient temperature attenuates METH-

induced hyperthermia and neurotoxicity (Bowyer et al., 1994; Ali et al., 1995). Prevention of METH-induced hyperthermia attenuates reactive species formation as well (Fleckenstein et al., 1997). Furthermore, selective inhibition of dopaminergic receptors by various agents also attenuates METH-induced hyperthermia and affords dopaminergic neuroprotection (Sonsalla et al., 1986). However, dopaminergic receptor antagonism fails to afford neuroprotection when animals receive METH in a high-temperature environment, indicating that protection is lost when the degree of dopaminergic damage is high (Broening et al., 2005). Current data indicate that METH administrations induce significant hyperthermia in animals pretreated with tap water or NIC water (Figure 2.2C). Furthermore, METH-induced hyperthermia was not different between rats pretreated with tap water (SM) and NIC water (NM), thus indicating that the NIC neuroprotection is not mediated by attenuation in METH-induced hyperthermia.

One possible explanation for NIC neuroprotection involves fundamental alterations in the nicotinic acetylcholine receptor (nAChR) system. The hypothesis is based on the findings that chronic NIC administration alters the expression and function of striatal nAChRs (Perez et al., 2008; Huang et al., 2009). Furthermore, NIC-induced changes in nAChRs differ between adolescent and adult rats; particularly, upregulation of the α4β2 subtypes and downregulation of the α6β2 subtypes of nAChRs are more robust in adolescent rats in comparison to adult rats (Doura et al., 2008). Assuming that these alterations in nAChRs contribute to protection, then the protection observed in current studies would be affected by age and could explain the shorter NIC

exposure necessary for neuroprotection to occur in adolescent versus adult rats. In other words, higher expression of α4β2 nAChR and/or lower expression of α6β2 nAChR potentially attenuate METH-induced neurotoxicity and in adolescents, NIC induction of this optimal balance is achieved sooner. Alterations in the nAChR system could also explain the absence of NIC neuroprotection when NIC was removed 3 weeks prior to METH administration as seen in the present study shown in Figure 2.4. For instance, studies by Pietila et al. (1998) demonstrated that in mice exposed to NIC via drinking water for 4 or 7 weeks, NIC-induced upregulation of nAChRs in the midbrain remained for 48–72 h, but it returned to baseline levels after 7 d of NIC removal. These findings suggest that if NIC-induced alterations in nAChRs play a role in neuroprotection, then the lack of change of nAChRs contribute to the lack of neuroprotection.

In summary, the present results demonstrate that long-term NIC administration, given to rats as to mimic cigarette smoking (Matta et al., 2007), attenuates the striatal dopaminergic deficits caused by METH. The relevance of these findings is at least three fold. First, since most METH abusers are exposed to NIC via cigarette smoking (~80%; McCann et al., 2008), preclinical studies such as these are clinically relevant. Secondly, the fact that chronic NIC administration attenuated the dopaminergic deficits caused by METH suggest that PD risk among METH abusers would be greater if most METH abusers were not cigarette smokers. These findings also suggest that METH abusers that are nonsmokers have higher indices of dopaminergic deficits than those who are cigarette smokers. Lastly, current data provide insights into the mechanism by

which NIC is neuroprotective to the dopamine system. Specifically, our findings indicate that age of NIC initiation and length of NIC exposure both play a role in this neuroprotection and that these neuroprotective effects go away after three weeks of NIC removal.

Figure 2.1 Experimental designs. A. Experiment A: rats received tap water or NIC water (10–75 µg/ml) from PND 40 to 96 and METH (4 x 7.5 mg/kg/injection, s.c., 2h-apart) or saline (1ml/kg/injection) at PND 89. B. Experiment B: rats received tap water containing 1% saccharin or NIC water (at 75 µg/ml) containing 1% saccharin from PND 40 to 61 and METH (4 x 7.5 mg/kg/injection, s.c., 2h-apart) or saline (1ml/kg/injection) at PND 54. C. Experiment C: rats received tap water containing 1% saccharin or NIC water (at 75 µg/ml) containing 1% saccharin from PND 40 to 61 and METH (4 x 7.5 mg/kg/injection, s.c., 2h-apart) or saline (1ml/kg/injection) at PND 82. D. Experiment D: rats received tap water containing 1% saccharin or NIC water (at 75 µg/ml) containing 1% saccharin from PND 61 to 82 and METH (4 x 7.5 mg/kg/injection, s.c., 2h-apart) or saline (1ml/kg/injection) at PND 75. E. Experiment E: rats received tap water containing 1% saccharin or NIC water (10–75 µg/ml) containing 1% saccharin from PND 61 to 100 and METH (4 x 7.5 mg/kg/injection, s.c., 2h-apart) or saline (1ml/kg/injection) at PND 93.

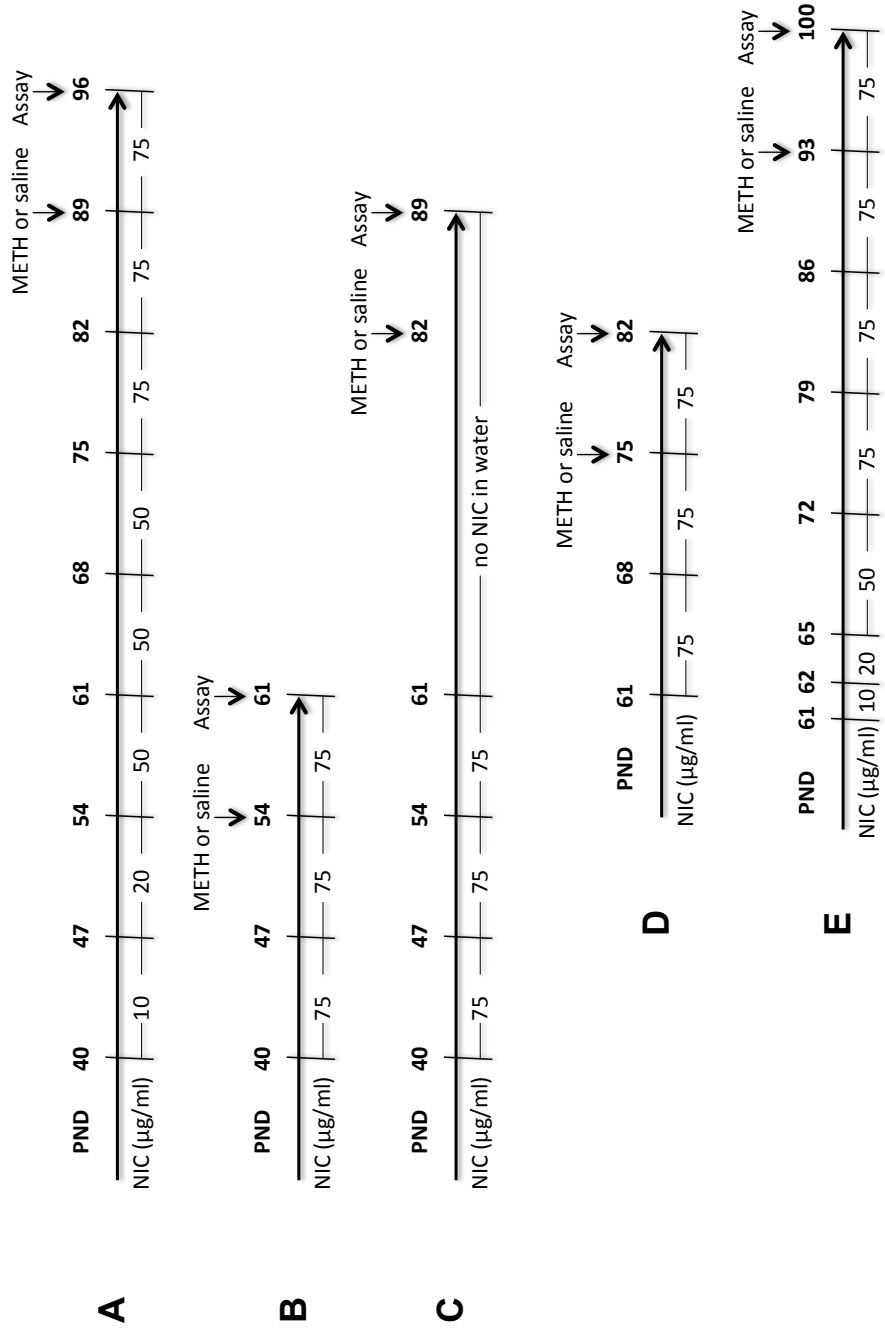
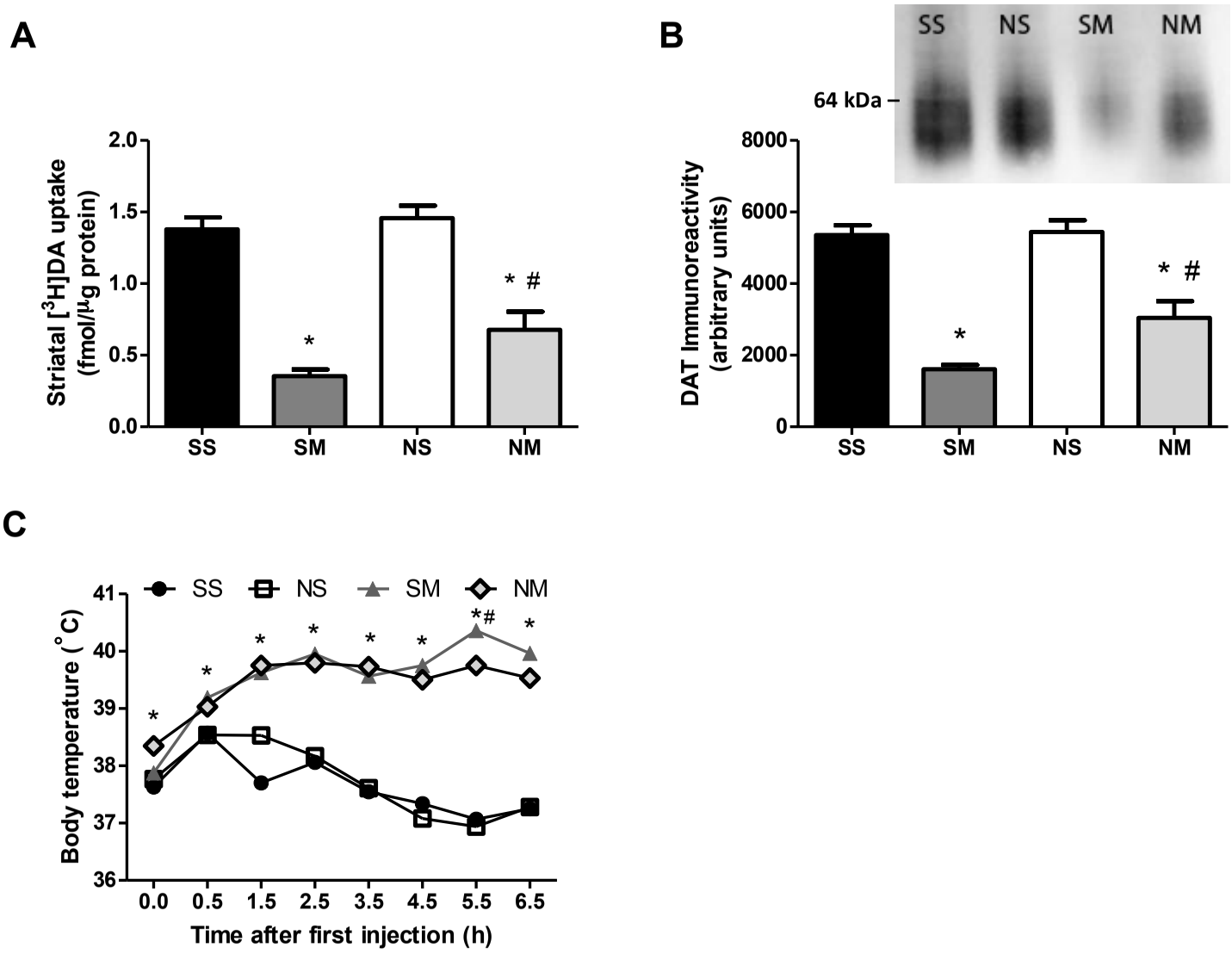

A
PND 40 47 54 61 68 75 82 89 96
NIC (µg/ml) 10 20 50 50 50 75 75 75
METH or saline
Assay
B
PND 40 47 54 61
NIC (µg/ml) 75 75 75
METH or saline
Assay
C
PND 40 47 54 61 82 89
NIC (µg/ml) 75 75 75 no NIC in water
METH or saline
Assay
D
PND 61 68 75 82
NIC (µg/ml) 75 75 75
METH or saline
Assay
E
PND 61 62 65 72 79 86 93 100
NIC (µg/ml) 10 20 50 75 75 75 75
METH or saline
Assay

Figure 2.2 Chronic NIC administration attenuates METH-induced deficits in striatal DAT function and expression with no change in METH-induced hyperthermia. This is experiment A described in Figure 2.1A. Data are expressed as mean values $\pm$ S.E.M. of n = 6–10 determinations. *Values that are significantly different from saline control. #Values that are significantly different from SM ($p < 0.05$). Legend: SS = tap water/saline injections; NS = NIC water/saline injections; SM = tap water/METH injections; NM = NIC water/METH injections.


A
Striatal [3H]DA uptake (fmol/µg protein)
2.0
1.5
1.0
0.5
0.0
SS
SM
NS
NM
* #
B
SS
NS
SM
NM
64 kDa –
DAT Immunoreactivity (arbitrary units)
8000
6000
4000
2000
0
C
SS
NS
SM
NM
Body temperature (°C)
41
40
39
38
37
36
0.0
0.5
1.5
2.5
3.5
4.5
5.5
6.5
Time after first injection (h)

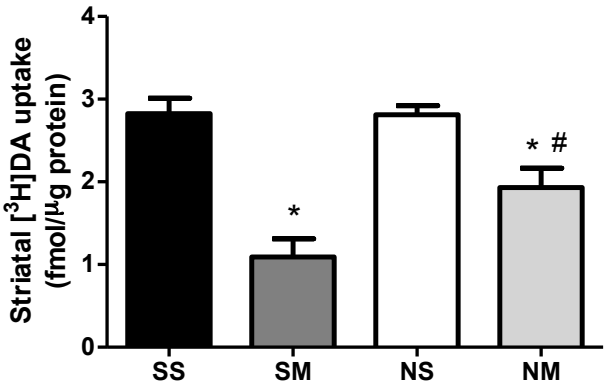


Figure 2.3 Short-term (i.e., 21 d) NIC administration starting in adolescence attenuates METH-induced deficits in striatal DAT function. This is experiment B described in Figure 2.1B. Data are expressed as mean values ± S.E.M. of n = 8–10 determinations. *Values that are significantly different from saline control. #Values that are significantly different from SM ($p < 0.05$). Legend: SS = tap water/saline injections; NS = NIC water/saline injections; SM = tap water/METH injections; NM = NIC water/METH injections.

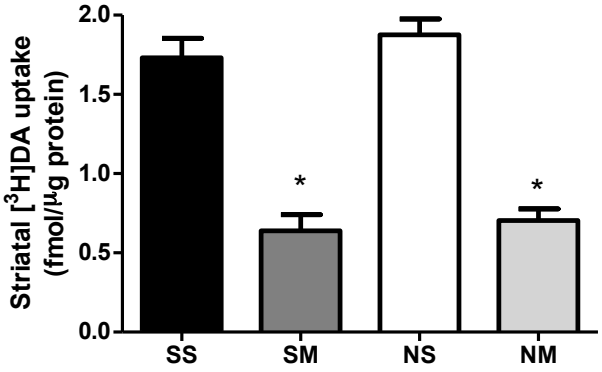


Figure 2.4 NIC neuroprotective effects on METH-induced deficits in striatal DAT function do not persist for 4 weeks. This is experiment C described in Figure 2.1C. Data are expressed as mean values ± S.E.M. of n = 8–11 determinations. *Values that are significantly different from saline control. Legend: SS = tap water/saline injections; NS = NIC water/saline injections; SM = tap water/METH injections; NM = NIC water/METH injections.

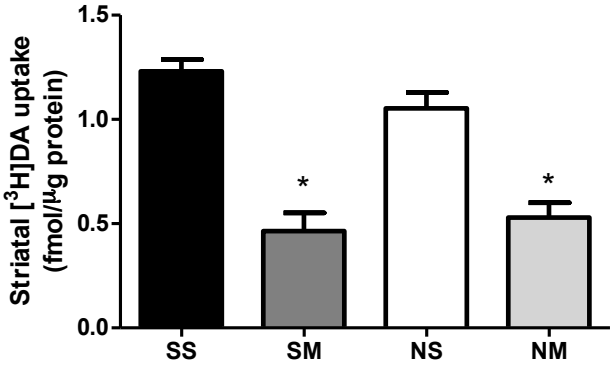


Figure 2.5 Short-term (i.e., 21 d) NIC administration starting in adulthood does not attenuate METH-induced deficits in striatal DAT function. This is experiment D described in Figure 2.1D. Data are expressed as mean values ± S.E.M. of n = 6–7 determinations. *Values that are significantly different from saline control. Legend: SS = tap water/saline injections; NS = NIC water/saline injections; SM = tap water/METH injections; NM = NIC water/METH injections.

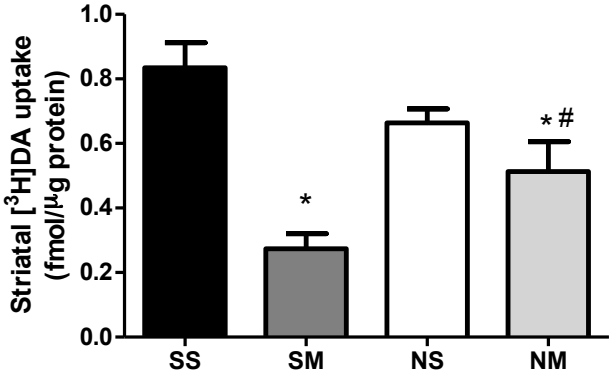


Figure 2.6 Long-term (i.e., 39 d) NIC administration starting in adulthood attenuates METH-induced deficits in striatal DAT function. This is experiment E described in Figure 2.1E. Data are expressed as mean values ± S.E.M. of n = 9–10 determinations. *Values that are significantly different from saline control. #Values that are significantly different from SM ($p < 0.05$). Legend: SS = tap water/saline injections; NS = NIC water/saline injections; SM = tap water/METH injections; NM = NIC water/METH injections.

CHAPTER 3

EFFECTS OF CHRONIC NICOTINE ADMINISTRATION ON METHAMPHETAMINE-INDUCED DOPAMINERGIC DEFICITS: ROLE OF α4β2 AND α6β2 NICOTINIC RECEPTOR SUBTYPES

Introduction

Repeated methamphetamine (METH) administrations in humans and laboratory animals cause long-term dopaminergic deficits in the striatum and nucleus accumbens and psychomotor impairment resembling some aspects of Parkinson's disease (PD) (Broening et al., 1997; McCann et al., 1998; Sekine et al., 2001; Volkow et al., 2001; Kousik et al., 2014). Recent clinical studies from others and us have indicated that METH/amphetamine abusers have a higher risk for developing PD (Callaghan et al., 2010; Callaghan et al., 2012; Curtin et al., 2014), and preclinical studies have shown similarities between these two neuropathologies, including reductions in tyrosine-hydroxylase and dopamine transporter (DAT) neurons in the striatum and nucleus accumbens core (Kousik et al., 2014). Furthermore, preclinical studies suggest that aberrant dopamine (DA) accumulation and release likely contribute to METH-induced dopaminergic deficits (Broening et al., 1997; Fleckenstein et al., 2007, for review).

Clinical and preclinical studies have demonstrated a neuroprotective effect of nicotine (NIC) on dopaminergic neurons in PD models (Ryan et al., 2001; Khwaja et al., 2007; Villafane et al., 2007; Huang et al., 2009; Takeuchi et al., 2009). For example, Vieira-Brock et al. (our companion manuscript) found that chronic NIC administration attenuates the persistent dopaminergic deficits caused by high-dose METH treatment as assessed by evaluating DAT function and immunoreactivity. Previous studies have suggested that α4β2 and α6β2 subtypes of nicotinic acetylcholine receptors (nAChRs) mediate this protection although other nicotinic subunits might also contribute (Ryan et al., 2001; Khwaja et al., 2007; Takeuchi et al., 2009; Quik et al., 2011). For example, the neuroprotective effect of NIC in rotenone-treated mice was inhibited when an α4β2 antagonist was administered (Takeuchi et al., 2009). Furthermore, the neuroprotective effect of chronic NIC was lost in α4-knockout mice lesioned with 6-hydroxy-DA, suggesting that α4β2 nAChRs might be crucial for protection (Ryan et al., 2001). However, other studies demonstrated that α6β2 nAChR binding is significantly increased in α4-knockout mice, suggesting that the loss of neuroprotection in α4-knockout mice could have occurred because of the increase in α6β2, as opposed to the loss of α4β2 nAChR sites (Perez et al., 2008). Similarly, others have suggested that NIC-induced reduction in α6β2 nAChRs mediate neuroprotection against paraquat-induced dopaminergic neurotoxicity (Khwaja et al., 2007). Overall, these studies suggest that α4β2 and/or α6β2 nAChRs mediate the neuroprotective effect of NIC to dopaminergic neurons.

The α4β2 and α6β2 nAChR subtypes are highly expressed on dopaminergic projections and regulate DA release in the striatum and nucleus accumbens (Champtiaux et al., 2002; Perez et al., 2012; Marks et al., 2014). Of relevance, chronic NIC administration increases the density of α4β2 nAChRs in human smokers (Benwell et al., 1988) and in experimental animal models (Marks et al., 1992; McCallum et al., 2006; Perez et al., 2008). In animal models, this upregulation is accompanied by an increase in electrically stimulated tonic DA release (Buisson and Bertrand, 2002, for review; Meyer et al., 2008; Perez et al., 2008). On the other hand, α6β2 nAChR expression is downregulated after chronic NIC administration (Lai et al., 2005; Perez et al., 2008), and this correlates with significant declines in phasic DA release in the striatum and nucleus accumbens (Lai et al., 2005; Perez et al., 2012; Exley et al., 2013). Given the role of aberrant DA accumulation and release in METH-induced dopaminergic deficits, the purpose of the present study was to investigate the potential role of these nAChR subtypes in the neuroprotection afforded by chronic NIC. The effect of age of onset of NIC exposure was also evaluated as it impacts the expression of nAChRs (Doura et al., 2008). Results revealed that chronic NIC exposure, independently of age of onset, differentially affects α4β2 and α6β2 nAChRs in METH-treated rats. Further, we speculate that increased α4β2 and/or decreased α6β2 nAChR levels may contribute to the protection against METH-induced dopaminergic deficits afforded by chronic NIC exposure.

Methods

Animals. Male Sprague-Dawley rats (Charles River Breeding Laboratories, Raleigh, NC) initially weighing 125–150 g (corresponding to postnatal day (PND) 40) or 245–270 g (corresponding to PND 60) (Spear, 2000; Tirelli et al., 2003) were housed 2–3 rats per cage and maintained under a controlled light/dark cycle (14:10 h) and in an ambient environment of 20 °C (with the exception of the 6-h period during which METH or saline vehicle was administered during which the ambient environment was maintained at 24 °C). During METH or saline administrations, core body (rectal) temperatures were measured using a digital thermometer (Physitemp Instruments, Clifton, NJ) every 1 h beginning 30 min before the first saline or METH administration and continuing until 30 min after the final saline or METH administration. Rats were placed on a cooler environment if their body temperature exceeded 40.5 °C and returned to their home cage once their body temperature dropped to 40 °C. Food and water were available *ad libitum*. All experiments were approved by the University of Utah Institutional Animal Care and Use Committee, in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals 8th Edition* (Institute of Laboratory Animal Resources, 2011).

Drug treatments. METH hydrochloride was provided by the National Institute on Drug Abuse (Research Triangle Institute; Research Triangle Park, NC) and administered at 4 x 7.5 mg/kg/s.c, 2-h intervals calculated as free-base. (-) NIC (1.010 g/ml; Sigma-Aldrich Co. LLC) was administered *ad libitum* p.o. at concentrations of 10, 20, 50, or 75 µg/ml via the water bottles. These dosing

protocols are delineated in Figure 2.1 of Chapter 2 (panels A, B, and E), and the same rats were utilized to generate the data presented in the current chapter. Briefly, for experiments in which NIC was given from PND 40–96, NIC concentrations were the following: 10 μg/ml from PND 40 to 46, 20 μg/ml from PND 47–53, 50 μg/ml from PND 54–74, and 75 μg/ml from PND 75–96. For experiments in which NIC was given from PND 40–61, NIC was given at 75 μg/ml for the entire time. For the experiment in which NIC was given from PND 61–100, NIC concentrations were the following: 10 μg/ml on PND 61, 20 μg/ml from PND 62–64, 50 μg/ml from PND 65–71 and 75 μg/ml from PND 72–100. To increase palatability, saccharin (Sweet & Low; Cumberland Packing Corporation, New York, 1%) was added to the animals' drinking water only in experiments in which the initial NIC concentration was 75 μg/ml, or during the highest escalating rate. These NIC doses in rats yield plasma concentrations similar to plasma NIC and cotinine concentrations typically found in human smokers (10–50 ng/ml for NIC and 300 ng/ml for cotinine) (Benowitz, 1994; Matta et al., 2007).

Tissue preparation. Rats were decapitated 7 d after METH treatment. Brains were hemisected, and the right side rapidly removed and frozen in isopentane on dry ice and stored at -80 °C. Frozen right hemisected brains were sliced at 12 μm thick at the level of the anterior striatum and nucleus accumbens (1.5 mm from bregma, Paxinos and Watson 6th edition) using a cryostat. Eight slices (four per rat) were mounted on each Superfrost® Plus glass micro slides (VWR International, Radnor, PA) and stored at -80 °C for subsequent use in autoradiography assays. The contralateral tissues of experiments PND 40–61

and PND 61–100 were analyzed as described in Chapter 2. Experiment PND 40–96 was a separate experiment from the one described in Chapter 2. Hippocampal and perirhinal cortex tissues were also analyzed and data reported in Chapter 5.

^{125}I-RTI-55 autoradiography. DAT density was used as a marker of dopaminergic integrity and assessed via ^{125}I-RTI-55 binding to striatal and nucleus accumbens core slices as previously described (O'Dell et al., 2012). Briefly, slides were thawed on a slide warmer (5–10 min) and preincubated in buffer-sucrose (10 mM sodium phosphate, 120 mM sodium chloride, 320 mM sucrose, pH 7.4) containing 100 nM fluoxetine at room temperature for 5 min, followed by a 2-h incubation in buffer-sucrose containing 25 pM ^{125}I-RTI-55 (2200 Ci/mmol, PerkinElmer, Watham, MA). Nonspecific binding was determined by slides incubated in buffer-sucrose containing 25 pM ^{125}I-RTI-55 and 100 nM fluoxetine plus 100 μM nomifensine. Slides were rinsed twice in ice-cold buffer and distilled water for 2 min and air-dried. Sample slides and standard ^{125}I microscale slides (American Radiolabeled Chemicals, St. Louis, MO) were placed on one cassette and exposed to the same Kodak MR film (Eastman Kodak Co., Rochester, NY, USA) for 24 h to keep variables constant.

^{125}I-epibatidine autoradiography. α4β2 nAChR density was assessed via ^{125}I-epibatidine binding to striatal and nucleus accumbens core slices as previously described (Lai et al., 2005; Huang et al., 2009). Briefly, slides were thawed on a slide warmer (5–10 min) and preincubated in binding buffer (50 mM Tris, 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, pH 7.5) plus 100

nM αCtxMII at room temperature for 30 min. The nonradiolabeled αCtxMII was used to inhibit epibatidine binding to α6β2 nAChR, followed by a 40-min incubation in binding buffer containing 0.015 nM ^{125}I-epibatidine (2200 Ci/mmol, PerkinElmer, Watham, MA) in the presence of 100 nM αCtxMII. Nonspecific binding was determined by slides incubated in binding buffer containing 0.015 nM ^{125}I-epibatidine plus 0.1 mM nicotine. Slides were rinsed twice in ice-cold buffer for 5 min followed by a 10 s rinse in distilled water. Slides were air-dried. Sample slides and standard ^{125}I microscale slides (American Radiolabeled Chemicals, St. Louis, MO) were placed on one cassette and exposed to the same Kodak MR film (Eastman Kodak Co., Rochester, NY, USA) for 24 h to keep variables constant.

^{125}I-αConotoxinMII (αCtxMII) autoradiography. α6β2 nAChR density was assessed via ^{125}I-αCtxMII binding to striatal and nucleus accumbens core slices as previously described (Lai et al., 2005; Huang et al., 2009). Briefly, slides were thawed on a slide warmer (5–10 min) and preincubated in buffer A (pH 7.5, 20 nM HEPES, 144 mM NaCl, 1.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 0.1% BSA, and 1 mM phenylmethylsulfonyl fluoride) at room temperature for 2 x 15 min, followed by a 1-h incubation in buffer B (pH 7.5, 20 nM HEPES, 144 mM NaCl, 1.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 0.2% BSA, 5 mM EDTA, 5 mM EGTA, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin A) containing 0.5 nM ^{125}I-αCtxMII (~2200 Ci/mmol that was synthesized as previously described) (Whiteaker et al., 2000). Nonspecific binding was determined by slides incubated in 0.5 nM ^{125}I-αCtxMII buffer B also containing 0.1 mM nicotine (Sigma-Aldrich,

St. Louis, MO). Slides were rinsed in room temperature buffer A for 10 min, then in ice-cold buffer A for another 10 min, followed by 2 x 10 min in 0.1x ice-cold buffer A, and finally in 4º C distilled water for 2 x 10 s. Slides were air-dried. Sample slides and standard ^{125}I microscale slides (American Radiolabeled Chemicals, St. Louis, MO) were placed on one cassette and exposed to same Kodak MR film (Eastman Kodak Co., Rochester, NY, USA) for 4 d to keep variables constant.

Data quantitation and statistical analyses. Optical densities from four replicate slices per rat were quantified using ImageJ software (National Institutes of Health, USA). Specific binding was obtained by subtracting film background from mean density values and converted to fmol/mg using the standard curve generated from ^{125}I standards. The optical densities of the samples were within the linear range of the standards. Statistical analyses were conducted using GraphPad Prism 5.01 software (La Jolla, CA). Mean concentrations $\pm$ standard error of the mean (SEM) were analyzed using one-way analysis of variance followed by a Newman-Keuls post hoc test. Differences among groups were considered significant if the probability of error was less than 5% ($p < 0.05$). Sample sizes are indicated in figure legends.

Results

Results presented in Figure 3.1 demonstrate that *ad libitum* exposure to NIC from PND 40–PND 96 (10–75 µg/ml; p.o; panel A), PND 40–PND 61 (75 µg/ml; p.o; panel B), or PND 61–PND 100 (10–75 µg/ml; p.o; panel C) attenuated the persistent METH (4 x 7.5 mg/kg/injection, s.c.)-induced striatal deficits in ^{125}I-

RTI-55 binding when METH was administered on PND 89, 54, and 93, respectively ($F_{3,37}$ = 39.54 #p < 0.05, $F_{3,31}$ = 36.93 #p < 0.05, and $F_{3,35}$ = 23.73 #p < 0.05, respectively). In other words, and in agreement with Chapter 2, long-term NIC administration attenuated the persistent METH-induced striatal loss of DAT under these conditions. These NIC regimens did not attenuate METH-induced hyperthermia (panel D for PND 40–96 regimen; hyperthermia data for PND 40–61 and PND 61–100 regimens are not shown). Likewise, *ad libitum* exposure to NIC from PND 40–PND 96 ($F_{3,33}$ = 24.46, #p < 0.05), PND 40–PND 61 ($F_{3,31}$ = 16.28, #p < 0.05) or PND 61–PND 100 ($F_{3,34}$ = 8.12, #p < 0.05) attenuated the METH-induced deficits in ^{125}I-RTI-55 binding in the nucleus accumbens core in these tissues (Table 3.1).

Using the same tissues described in Figure 3.1, results presented in Figure 3.2A demonstrate that *ad libitum* exposure to an escalating-dose of NIC from PND 40–PND 96 attenuated the persistent (e.g., 7-d) METH-induced deficits in striatal ^{125}I-epibatidine binding ($F_{3,36}$ = 20.98 #p < 0.05) when METH was administered at PND 89. METH did not alter striatal ^{125}I-epibatidine binding when administered at PND 54 or 93 (panels B and C, respectively). In each experiment, NIC administration *per se* increased striatal ^{125}I-epibatidine binding in both saline- and METH-treated rats (panel A, $F_{3,36}$ = 20.98 *p < 0.05; panel B, $F_{3,31}$ = 16.14 *p < 0.05; panel C, $F_{3,36}$ = 25.42 *p < 0.05). Likewise, in the nucleus accumbens core, *ad libitum* exposure to NIC from PND 40–PND 96 attenuated METH-induced deficits in ^{125}I-epibatidine binding ($F_{3,34}$ = 19.71, #p < 0.05), and NIC administration *per se* increased ^{125}I-epibatidine binding in both saline- and

METH-treated rats (PND 40–96, $F_{3, 34}$ = 19.71 *p < 0.05; PND 40–61, $F_{3,30}$ = 19.90 *p < 0.05; PND 61–100, $F_{3,35}$ = 34.43 *p < 0.05) (Table 3.2).

Again using the same tissues obtained in experiments presented for Figure 3.1, results presented in Figure 3.3 demonstrate that both METH and NIC treatments *per se* decreased striatal ^{125}I-αCtxMII binding (panel A, $F_{3,34}$ = 11.98 *p < 0.05; panel B, $F_{3,31}$ = 13.73 *p < 0.05; and panel C, $F_{3,36}$ = 16.96, *p < 0.05). However, NIC treatment did not alter α6β2 nAChR binding in METH-treated rats (i.e., ^{125}I-αCtxMII binding was not statistically different between METH-treated rats exposed to tap water or NIC water). Similarly, in the nucleus accumbens core, METH *per se* decreased ^{125}I-αCtxMII binding (PND 40–96, $F_{3,31}$ = 4.54, *p < 0.05; PND 40–61, $F_{3,32}$ = 11.68 ,*p < 0.05; and PND 61–100, $F_{3,35}$ = 7.63, *p < 0.05) (Table 3.3). In contrast to the striatal data, NIC *per se* did not consistently downregulate α6β2 nAChR binding in the nucleus accumbens core. Furthermore, NIC did not alter α6β2 nAChR binding in the presence of METH when given from PND 40–96 or PND 61–100 (i.e., ^{125}I-αCtxMII binding was not statistically different between METH-treated rats exposed to tap water or NIC water). However, NIC attenuated METH-induced deficits in α6β2 nAChR binding when given from PND 40–61 (SM vs. NM, #p < 0.05). In other words, METH *per se* consistently decreased α6β2 nAChR binding in the striatum in the presence or absence of NIC. But in the nucleus accumbens core, METH *per se* only reduced α6β2 nAChR binding in the absence of NIC. Representative autoradiograms associated with Figures 3.1–3.3 are presented in Figure 3.4.

Discussion

The current studies demonstrate that long-term oral NIC administration, when available during adolescence or adulthood to rats, attenuates METH-induced dopaminergic deficits in the striatum and nucleus accumbens core. Furthermore, high-dose METH administration causes a persistent reduction in α6β2 nAChR binding sites in the striatum and nucleus accumbens core. These data are consistent with previous studies that showed that lesioning with the dopaminergic neurotoxins 6-hydroxy-DA or MPTP in rodents reduced striatal α6β2 nAChR density (Quik et al., 2003; Huang et al., 2009). Likewise, NIC administration *per se* reduced striatal α6β2 nAChR density as previously reported (Lai et al., 2005; Khwaja et al., 2007). The combination of NIC and METH did not further reduce α6β2 nAChR density in the striatum and nucleus accumbens. Conversely, high-dose METH administration caused either a reduction or no change in α4β2 nAChR density in both regions. These effects are similar to previous studies involving paraquat-induced damage in rodents in which striatal α4β2 nAChR density was unchanged (Khwaja et al., 2007) and in studies with 6-hydroxy-DA or MPTP administration to rodents in which striatal α4β2 nAChR density was reduced (Quik et al., 2003; Huang et al., 2009). Finally, chronic NIC administration increased α4β2 nAChR density in the striatum and nucleus accumbens core in both saline-treated rats and METH-treated rats. Overall, these data suggest that terminals containing α6β2 nAChRs are susceptible to the long-term deficits caused by METH and that either increasing α4β2 nAChRs and/or reducing α6β2 nAChRs sites may protect DA terminals

from neurotoxicity.

The present data are consistent with previous preclinical studies linking NIC-induced protection of dopaminergic neurons to alterations in nAChR expression (Pietila et al., 1998; Slotkin et al., 2004; Lai et al., 2005; Khwaja et al., 2007; Even et al., 2008; Perez et al., 2008; Huang et al., 2009). For example, long-term NIC pretreatment via drinking water to rats (i.e., at least 3 weeks) starting at PND 30 attenuated the dopaminergic deficits caused by 6-hydroxy-DA lesion when assessed by ^{125}I-RTI-121 autoradiography (Huang et al., 2009). In these studies, chronic NIC *per se* did not affect the density of α4β2 and α6β2 nAChRs, but rather attenuated the declines in the density of α4β2 and α6β2 nAChRs caused by 6-hydroxy-DA. Similarly, NIC administration via drinking water to mice for 6 weeks starting during adulthood attenuated paraquat-induced dopaminergic deficits (Khwaja et al., 2007). In these latter studies, NIC *per se* caused an upregulation of α4β2 and a downregulation of α6β2 nAChRs when assessed via ^{125}I-epibatidine and ^{125}I-CtxMII autoradiography, respectively (Khwaja et al., 2007).

Our current data indicate that chronic NIC reduces α6β2 nAChR density, suggesting that chronic NIC administration protects dopaminergic neurons against METH-induced toxicity via downregulation of α6β2 nAChRs. In other words, whenever downregulation of α6β2 nAChRs occurred, as seen in animals pretreated with NIC alone (i.e., NS) or in combination with METH (i.e., NM), dopaminergic neuroprotection was observed. The mechanism whereby downregulation of α6β2 nAChRs might protect dopaminergic neurons potentially

involves the role of this receptor subtype in mediating phasic DA release (Meyer et al., 2008; Perez et al., 2008; Perez et al., 2009; Exley et al., 2013). Particularly, chronic NIC administration reduces phasic DA signaling via downregulation of α6β2 nAChRs in the striatum and nucleus accumbens (Perez et al., 2008; Perez et al., 2009; Perez et al., 2012; Exley et al., 2013). Of note, in the striatum, the α6β2 nAChR subtypes are selectively located on dopaminergic terminals (see review by Quik and McIntosh, 2006). Previous findings have demonstrated that amphetamine/METH administration mainly disrupts DA signaling by significantly augmenting phasic DA release (Howard et al., 2011; Covey et al., 2013; Daberkow et al., 2013; Howard et al., 2013). These studies have demonstrated that these immediate increases in phasic DA release during amphetamine/METH administration lead to exocytotic events that likely contribute to the long-term dopaminergic damage. In fact, our current data demonstrate that METH consistently reduces α6β2 nAChR density 7 days after last administration. Thus, in line with previous evidence, our data reveal that dopaminergic terminals containing α6β2 nAChRs, which mediate phasic DA release, are more susceptible to the neurotoxic effects of METH. Overall, these findings suggest that the NIC-induced reduction in dopaminergic terminals that mediate phasic DA release (i.e., reduction in α6β2 nAChR density) might afford neuroprotection by offsetting the METH-induced increases in phasic DA release.

Another possible mechanism by which NIC affords neuroprotection might be because of upregulation of α4β2 nAChRs. As current and previous studies have shown, chronic NIC administration to rodents upregulates α4β2 nAChRs

binding sites (and upregulation was accompanied by increase in function; see Buisson and Bertrand, 2002, for review). The α4β2 nAChRs are located on dopaminergic terminals as well as in GABAergic neurons in the striatum (Quik and McIntosh, 2006, for review; English et al., 2012; Luo et al., 2013). In contrast to α6β2 nAChRs, the α4β2 subtypes in dopaminergic terminals mediate tonic DA release (Meyer et al., 2008). Chronic NIC could protect against METH-induced dopaminergic deficits through increased release of tonic DA (or basal firing). Activation of α4β2 nAChRs by acetylcholine or NIC causes tonic DA release (Buisson and Bertrand, 2002, for review; Meyer et al., 2008). Another accepted mechanism by which METH causes long-term dopaminergic deficits is through accumulation of cytoplasmic DA that readily oxidizes and forms reactive species (Cubells et al., 1994; Fleckenstein et al., 2007, for review). Thus, activation of α4β2 nAChRs could lead to elimination of the excess of intracellular DA caused by METH and consequent reduction in reactive species formation. In fact, activation of α4β2 nAChRs by NIC has been shown to have antioxidant properties (Linert et al., 1999), and its administration to rats has specifically shown to suppress the formation of dihydrobenzoacetic acid (DHBA) (Obata et al., 2002) (DHBA; an index of hydroxyl radical formation that is increased after high-dose METH treatment) (Fleckenstein et al., 1997). Furthermore, others have suggested that NIC-mediated neuroprotection against paraquat-induced dopaminergic damage might occur via an increase of DA release, whereby reactive oxygen species might be reduced (Khwaja et al., 2007). Of note, METH administration causes acetylcholine release providing evidence that METH

indirectly activates nAChRs (Tsai and Chen, 1994; Taguchi et al., 1998; Dobbs and Mark, 2008). In case of α4β2 nAChRs located on GABAergic neurons, previous findings have demonstrated that chronic NIC administration increases basal firing of GABA neurons, thereby increasing inhibition of postsynaptic events (Nashmi et al., 2007). Thus, NIC neuroprotection via upregulation of α4β2 nAChRs could potentially occur by offsetting METH-induced postsynaptic excitotoxicity events. Present data showed that NIC water intake increased α4β2 nAChR density, suggesting that at the time of METH injections, more tonic DA release and basal firing of GABA neurons potentially occurred in the NIC-treated METH group in comparison to rats that drank tap water (i.e., NIC-naïve METH group). Furthermore, data also showed that DAT density was greater in the NIC-treated METH group in comparison to the NIC-naïve METH group. These data are consistent with the hypothesis that increases in α4β2 contributed to dopaminergic neuroprotection.

Previous studies have shown that NIC causes α4β2 nAChR upregulation by inducing higher assembly of β2 subunits with α4 subunits and consequently reducing assembly of β2 subunits with α6 subunits (Kuryatov et al., 2005; Sallette et al., 2005; Colombo et al., 2013). In fact, α6 subunits are highly degraded during chronic NIC treatment, supporting the idea that chronic NIC treatment alters subunit assembly. Thus, because α4β2 upregulation occurs at the cost of α6β2 downregulation, chronic NIC exposure reduces α6β2-containing terminals and augments α4β2-containing terminals. At the time of METH administration, animals that were pre-exposed to NIC presumably had fewer DA

terminals expressing α6β2 nAChRs and more DA terminals expressing α4β2 nAChRs. Furthermore, previous studies have provided evidence of distinct populations of dopaminergic terminals in the striatum, i.e., some expressing α4β2 and others expressing α6β2 nAChRs (Meyer et al., 2008). Current data show greater α4β2 nAChR density (or α4β2-containing terminals) in METH-treated rats pre-exposed to NIC in comparison to METH-treated rats not exposed to NIC, supporting the hypothesis that α4β2-containing terminals are protected. Of note, not all dopaminergic terminals express nAChRs, demonstrated in studies showing that mecamylamine (a nonselective nAChR antagonist) does not inhibit 100% of electrically stimulated DA release (Quik et al., 2011). Thus, the 60–70% METH-induced DAT density deficits showed in current studies are greater than any effect of METH on α6β2 or α4β2 nAChRs. These findings support current and previous data demonstrating that NIC neuroprotection is a small effect (~35%); thus, terminals not expressing nAChRs are likely not involved in NIC neuroprotection. In summary, as shown in Figure 3.5, the proposed model for NIC neuroprotection is that either adding α4β2 or removing α6β2 nAChRs from dopaminergic terminals protect these terminals against METH toxicity, and these effects can be induced by long-term NIC exposure.

Figure 3.1 Long-term NIC administration attenuates METH-induced deficits in striatal ^{125}I-RTI-55 binding. A. Rats received tap water or NIC water (10–75 µg/ml) from PND 40 to 96 and METH (4 x 7.5 mg/kg/injection, s.c., 2h-apart) or saline (1ml/kg/injection) at PND 89. B. Rats received tap water or NIC water (75 µg/ml) from PND 40 to 61 and METH (4 x 7.5 mg/kg/injection, s.c., 2h-apart) or saline (1ml/kg/injection) at PND 54. C. Rats received tap water or NIC water (10–75 µg/ml) from PND 61 to 100 and METH (4 x 7.5 mg/kg/injection, s.c., 2h-apart) or saline (1ml/kg/injection) at PND 93. Brains were harvested 7 d after METH and DAT density was assessed via ^{125}I-RTI-55 autoradiography. Data are expressed as mean values $\pm$ S.E.M. of A. n = 8–12 determinations, B. n = 8–10 determinations, C. n = 9–10 determinations. *Values that are significantly different from SS ($p < 0.05$). #Values that are significantly different from SM ($p < 0.05$). ^Values that are significantly different from NS ($p < 0.05$). Legend: SS = tap water/saline injections; SM = tap water/METH injections; NS = NIC water/saline injections; NM = NIC water/METH injections.

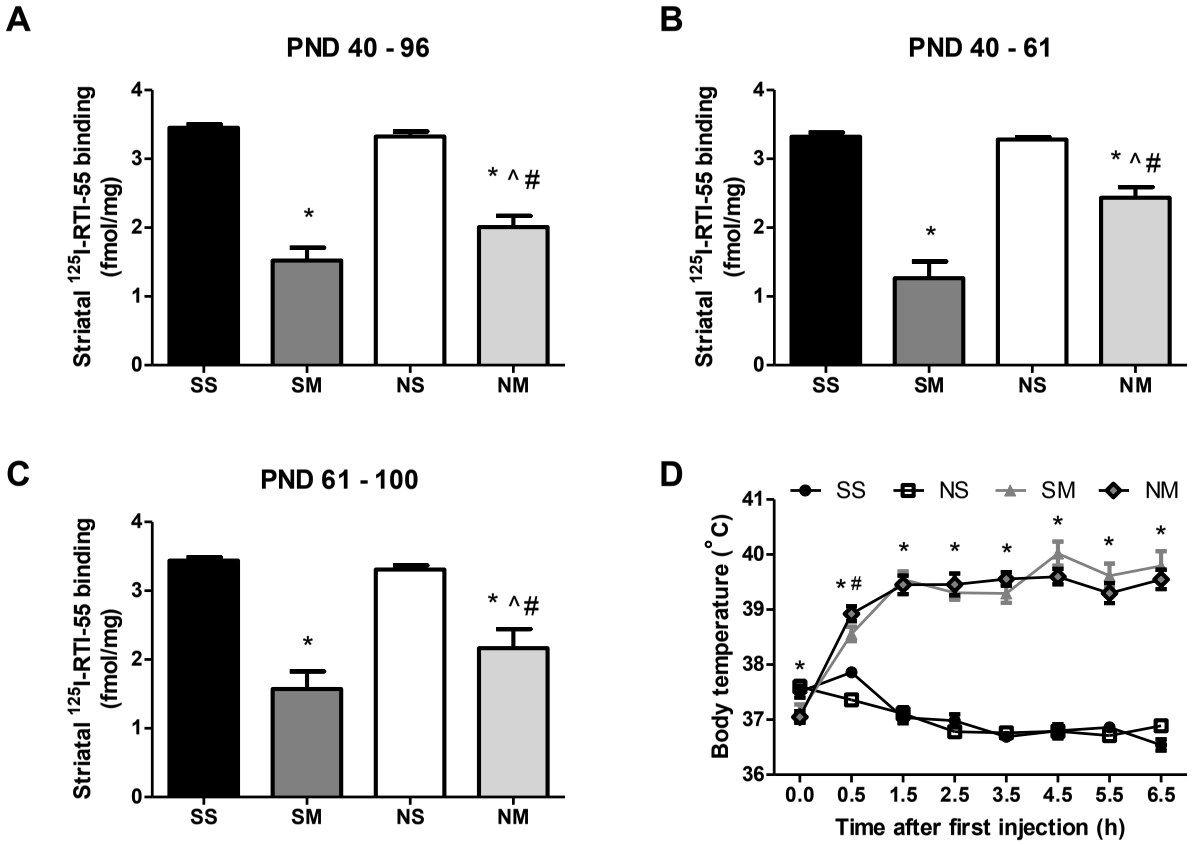

A
PND 40 - 96
Striatal 125I-RTI-55 binding (fmol/mg)
SS
SM
NS
NM
B
PND 40 - 61
C
PND 61 - 100
D
Body temperature (°C)
Time after first injection (h)
0.0
0.5
1.5
2.5
3.5
4.5
5.5
6.5

Table 3.1 Long-term NIC administration attenuates METH-induced deficits in ^{125}I-RTI-55 binding to nucleus accumbens core.

	^{125}I-RTI-55 binding to nucleus accumbens core (fmol/mg)			
Experiment	SS	SM	NS	NM
PND 40–96	2.53 ± 0.07	1.37 ± 0.17*	2.46 ± 0.11	1.85 ± 0.13*^#
PND 40–61	2.61 ± 0.08	1.22 ± 0.10*	2.50 ± 0.11	2.18 ± 0.11#
PND 61–100	2.65 ± 0.08	1.74 ± 0.21*	2.58 ± 0.10	2.19 ± 0.14#

*Rats received NIC in drinking water from PND 40–96 (10–75 µg/ml), PND 40–61 (75 µg/ml) or PND 61–100 (10–75 µg/ml). Brains were harvested 7 d after METH treatment. ^{125}-RTI-55 binding to nucleus accumbens core was performed as described in methods. Data are expressed as mean values ± S.E.M. *Values significant different from SS ($p < 0.05$). ^Values significant different from NS ($p < 0.05$). #Values significant different from SM ($p < 0.05$). Legend: SS = tap water/saline injections; SM = tap water/METH injections; NS = NIC water/saline injections; NM = NIC water/METH injections.*

Figure 3.2 Long-term NIC administration increases striatal α4β2 nAChR binding in saline-treated and METH-treated rats. A. Rats received tap water or NIC water (10–75 µg/ml) from PND 40 to 96 and METH (4 x 7.5 mg/kg/injection, s.c., 2h-apart) or saline (1ml/kg/injection) at PND 89. B. Rats received tap water or NIC water (75 µg/ml) from PND 40 to 61 and METH (4 x 7.5 mg/kg/injection, s.c., 2h-apart) or saline (1ml/kg/injection) at PND 54. C. Rats received tap water or NIC water (10–75 µg/ml) from PND 61 to 100 and METH (4 x 7.5 mg/kg/injection, s.c., 2h-apart) or saline (1ml/kg/injection) at PND 93. Brains were harvested 7 d after METH and α4β2 density was assessed via ^{125}I-epibatidine autoradiography. Data are expressed as mean values $\pm$ S.E.M. of A. n = 8–12 determinations, B. n = 8–10 determinations, C. n = 10 determinations. *Values that are significantly different from SS ($p < 0.05$). #Values that are significantly different from SM ($p < 0.05$). Legend: SS = tap water/saline injections; SM = tap water/METH injections; NS = NIC water/saline injections; NM = NIC water/METH injections.

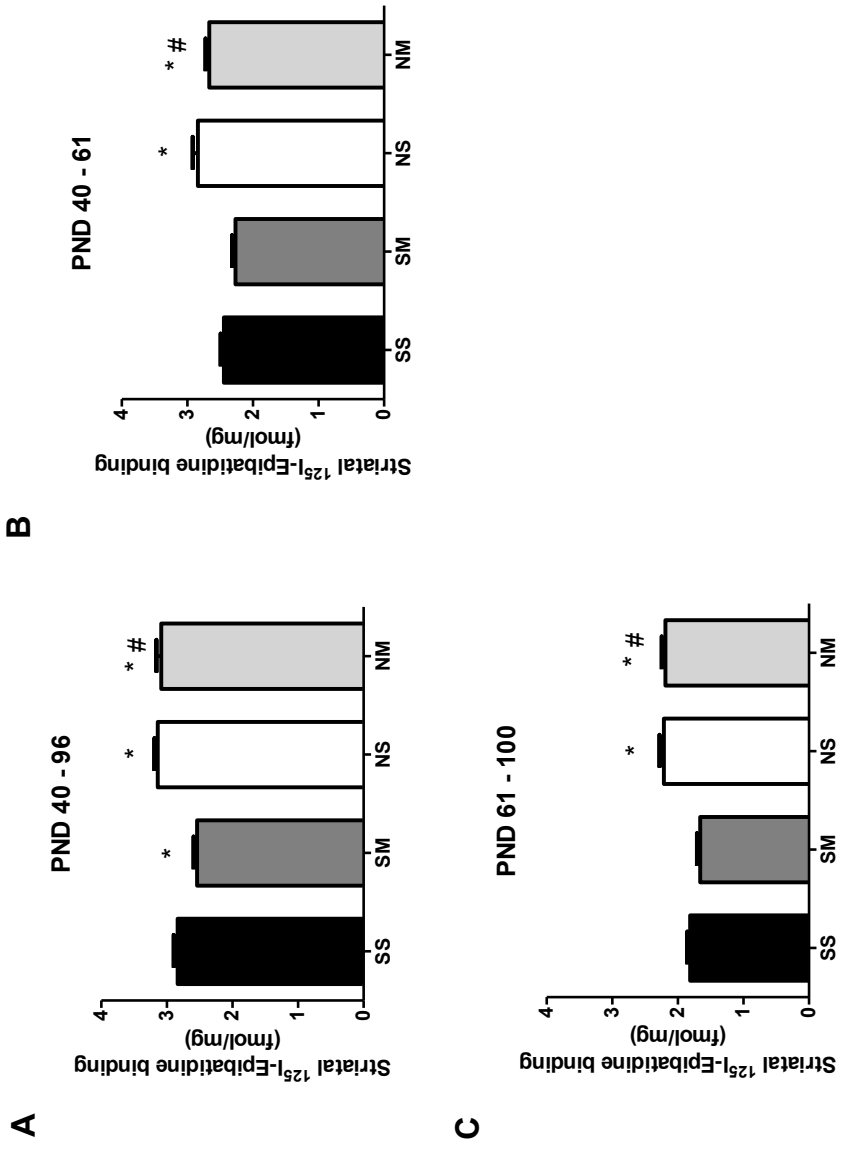

A
PND 40 - 96
Striatal 125I-Epibatidine binding
(fmol/mg)
0
1
2
3
4
SS
SM
NS
NM
*
*
* #
B
PND 40 - 61
Striatal 125I-Epibatidine binding
(fmol/mg)
0
1
2
3
4
SS
SM
NS
NM
*
* #
C
PND 61 - 100
Striatal 125I-Epibatidine binding
(fmol/mg)
0
1
2
3
4
SS
SM
NS
NM
*
* #

Table 3.2 Long-term NIC administration increases ^{125}I-epibatidine binding to nucleus accumbens core.

	^{125}I-epibatidine binding to nucleus accumbens core (fmol/mg)			
Experiment	SS	SM	NS	NM
PND 40–96	2.65 ± 0.07	2.37 ± 0.06*	2.94 ± 0.11*	3.17 ± 0.09*#
PND 40–61	2.35 ± 0.04	2.24 ± 0.05	2.79 ± 0.06*	2.65 ± 0.06*#
PND 61–100	1.66 ± 0.05	1.57 ± 0.06	2.16 ± 0.07*	2.16 ± 0.04*#

*Rats received NIC in drinking water from PND 40–96 (10–75 µg/ml), PND 40–61 (75 µg/ml) or PND 61–100 (10–75 µg/ml). Brains were harvested 7 d after METH treatment. ^{125}-epibatidine binding to nucleus accumbens was performed as described in methods. Data are expressed as mean values ± S.E.M. *Values significant different from SS ($p < 0.05$). #Values significant different from SM ($p < 0.05$). Legend: SS = normal water/saline injections; SM = normal water/METH injections; NS = NIC water/saline injections; NM = NIC water/METH injections.*

Figure 3.3 NIC or METH administration reduces striatal α6β2 nAChR binding. A. Rats received tap water or NIC water (10–75 µg/ml) from PND 40 to 96 and METH (4 x 7.5 mg/kg/injection, s.c., 2h-apart) or saline (1ml/kg/injection) at PND 89. B. Rats received tap water or NIC water (75 µg/ml) from PND 40 to 61 and METH (4 x 7.5 mg/kg/injection, s.c., 2h-apart) or saline (1ml/kg/injection) at PND 54. C. Rats received tap water or NIC water (10–75 µg/ml) from PND 61 to 100 and METH (4 x 7.5 mg/kg/injection, s.c., 2h-apart) or saline (1ml/kg/injection) at PND 93. Brains were harvested 7 d after METH and α6β2 density was assessed via ^{125}I-αCtxMII autoradiography. Data are expressed as mean values $\pm$ S.E.M. of A. n = 8–12 determinations, B. n = 8–10 determinations, C. n = 10 determinations. *Values that are significantly different from SS ($p < 0.05$). #Values that are significantly different from SM ($p < 0.05$). ^Values that are significantly different from NS ($p < 0.05$). Legend: SS = tap water/saline injections; SM = tap water/METH injections; NS = NIC water/saline injections; NM = NIC water/METH injections.

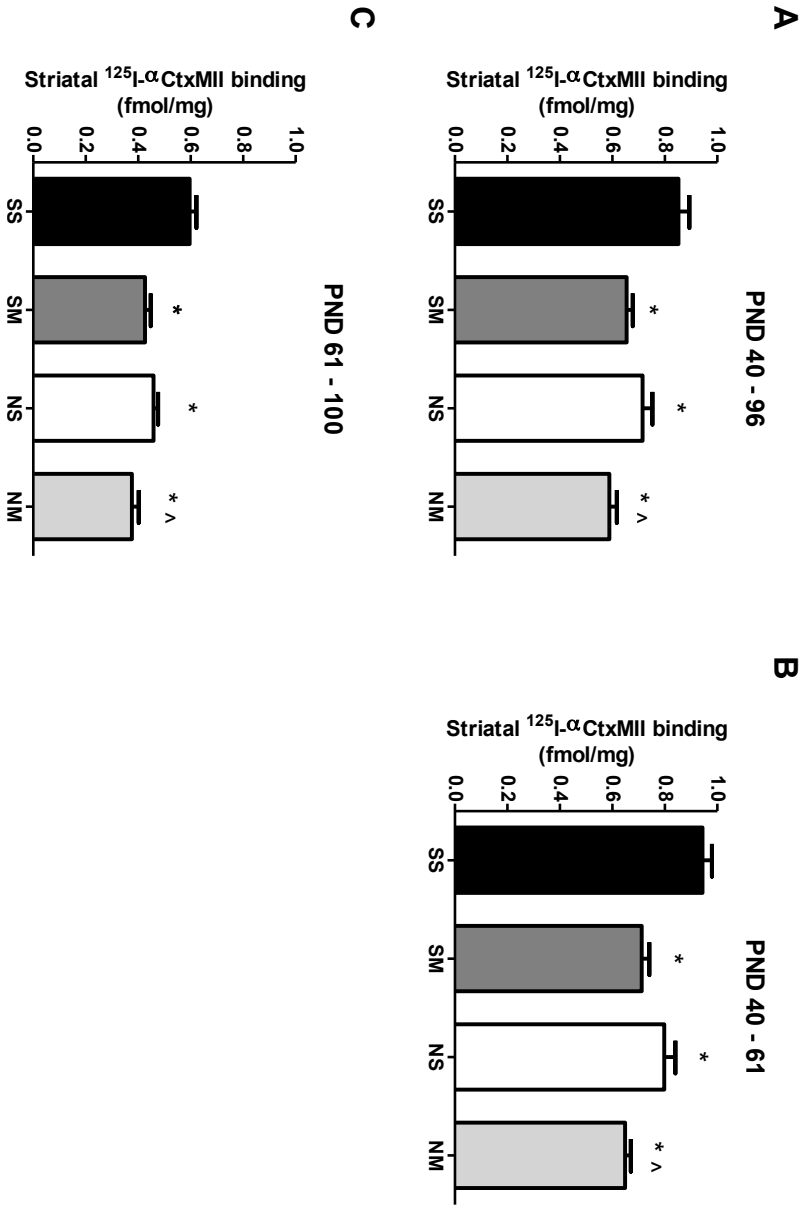

A
Striatal 125I-αCtxMII binding (fmol/mg)
0.0
0.2
0.4
0.6
0.8
1.0
SS
SM
NS
NM
*
*
*
PND 40 - 96
B
Striatal 125I-αCtxMII binding (fmol/mg)
0.0
0.2
0.4
0.6
0.8
1.0
SS
SM
NS
NM
*
*
*
PND 40 - 61
C
Striatal 125I-αCtxMII binding (fmol/mg)
0.0
0.2
0.4
0.6
0.8
1.0
SS
SM
NS
NM
*
*
*
PND 61 - 100

Table 3.3 NIC or METH administration reduces ^{125}I-αCtxMII binding to nucleus accumbens core.

	^{125}I-αCtxMII binding to nucleus accumbens core (fmol/mg)			
Experiment	SS	SM	NS	NM
PND 40–96	0.73 ± 0.03	0.56 ± 0.02*	0.61 ± 0.06*	0.58 ± 0.03*
PND 40–61	0.87 ± 0.04	0.58 ± 0.04*	0.84 ± 0.05	0.72 ± 0.04*^#
PND 61–100	0.53 ± 0.02	0.38 ± 0.03*	0.49 ± 0.02	0.38 ± 0.04*^

*Rats received NIC in drinking water from PND 40–96 (10–75 µg/ml), PND 40–61 (75 µg/ml) or PND 61–100 (10–75 µg/ml). Brains were harvested 7 d after METH treatment. ^{125}-αCtxMI binding to nucleus accumbens core was performed as described in methods. Data are expressed as mean values ± S.E.M. *Values significant different from SS ($p < 0.05$). ^Values significant different from NS ($p < 0.05$). #Values significant different from SM ($p < 0.05$). Legend: SS = normal water/saline injections; SM = normal water/METH injections; NS = NIC water/saline injections; NM = NIC water/METH injections.*

Figure 3.4 Representative autoradiographs depicting the effects of NIC and METH treatments on striatal and nucleus accumbens. A. DAT (^{125}I-RTI-55 binding), B. α4β2 nAChR (^{125}I-epibatidine binding) and C. α6β2 nAChR (^{125}I-αCtxMII) densities. Legend: SS = tap water/saline injections; SM = tap water/METH injections; NS = NIC water/saline injections; NM = NIC water/METH injections; Blank = nonspecific binding.

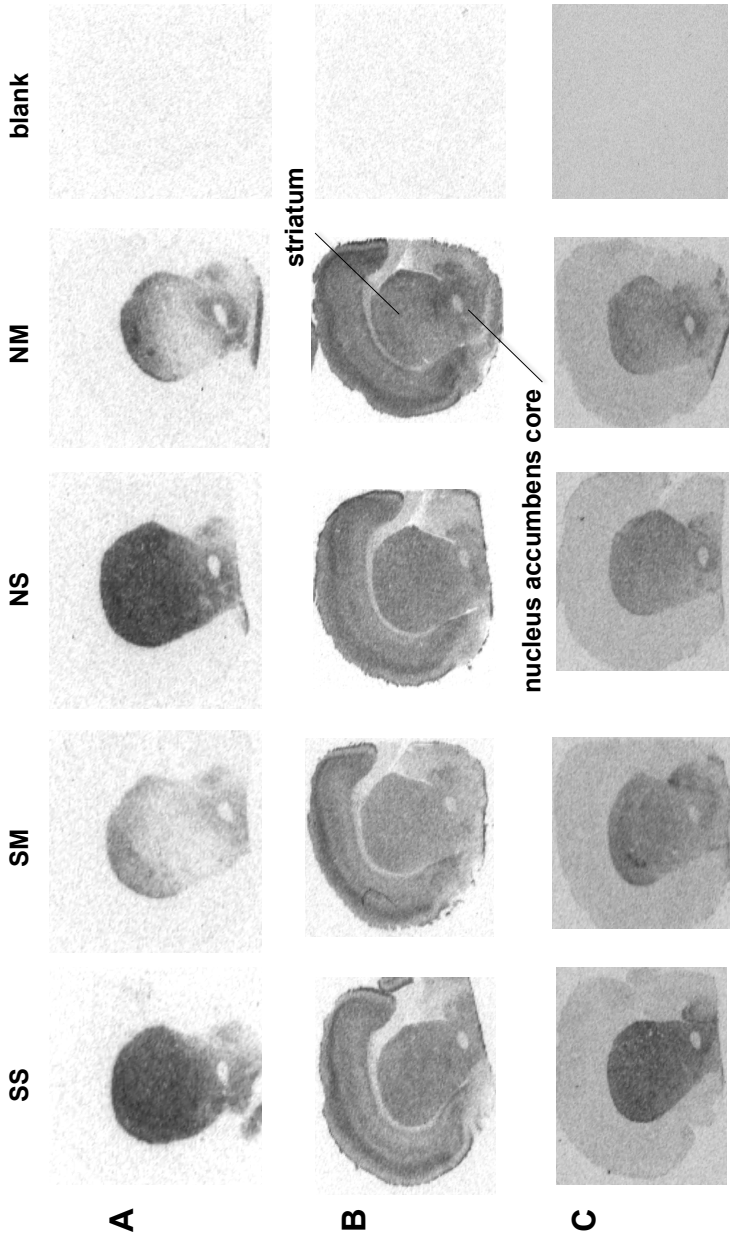
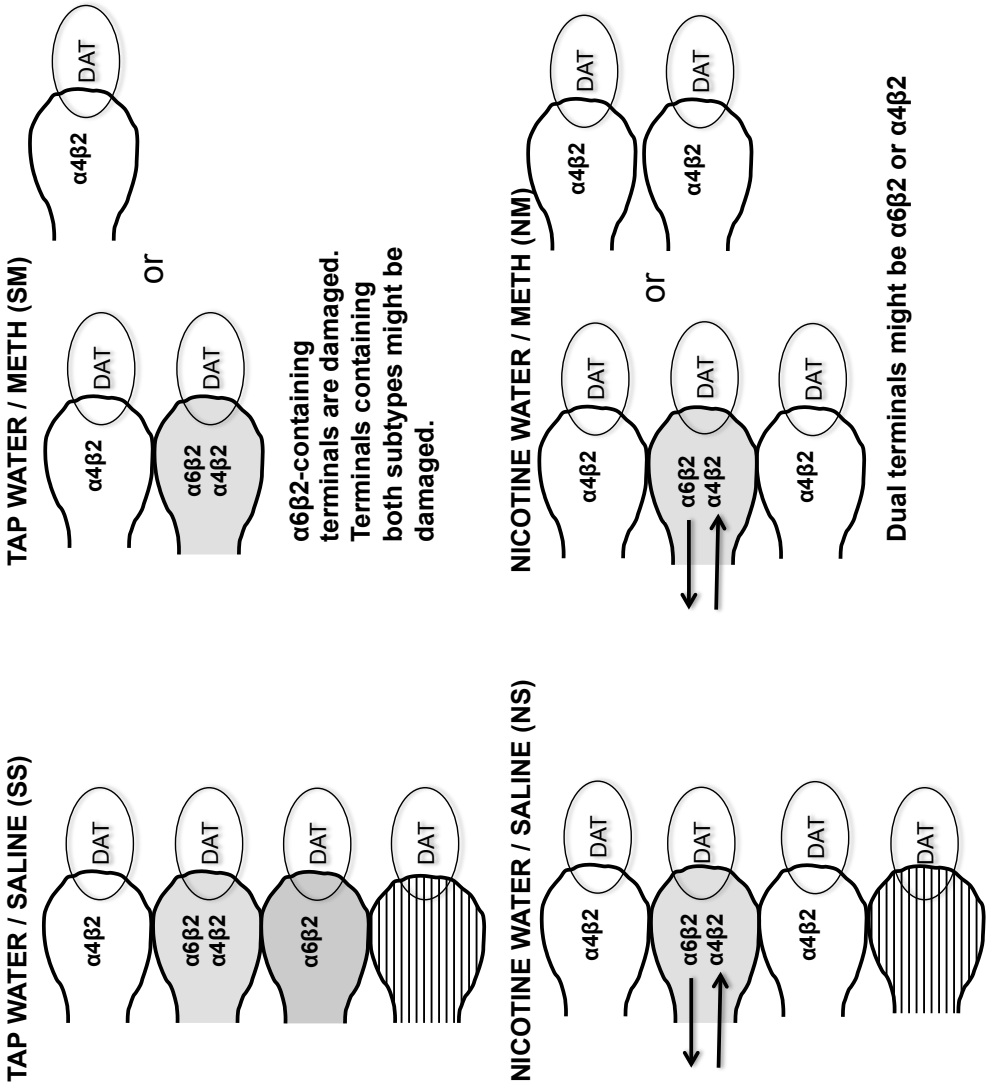

SS
SM
NS
NM
blank
striatum
nucleus accumbens core
A
B
C

Figure 3.5 Schematic diagram illustrating a proposed model for NIC neuroprotection to METH-induced dopaminergic deficits. Dopaminergic terminals are represented in white (α4β2-containing terminals), light gray (terminals containing α4β2 and α6β2), dark gray (α6β2-containing terminals), or white shaded (non-nAChRs terminals). The arrows represent possible exchange of nAChR subunits.


TAP WATER / SALINE (SS)
α4β2
α6β2 α4β2
α6β2
DAT
TAP WATER / METH (SM)
α4β2
Or
α6β2 α4β2
α6β2-containing terminals are damaged. Terminals containing both subtypes might be damaged.
NICOTINE WATER / SALINE (NS)
NICOTINE WATER / METH (NM)
Dual terminals might be α6β2 or α4β2

CHAPTER 4

CHRONIC NICOTINE ADMINISTRATION PRIOR TO METHAMPHETAMINE PROTECTS AGAINST DEFICITS IN DOPAMINE TRANSPORTER AND α4β2 NICOTINIC RECEPTORS IN THE STRIATUM

Introduction

Methamphetamine (METH) is a psychostimulant, and its abuse can lead to long-lasting dopaminergic deficits (Volkow et al., 2001). Recent clinical evidence from others and us indicates that METH abusers are at greater risk for developing dopaminergic neurodegenerative disorders such as Parkinson's disease (PD) (Callaghan et al., 2012; Curtin et al., 2014). Epidemiological studies demonstrate that lifetime exposure to cigarette smoking is inversely associated with PD risk (Chen et al., 2010). Furthermore, the duration (years of smoking) rather than intensity (daily number of cigarettes) of smoking is correlated with this lower PD risk, suggesting that neuroprotection comes from neuroadaptations induced by long-term, rather than the direct effects of, smoking (Chen et al., 2010). As reviewed by Ritz and Rhodes (2010), it is unclear whether smoking is suppressing/delaying the development of PD or slowing its progression. Thus, it is of relevance to investigate whether smoking exposure before or after disease onset is important for neuroprotection.

One likely neuroprotective agent in smoking is nicotine (NIC), as extensive

preclinical research has indicated that NIC protects against nigrostriatal dopaminergic deficits (for review, see Quik et al., 2009). Specifically, NIC protects when given chronically before and after, but not when given only after lesion with the dopaminergic neurotoxins 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,3,6-tetrahydro pyridine (MPTP) to rats or monkeys, respectively (Huang et al., 2009). These findings suggest that posttreatment alone is not sufficient for neuroprotection. Thus, since others have demonstrated that posttreatment alone is not sufficient for NIC neuroprotection, one of the hypotheses tested herein is that pretreatment alone is sufficient to afford dopaminergic neuroprotection against METH-induced deficits.

NIC modulates nigrostriatal dopaminergic function via nicotinic acetylcholine receptors (nAChRs) (Zhou et al., 2002; Quik and McIntosh, 2006; Grady et al., 2007; Colombo et al., 2013). Our previous preclinical data demonstrate that chronic NIC exposure, beginning during adolescence (i.e., the corresponding age in humans at which cigarette smoking is generally initiated (Kandel and Logan, 1984; Chen and Kandel, 1995; Breslau and Peterson, 1996), protects against the persistent dopamine transporter (DAT) density and function deficits caused by METH. This neuroprotection was accompanied by increased α4β2 and reduced α6β2 nAChRs densities. However, in these studies, rats were exposed to NIC before and after METH treatment (see Chapter 3). The purpose of the present study was to determine if NIC must be present during and after METH exposure in order to afford protection. Results revealed that long-term NIC pretreatment *per se* is sufficient and that NIC does not need to be present at the

time of METH treatment to afford protection. The current data also suggest that NIC-induced increases in α4β2 and decreases in α6β2 nAChR levels may contribute to this protection.

Methods

Animals. Male Sprague-Dawley rats (Charles River Breeding Laboratories, Raleigh, NC) initially weighing 125–180 g (corresponding to postnatal day (PND) 40) (Tirelli et al., 2003) were housed 2–3 rats per cage and maintained under a controlled light/dark cycle (14:10 h) and in an ambient environment of 20 °C (with the exception of the 6-h period during which METH or saline vehicle was administered during which the ambient environment was maintained at 24 °C). During METH or saline administrations, core body (rectal) temperatures were measured using a digital thermometer (Physitemp Instruments, Clifton, NJ) every 1 h beginning 30 min before the first saline or METH administration and continuing until 30 min after the final saline or METH administration. Rats were placed on a cooler environment if their body temperature exceeded 40.5 °C and returned to their home cage once their body temperature dropped to 40 °C. Food and water were available *ad libitum*. All experiments were approved by the University of Utah Institutional Animal Care and Use Committee, in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals 8th Edition* (Institute of Laboratory Animal Resources, 2011).

Drug treatments. METH hydrochloride was provided by the National Institute on Drug Abuse (Research Triangle Institute; Research Triangle Park,

NC) and administered (s.c.) at 4 x 7.5 mg/kg/injection, (2-h intervals) calculated as free-base. (-) NIC (1.010 g/ml; Sigma-Aldrich Co. LLC) was administered *ad libitum* p.o. via the water bottles. NIC dose began at a low concentration of 10 µg/ml for the first week and was raised to 20 µg/ml for the second week, 50 µg/ml for the third and fourth weeks, and 75 µg/ml for the remaining period, a dose that corresponds to moderate-heavy human smoking (Matta et al., 2007) and previously used in rats and known to affect nAChRs (Huang et al., 2009). NIC was maintained throughout the entire testing period (i.e., from PND 40 to 96) in only one group of animals, the NM(1) group. In order to test whether NIC preadministration is sufficient to afford neuroprotection, but without the potential confounder of NIC withdrawal during METH administrations, NIC water was replaced with tap water 2 h prior to saline or METH administrations in the NS and NM(2) groups, respectively. In order to test whether the absence of NIC during METH administrations impacts neuroprotection, NIC water was replaced with tap water 24 h prior to METH administrations in the NM(3) group. Of note, in the NS, NM(2) and NM(3) groups, NIC was not administered during the weeklong after METH.

Tissue preparation. Rats were decapitated 7 d after METH treatment. Brains were hemisected, and the right side rapidly removed and frozen in isopentane on dry ice and stored at -80°C. Frozen right hemisected brains were sliced at 12 µm thick at the level of the anterior striatum (1.5 mm from bregma, Paxinos and Watson 6th edition) using a cryostat. Eight slices (four per rat) were mounted on each Superfrost® Plus glass micro slides (VWR International,

Radnor, PA) and stored at -80°C for subsequent use in autoradiography assays as described below. The hippocampal and cortex tissues from these animals were also processed and data were reported in Chapter 5.

^{125}I-RTI-55 autoradiography. DAT density was used as a marker of dopaminergic integrity and assessed via ^{125}I-RTI-55 binding to striatal slices as previously described (O'Dell et al., 2012). Briefly, slides were thawed on a slide warmer (5–10 min) and preincubated in buffer-sucrose (10 mM sodium phosphate, 120 mM sodium chloride, 320 mM sucrose, pH 7.4) containing 100 nM fluoxetine at room temperature for 5 min followed by a 2-h incubation in buffer-sucrose containing 25 pM ^{125}I-RTI-55 (2200 Ci/mmol, PerkinElmer, Watham, MA). Slides were rinsed twice in ice-cold buffer and distilled water for 2 min and air-dried. Sample slides and standard ^{125}I microscale slides (American Radiolabeled Chemicals, St. Louis, MO) were placed on one cassette and exposed to the same Kodak MR film (Eastman Kodak Co., Rochester, NY, USA) for 24 h to keep variables constant.

^{125}I-epibatidine autoradiography. α4β2 nAChR density was assessed via ^{125}I-epibatidine binding to striatal slices as previously described (Huang et al., 2009). Briefly, slides were thawed on a slide warmer (5–10 min) and preincubated in binding buffer (50 mM Tris, 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, pH 7.5) plus 100 nM αCtxMII at room temperature for 30 min. The nonradiolabeled αCtxMII was used to inhibit epibatidine binding to α6β2 nAChRs followed by a 40-min incubation in binding buffer containing 0.015 nM ^{125}I-epibatidine (2200 Ci/mmol, PerkinElmer, Watham, MA) in the presence of

100 nM αCtxMII. Slides were rinsed twice in ice-cold buffer for 5 min followed by a 10 s rinse in distilled water. Slides were air-dried. Sample slides and standard ^{125}I microscale slides (American Radiolabeled Chemicals, St. Louis, MO) were placed on one cassette and exposed to the same Kodak MR film (Eastman Kodak Co., Rochester, NY, USA) for 24 h to keep variables constant.

^{125}I-αConotoxinMII (αCtxMII) autoradiography. α6β2 nAChR density was assessed via ^{125}I-αCtxMII binding to striatal slices as previously described (Huang et al., 2009). Briefly, slides were thawed on a slide warmer (5–10 min) and preincubated in buffer A (pH 7.5, 20 nM HEPES, 144 mM NaCl, 1.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 0.1% BSA, and 1 mM phenylmethylsulfonyl fluoride) at room temperature for 2 x 15 min followed by a 1-h incubation in buffer B (pH 7.5, 20 nM HEPES, 144 mM NaCl, 1.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 0.2% BSA, 5 mM EDTA, 5 mM EGTA, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin A) containing 0.5 nM ^{125}I-αCtxMII (~2200 Ci/mmol that was synthesized as previously described by Whiteaker et al. (2000). Slides were rinsed in room temperature buffer A for 10 min, then in ice-cold buffer A for another 10 min, followed by 2 x 10 min in 0.1x ice-cold buffer A, and finally in 4 ºC distilled water for 2 x 10 s. Slides were air-dried. Sample slides and standard ^{125}I microscale slides (American Radiolabeled Chemicals, St. Louis, MO) were placed on one cassette and exposed to the same Kodak MR film (Eastman Kodak Co., Rochester, NY, USA) for 4 days to keep variables constant.

NIC and cotinine concentration. NIC and cotinine (COT) were assessed using a liquid chromatography tandem mass spectrometry method previously

developed and validated (Miller et al., 2010; Vieira-Brock et al., 2013). Briefly, trunk blood was collected in sodium heparin-containing tubes and centrifuged at 3000 × g for 15 min to obtain plasma. One ml of test samples or analyte-free plasma for standards and quality controls were used. Standards were fortified with NIC (Sigma-Aldrich Co. LLC) and COT (Toronto Research Chemicals, Canada), making concentrations ranging from 1 ng/ml to 100 ng/ml. Quality controls contained a low (2 ng/ml), a medium (10 ng/ml), and a high (80 ng/ml) concentration of NIC and COT. Analytes extraction was performed using preconditioned Oasis® MCX cartridges (3 cm^3, 60 mg; Waters®, Millford, MA). Analytes were eluted with 2 ml methanol containing 5% (v/v) concentrated aqueous ammonium hydroxide and reconstituted with 150 µl of 10 mM ammonium acetate (pH 5.0) + 0.001 % formic acid/HPLC grade methanol (85:15 v/v). Analytes were resolved on a Discovery® HS F5 HPLC column (10 cm × 4 mm x 3 µm, Supelco®, Bellefonte, PA) and Acquity UPLC® system (Waters®, Millford, MA) with a flow rate of 0.6 ml/min. The mobile phase consisted of a gradient elution of 10 mM ammonium acetate with 0.001% formic acid at pH 5.0 (aqueous) and methanol (organic). The lower limit of quantification (LOQ) of this analysis was 1.0 ng/ml for NIC and 2.5 ng/ml for COT.

Data quantitation and statistical analyses. Optical densities from four replicate slices per rat were quantified using ImageJ software (National Institutes of Health, USA). Specific binding was obtained by subtracting film background from mean density values and converted to fmol/mg using the standard curve generated from ^{125}I standards. The optical densities of the samples were within

the linear range of the standards. Statistical analyses were conducted using GraphPad Prism 5.01 software (La Jolla, CA). Mean concentrations ± the standard error of the mean (SEM) were analyzed using an one-way analysis of variance followed by a Newman-Keuls post hoc test. Differences among groups were considered significant if the probability of error was less than 5% ($p < 0.05$). Sample sizes are indicated in figure legends.

Results

Results presented in Table 4.1 demonstrate that the oral NIC doses used in our rat studies yield plasma NIC and COT concentrations similar to plasma NIC and COT concentrations typically found in human smokers (5–50 ng/ml for NIC and ~300 ng/ml for COT) (Benowitz, 1994; Matta et al., 2007). NIC and COT were <LOQ in plasma from NIC-naïve rats and from NIC-treated rats in which NIC was removed 7–8 d prior to blood collection.

Results presented in Figure 4.1 demonstrate that *ad libitum* exposure to an escalating-dose regimen of NIC (see Methods for details) from PND 40–96 attenuated the persistent (i.e., 7-d) METH-induced decrease in striatal DAT density as assessed by ^{125}I-RTI-55 binding ($F_{5,43} = 23.00$, #$p < 0.05$) when METH was administered on PND 89. This same escalating-dose regimen of oral NIC also attenuated the persistent METH-induced decrease in striatal DAT density as assessed by ^{125}I-RTI-55 binding when NIC was administered from PND 40–89 and METH at PND 89, with NIC exposure ending 2 h prior to the first METH injection (#$p < 0.05$). Similarly, oral NIC attenuated the persistent METH-induced decrease in striatal DAT density as assessed by ^{125}I-RTI-55 binding when NIC

was administered from PND 40–88 and METH at PND 89 (i.e., NIC exposure ended 24 h prior to the first METH injection; #$p < 0.05$).

Using the same tissues described in Figure 4.1, results presented in Figure 4.2 demonstrate that NIC exposure from PND 40–PND 96 attenuated the persistent (e.g., 7-d) METH-induced deficits in striatal ^{125}I-epibatidine binding when METH was administered at PND 89 ($F_{5,44} = 6.78$, #$p < 0.05$). NIC also attenuated the persistent (e.g., 7-d) METH-induced decrease in striatal ^{125}I-epibatidine binding when NIC was administered from PND 40–89 and METH at PND 89, with NIC exposure ending 2 h prior to the first METH exposure (#$p < 0.05$). Similarly, NIC attenuated the persistent (e.g., 7-d) METH-induced decrease in striatal ^{125}I-epibatidine binding when NIC was administered from PND 40–88 and METH at PND 89, with NIC exposure ending 24 h prior to the first METH exposure (#$p < 0.05$). NIC treatment *per se* increased striatal ^{125}I-epibatidine binding when given throughout (as seen in NM(1) group; *p<0.05), but this effect did not persist after 7 or 8 d of NIC abstinence (as seen in NS, NM(2), and NM(3) groups; ns in comparison to SS).

Again using the same tissues obtained in experiments presented for Figure 4.1, results presented in Figure 4.3 demonstrate *ad libitum* exposure to an escalating-dose of NIC from PND 40–PND 96 did not attenuate the persistent (e.g., 7-d) METH-induced deficits in striatal ^{125}I-αCtxMII binding when METH was administered at PND 89 ($F_{5,43} = 3.33$, SM vs NM(1) ns). This same escalating-dose regimen of oral NIC also did not attenuate the persistent (e.g., 7-d) METH-induced decrease in striatal ^{125}I-αCtxMII binding when NIC was administered

from PND 40–89 and METH at PND 89 (SM vs NM(2), ns). Similarly, oral NIC did not attenuate the persistent (e.g., 7-d) METH-induced decrease in striatal ^{125}I-αCtxMII binding when NIC was administered from PND 40–88 and METH at PND 89 (SM vs NM(3), ns). Seven days of NIC abstinence *per se* did not decrease striatal ^{125}I-αCtxMII binding (SS vs NS, ns).

Representative autoradiograms associated with Figures 4.1–4.3 are presented in Figure 4.4.

Discussion

NIC activates nAChRs affecting several downstream pathways that might be involved in NIC neuroprotection, including regulation of DA release (Zhou et al., 2001; Marks et al., 2014), reduction of oxidative stress (Linert et al., 1999; Soto-Otero et al., 2002; Egea et al., 2007), modulation of mitochondrial complex I function (Xie et al., 2005), and augmentation of neurotrophic factors (Maggio et al., 1998; Belluardo et al., 1999; Belluardo et al., 2008; Takarada et al., 2012). However, the specific NIC-induced change in nAChRs that mediates this neuroprotection and timing in which this effect occurs are unknown. Previously, we have shown that long-term NIC pre- plus posttreatment attenuates the persistent striatal dopaminergic deficits caused by METH administrations. NIC neuroprotection was accompanied by an increase in α4β2 and a decrease in α6β2 nAChR density. These data suggested that NIC-induced upregulation in α4β2 and downregulation of α6β2 nAChR affords neuroprotection. Furthermore, because others have shown that these effects of NIC on nAChRs levels occur via exchange of α4 for α6 subunits (for review, see Colombo et al., 2013), data also

suggested that α4β2-, but not α6β2-, containing dopaminergic terminals are protected. However, the effects on nAChRs seen 7 d after METH administration could have occurred due to the effects of NIC *per se* on the trafficking of these receptors as opposed to protection of dopaminergic terminals. In order to exclude this potential problem, in current experiments NIC was absent during the weeklong period after METH. Overall, current studies demonstrate that long-term NIC preadministration via drinking water attenuates the striatal dopaminergic deficits caused by a subsequent high-dose METH administration; particularly, NIC protects α4β2-containing, but not α6β2-containing, striatal neurons.

It is of clinical and scientific interest to understand whether any potential NIC neuroprotection occurs when NIC is given before or after dopaminergic lesion (reviewed by Ritz and Rhodes, 2010). Previous preclinical studies showed that pre- plus posttreatment with NIC for several weeks attenuate dopaminergic deficits caused by 6-hydroxydopamine or MPTP (Huang et al., 2009). However, long-term NIC posttreatment alone did not attenuate dopaminergic deficits, suggesting that pretreatment is necessary for NIC neuroprotection (Huang et al., 2009). The present results reveal that 49 d pretreatment with NIC beginning in adolescence and terminated 2 or 24 h before METH administrations attenuates the persistent METH-induced dopaminergic deficits as assessed by ^{125}I-RTI-55 binding in the striatum. These data indicate that NIC pretreatment, as opposed to posttreatment, is responsible for dopaminergic neuroprotection afforded by NIC in the METH model of dopaminergic dysfunction. Furthermore, data suggest that the presence of NIC during METH administrations is not necessary for

neuroprotection since the plasma half-life of NIC in rats is approximately 1 h (Ghosheh et al., 1999; Vieira-Brock et al., 2013), and in current studies NIC was removed 24 h prior to METH. Additionally, current data showed that plasma NIC and metabolites were lower than LOQ in these animals 7–8 d abstinent from NIC, indicating that the observed effects in DAT and nAChRs density are reflective of persistent effects, as opposed to temporary effects, of NIC.

As observed in current studies, chronic NIC administration afforded dopaminergic neuroprotection even when NIC was removed 24-h prior to METH administrations. Studies from others have demonstrated that 24 h NIC abstinence after chronic dosing to rats increases vesicular monoamine transporter-2 (VMAT-2) mRNA and protein expression in the substantia nigra and striatum, respectively (Duchemin et al., 2009), and these effects have been implicated in NIC neuroprotection against MPTP-induced damage in mice (Singh et al., 2008). VMAT-2 mediates the sequestration of DA from the cytosol into synaptic vesicles. In laboratory animals, METH administrations reduce VMAT-2 function, which is thought to contribute to METH neurotoxicity via accumulation of cytosolic DA and formation of reactive species (for review, see Fleckenstein et al., 2009; Chu et al., 2010). Thus, the current observation that NIC neuroprotection remained even after 24-h NIC abstinence before METH administrations could have occurred because of increases in VMAT-2.

Extensive clinical and preclinical evidence demonstrates that chronic NIC exposure causes upregulation of β2-containing nAChRs sites (Perez et al., 2008; Cosgrove et al., 2012; Marks et al., 2014). In rodents, NIC-induced upregulation

of nAChRs density in the striatum after several weeks of administration via drinking water remained elevated up to 72 h after NIC administration was stopped and returned to baseline after 7 d (Pietila and Ahtee, 2000; Natividad et al., 2010). Similarly, our current data demonstrate that chronic NIC administration leads to upregulation in striatal nAChRs density, particularly the α4β2 subtype, as assessed by ^{125}I-epibatidine binding as seen in rats that received NIC continuously for 56 d. After 7 d NIC abstinence, α4β2 nAChR density returned to baseline levels. Nevertheless, the density of striatal α4β2 nAChR is statistically greater in METH-treated rats 7 or 8 d abstinent from chronic NIC administration in comparison to METH-treated rats that had never received NIC. In other words, data suggest that striatal neurons expressing α4β2 nAChRs were protected from METH-induced neurotoxicity by chronic NIC preadministration.

In contrast to α4β2 nAChRs, long-term NIC administration leads to downregulation of α6β2 nAChRs in the striatum as shown by others (Perez et al., 2008; Marks et al., 2014) and us (see Chapter 3). In the striatum, the α6β2 subtype is expressed predominantly in dopaminergic terminals that innervate the striatum (for review, see Quik and McIntosh, 2006). Similar to our previous studies, in current studies striatal α6β2 nAChRs density was reduced in METH-treated rats naïve to NIC, suggesting that α6β2-containig terminals are damaged by METH. Current data also reveal that NIC preadministration for 49 d lead to significant deficits in α6β2 nAChRs density in METH-treated rats. These deficits persisted even after 7–8 d NIC administration was stopped, suggesting that these reductions in α6β2 density reflect a loss of dopaminergic terminals

expressing α6β2 as opposed to NIC-induced downregulation of α6β2.

Previously we proposed a model for NIC neuroprotection against METH-induced striatal dopaminergic deficits that consisted of increasing α4β2 and reducing α6β2 nAChRs sites (see Chapter 3). Previous studies have demonstrated that upregulation of α4 and downregulation of α6 nAChR subunits occur due to NIC-induced modulation of β2 subunits assembly in the endoplasmic reticulum (Kuryatov et al., 2005; Sallette et al., 2005; for review, see Colombo et al., 2013; Srinivasan et al., 2014). A substitution of α6β2 by α4β2 induced by NIC might afford protection via modulation of a diverse system including phasic/tonic DA release (Meyer et al., 2008; Howard et al., 2011), glutamate release (Xiao et al., 2009; Northrop et al., 2011) and GABAergic inhibition (Nashmi et al., 2007). Specifically, NIC-induced exchange of α6β2 by α4β2 potentially lead to reduction in phasic and increase in tonic DA release, reduction in glutamate release and augmentation in GABAergic inhibition of excitotoxicity. All of these effects would likely lead to attenuation of METH-induced neurotoxicity based upon evidence indicating that METH causes phasic, more than tonic, DA release (Howard et al., 2013) and METH increases glutamate release and causes excitotoxicity (Mark et al., 2004; Halpin et al., 2014, for review). In summary, current studies indicate that long-term NIC pretreatment is sufficient to afford dopaminergic neuroprotection against METH-induced neurotoxicity, and NIC might do so by increasing α4β2-containing and reducing α6β2-containing striatal neurons.

Table 4.1 Plasma NIC and COT concentrations from rats exposed to tap or NIC water and treated with saline or METH.

	Mean Concentration (ng/ml plasma) *(SEM)*					
Analyte	SS	SM	NS	NM(1)	NM(2)	NM(3)
NIC	<LOQ	<LOQ	<LOQ	25.4 *(8.2)*	<LOQ	<LOQ
COT	<LOQ	<LOQ	<LOQ	369.3 *(41.3)*	<LOQ	<LOQ

Nicotine (NIC) and cotinine (COT) were measured from rat plasma collected at the end of experiment as described in Methods. Legend: LOQ = lower limit of quantification; SS = tap water/saline injections; SM = tap water/METH injections; NS = NIC water/saline injections; NM = NIC water/METH injections.

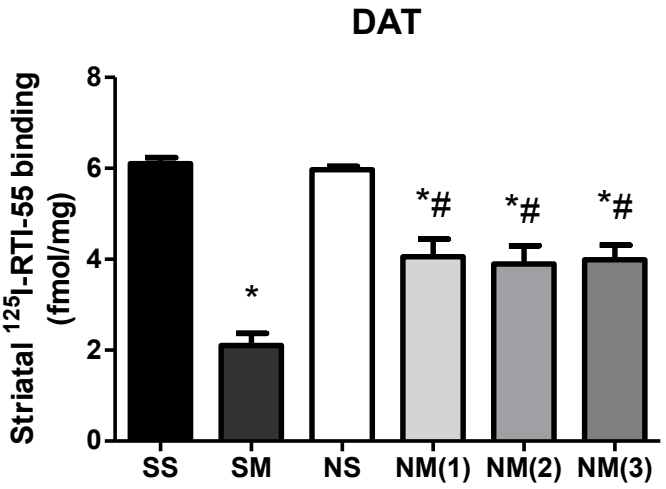


Figure 4.1 Long-term NIC preadministration protects dopaminergic terminals. Rats received oral NIC (N) or tap water (S) from PND 40 to 96 (NM(1)), PND 40 to 89 (NM(2)) or PND 40 to 88 (NM(3)) and either saline (S) or METH (M) administrations at PND 89 as delineated in Methods. Striatal ^{125}I-RTI-55 binding was assessed 7 d after METH or saline injections. Data are expressed as mean $\pm$ S.E.M. of n = 7–9 determinations. *Represent values statistically different from saline-controls ($p < 0.05$). # Represent values statistically different from SM ($p < 0.05$). Legend: SS = tap water/saline injections; SM = tap water/METH injections; NS = NIC water/saline injections; NM = NIC water/METH injections.

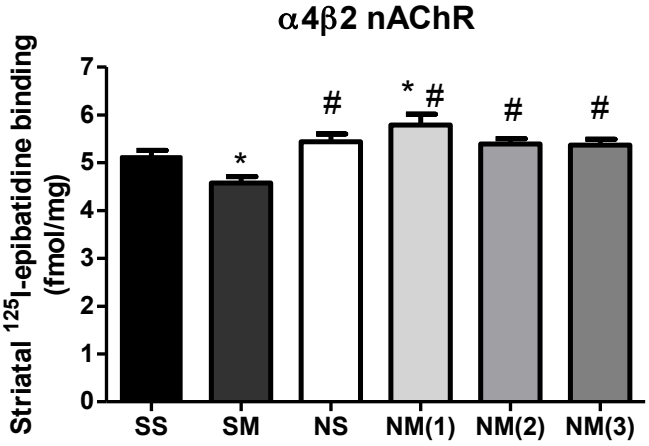


Figure 4.2 Long-term NIC preadministration protects α4β2-containing dopaminergic terminals. Rats received NIC (N) or tap water (S) from PND 40 to 96 (NM(1)), PND 40 to 89 (NM(2)), or PND 40 to 88 (NM(3)) and either saline (S) or METH (M) administrations at PND 89 as delineated in Methods. Striatal ^{125}I-epibatidine binding was assessed 7 d after METH or saline injections. Data are expressed as mean $\pm$ S.E.M. of n = 7–9 determinations. *Represent values statistically different from SS ($p < 0.05$). # Represent values statistically different from SM ($p < 0.05$). Legend: SS = tap water/saline injections; SM = tap water/METH injections; NS = NIC water/saline injections; NM = NIC water/METH injections.

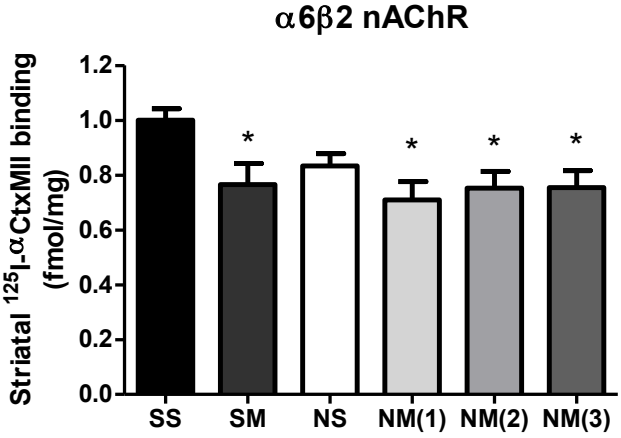
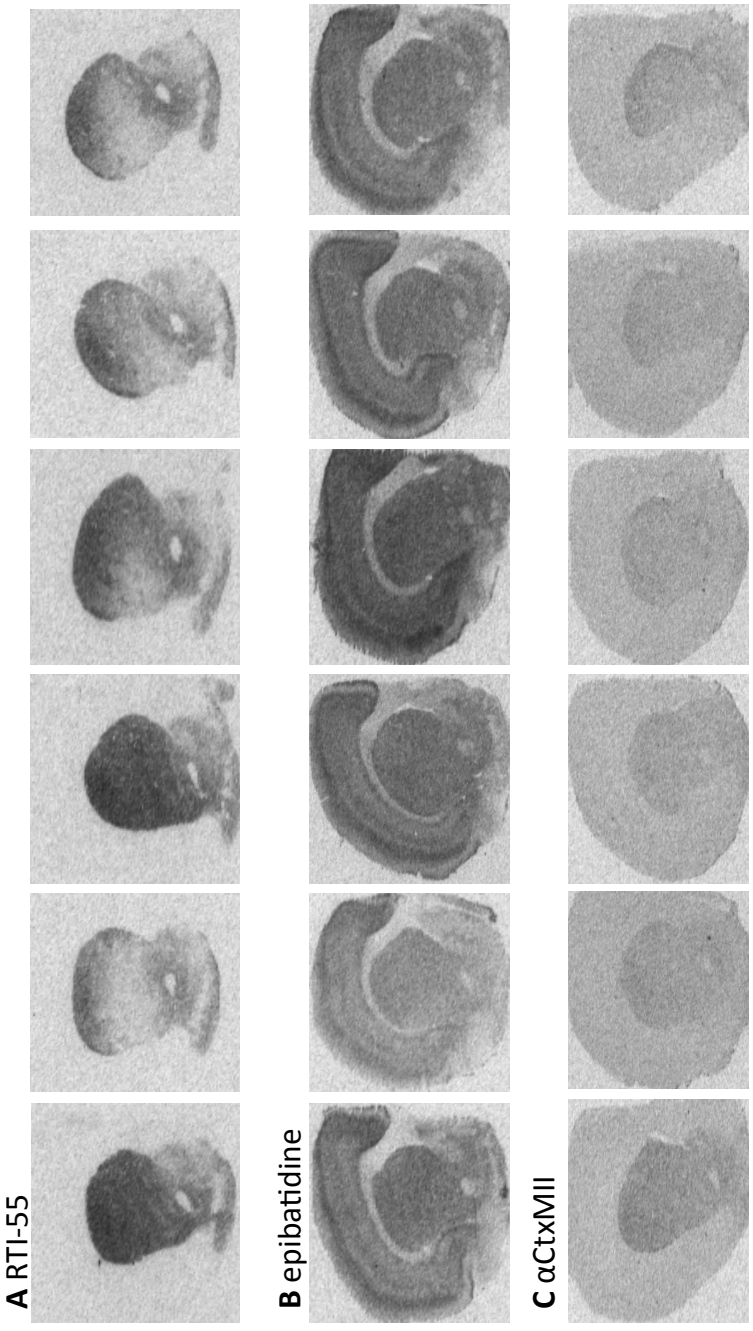


Figure 4.3 Long-term NIC preadministration does not protect α6β2-containing dopaminergic terminals. Rats received oral NIC (N) or tap water (S) from PND 40 to 96 (NM(1)), PND 40 to 89 (NM(2)), or PND 40 to 88 (NM(3)) and either saline (S) or METH (M) administrations at PND 89 as delineated in Methods. Striatal ^{125}I-αCtxMII binding was assessed 7 d after METH or saline injections. Data are expressed as mean $\pm$ S.E.M. of n = 7–9 determinations. *Represent values statistically different from SS ($p < 0.05$). Legend: SS = tap water/saline injections; SM = tap water/METH injections; NS = NIC water/saline injections; NM = NIC water/METH injections.

Figure 4.4 Representative autoradiographs depicting the effects of NIC and METH treatments on the striatum. A. DAT (^{125}I-RTI-55 binding), B. α4β2 nAChR (^{125}I-epibatidine binding) and C. α6β2 nAChR (^{125}I-αCtxMII) densities. Legend: SS = tap water/saline injections; SM = tap water/METH injections; NS = NIC water/saline injections; NM = NIC water/METH injections.


SS
SM
NS
NM(1)
NM(2)
NM(3)
A RTI-55
B epibatidine
C αCtxMII

CHAPTER 5

NICOTINE ADMINISTRATION ATTENUATES METHAMPHETAMINE-INDUCED NOVEL OBJECT RECOGNITION DEFICITS

Introduction

Methamphetamine (METH) abuse is a significant public health problem with annual prevalence rate of abuse in 2013 over 1% among adolescents and young adults (Johnston et al., 2014). Extensive clinical evidence indicates that METH abuse causes significant neurocognitive deficits (Kalechstein et al., 2003; Gonzalez et al., 2004; Hoffman et al., 2006; Kalechstein et al., 2009; Cherner et al., 2010; Casaletto et al., 2014). For example, episodic memory is reduced among participants with a history of METH abuse (~11 years), as assessed by performance in learning and recall tests (Casaletto et al., 2014). METH users also present with deficits in learning, motor ability, and working memory tests (Cherner et al., 2010). Neurocognitive deficits occur not only in individuals currently using METH (Simon et al., 2000), but can also persist long after METH is discontinued (4 d–7 months) (Kalechstein et al., 2003; Gonzalez et al., 2004; Hoffman et al., 2006; Kalechstein et al., 2009; Cherner et al., 2010; Casaletto et al., 2014). Among the different types of neurocognitive deficits caused by METH abuse, METH-associated neurocognitive deficits are greater for episodic

memory, executive functions, information processing speed and motor skills, and lesser for attention, working memory and verbal fluency (Scott et al., 2007). Notably, relapse is associated with episodic memory deficits, but not other types of cognitive dysfunction among METH abusers (Simon et al., 2004).

In addition to its impact on cognition, METH abuse causes brain abnormalities in areas important for episodic memory, such as the hippocampus and cortex. For example, Thompson et al. (2004) reported that METH abusers have 7.8% smaller hippocampal volumes than control subjects as assessed by MRI, and these deficits correlated with deficits in episodic memory. The integrity of hippocampal and cortical neurons can also be assessed by the binding of the serotonin transporter (SERT), a marker highly expressed in these neuronal regions (Lawrence et al., 1993; Meneses et al., 2011, for review). Loss of presynaptic serotonergic markers, such as SERT, indicates loss of this population of neurons. Studies have reported significant loss of serotonergic markers in the hippocampus and cortex of individuals with cognitive dysfunction, such as in METH abuse or Alzheimer's disease (Chen et al., 1996; Sekine et al., 2006; Ouchi et al., 2009). For example, positron emission tomography scan revealed that SERT densities are reduced in several brain regions of abstinent METH abusers (Sekine et al., 2006).

Novel object recognition (NOR) is an established preclinical model for evaluating episodic memory (see review by Kinnavane et al., 2014). This test relies on the instinct of rats to preferentially explore novel objects over familiar objects thus, requiring the animals to remember which object is familiar. The

perirhinal cortex (PRh) and the hippocampal regions CA1 and CA3 and dentate gyrus (DG) are important mediators of NOR (Melichercik et al., 2012; Kinnavane et al., 2014, for review). Specifically, the PRh-CA1 pathway is important for familiarization of objects, and the PRh-DG-CA3 pathway is important during exploration of novel objects (Kinnavane et al., 2014, for review). Overall, intact functions of these regions are required for NOR.

In preclinical studies, both contingent and/or noncontingent METH administrations have been shown to impair NOR (McCabe et al., 1987; Belcher et al., 2008; Herring et al., 2008; Tellez et al., 2010; Reichel et al., 2011; Reichel et al., 2012). Besides deficits in NOR, these studies in rats revealed significant deficits in SERT density in the hippocampus and PRh (Belcher et al., 2008; Reichel et al., 2012). Both clinical and preclinical evidence suggest that cortical and hippocampal SERT sites are important for learning and memory (Meneses et al., 2011, for review). Furthermore, preclinical evidence suggests a link between SERT sites and NOR. For example, significant NOR deficits occurred in SERT knockout mice (Olivier et al., 2008). Similarly, pretreatment with a selective SERT inhibitor, such as fluoxetine, attenuated both NOR deficits and hippocampal/cortical SERT density deficits in METH-treated rats (Tellez et al., 2010). These data suggest that abnormalities in SERT density in the hippocampus and/or PRh might mediate NOR deficits in rats. Overall, METH-induced NOR deficits might be related to abnormalities to the hippocampus, and PRh and these can be assessed via SERT densities.

Extensive evidence from clinical (Jubelt et al., 2008; Newhouse et al.,

2012; Sofuoglu et al., 2013; Kalechstein et al., 2014) and preclinical studies (Mizoguchi et al., 2011; Gould et al., 2013) has revealed that nicotinic acetylcholine receptor (nAChR) agonists have cognitive-enhancing properties. This is of importance because many METH abusers smoke cigarettes (~80%; Thompson et al., 2004; McCann et al., 2008) and are thus exposed to nicotine (NIC), a nAChR agonist; however, few studies have investigated the impact of NIC exposure onto METH-induced episodic memory deficits. Among these, in clinical trials, NIC patch application improved episodic memory or attention in patients with schizophrenia or Alzheimer's disease in comparison to placebo-treated patients (Jubelt et al., 2008; Newhouse et al., 2012). Activation of α4β2 subtypes of nAChRs also improved working memory in rhesus monkeys that self-administered cocaine (Gould et al., 2013). Further, preclinical studies with systemic or local infusion of NIC indicate the important role of α4β2 subtypes of nAChRs and the hippocampus and PRh in NOR (Melichercik et al., 2012).

The aim of current studies was three-fold. First, we investigated whether NIC impacts the episodic memory deficits caused by METH in rats chronically pre- or posttreated with NIC. Second, we investigated whether any potential cognitive neuroprotection afforded by NIC is mediated by protection of hippocampal and/or PRh serotonergic neurons. Finally, we explored whether α4β2 nAChRs contribute to the impact of NIC on cognitive performance in the NOR test. Results revealed that both NIC pre- and posttreatment attenuates METH-induced episodic memory deficits as assessed by NOR. This protection was accompanied by an increase in α4β2 nAChR binding, but not an attenuation

of METH-induced SERT density deficits in the hippocampus and PRh. These findings suggest that NIC-induced increases in α4β2 nAChR binding in the hippocampus and PRh may contribute to the NIC-induced attenuation of episodic memory deficits caused by METH.

Methods

Animals. Male Sprague-Dawley rats (Charles River Breeding Laboratories, Raleigh, NC), initially weighing 125–150 g (corresponding to postnatal day (PND) 40), 245–270 g (corresponding to PND 60) (Spear, 2000; Tirelli et al., 2003), or 350–415 g (corresponding to PND 89) were housed 2–3 rats per cage and maintained under a controlled light/dark cycle (14:10 h) and in an ambient environment of 20 °C (with the exception of the 6-h period during which METH or saline vehicle was administered during which the ambient environment was maintained at 24 °C). During METH or saline administrations, core body (rectal) temperatures were measured using a digital thermometer (Physitemp Instruments, Clifton, NJ) every 1 h beginning 30 min before the first saline or METH administration and continuing until 30 min after the final saline or METH administration. Rats were placed on a cooler environment if their body temperature exceeded 40.5 °C and returned to their home cage once their body temperature dropped to 40 °C. Food and water were available *ad libitum*. All experiments were approved by the University of Utah Institutional Animal Care and Use Committee, in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals 8th Edition* (Institute of Laboratory Animal Resources, 2011).

Drug treatments. METH hydrochloride was provided by the National Institute on Drug Abuse (Research Triangle Institute; Research Triangle Park, NC) and administered at 4 x 7.5 mg/kg/injection, s.c, 2-h intervals, with doses calculated as free-base. (-) NIC (1.010 g/ml; Sigma-Aldrich Co. LLC) was administered *ad libitum* p.o. at concentrations of 10, 20, 50, or 75 μg/ml via the water bottles. Dosing protocols are delineated in the figures. To increase palatability, saccharin (Sweet & Low; Cumberland Packing Corporation, New York, 1%) was added to the animals' drinking water only in experiments in which the initial NIC concentration was 75 μg/ml, or during the highest escalating rate (i.e., Figures 5.1B and C and Figure 5.2B). In our current studies, NIC water consumption was ~34 ml/rat/day, and tap water consumption was ~47 ml/rat/day, similarly to previous reports (Bordia et al., 2008). These NIC doses in rats yield plasma concentrations similar to plasma NIC and cotinine concentrations typically found in human smokers (10–50 ng/ml for NIC and 300 ng/ml for cotinine) (Benowitz, 1994; Matta et al., 2007).

NOR. After 3 days of recovery from METH or saline administrations, rats underwent a 5-min habituation session in test apparatus (clear plexiglas open field 45-cm wide x 26-cm height) in which they were allowed to explore the environment without the objects. On the following day (i.e., 5 d after METH) and during the familiarization phase, the rats explored two identical objects (plastic water bottles 12 cm tall) for 3 min. NOR test was conducted 90 min later by allowing rats to explore an object from the familiarization phase and a novel object for 3 min (polyvinyl chloride (PVC) pipe, 9 cm tall x 5 cm wide). The

position of the objects was counterbalanced between morning and afternoon sessions. The selection of these objects is based upon previously published research (Besheer and Bevins, 2000; Reichel et al., 2012) in which no difference in novel-object preference was observed with this pair of objects. The apparatus and objects were cleaned with CaviCide™ (Metrex Research, LLC, California) between each rat session. Each session was video recorded for later analysis by experimenters blinded to group treatment. Exploration was defined as sniffing or touching the object with the nose; sitting on or leaning against the object was not counted as exploration.

Tissue preparation. Rats were decapitated 7 d after METH treatment. Brains were hemisected, and the right side rapidly removed and frozen in isopentane on dry ice and stored at -80 °C. Frozen right hemisected brains were sliced at 12 µm thick at the level of the dorsal hippocampus/PRh (3.5 mm from bregma, Paxinos and Watson 6th edition) using a cryostat. Eight slices (four per rat) were mounted on each Superfrost® Plus glass micro slides (VWR International, Radnor, PA) and stored at -80 °C for subsequent use in autoradiography assays. The left hippocampus was dissected out on ice, placed in cold sucrose buffer (0.32 M sucrose, 3.8 mM NaH_2PO_4, and 12.7 mM Na_2HPO_4) and used for [^{3}H]5-HT uptake as described below. The striatal tissues from these animals were also processed, and data are presented in Chapters 3 and 4.

[^{125}I]RTI-55 autoradiography. SERT density was assessed via [^{125}I]RTI-55 binding to dorsal hippocampus and PRh slices as previously described (O'Dell

et al., 2012). Briefly, slides were thawed on a slide warmer (5–10 min) and incubated in sucrose buffer (10 mM sodium phosphate, 120 mM sodium chloride, 320 mM sucrose, pH 7.4) containing 21 pM [^{125}I]RTI-55 (2200 Ci/mmol, PerkinElmer, Watham, MA). Nonspecific binding was determined by slides incubated in sucrose buffer containing 21 pM [^{125}I]RTI-55 and 100 nM fluoxetine. Slides were rinsed twice in ice-cold buffer and distilled water for 2 min and air-dried. Sample slides and standard ^{125}I microscale slides (American Radiolabeled Chemicals, St. Louis, MO) were placed on one cassette and exposed to the same Kodak MR film (Eastman Kodak Co., Rochester, NY, USA) for 24 h to keep variables constant.

[^{125}I]-Epibatidine autoradiography. α4β2 nAChR density was assessed via [^{125}I]-epibatidine binding to dorsal hippocampus and perirhinal cortex (PRh) slices as previously described (Lai et al., 2005; Huang et al., 2009). Briefly, slides were thawed on a slide warmer (5–10 min) and preincubated in binding buffer (50 mM Tris, 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, pH 7.5) at room temperature for 30 min, followed by a 40-min incubation in binding buffer containing 0.015 nM [^{125}I]-epibatidine (2200 Ci/mmol, PerkinElmer, Watham, MA) in the presence of 100 nM αCtxMII. Nonspecific binding was determined by slides incubated in binding buffer containing 0.015 nM [^{125}I]-epibatidine plus 0.1 mM nicotine. Slides were rinsed twice in ice-cold buffer for 5 min followed by a 10 s rinse in distilled water. Slides were air-dried. Sample slides and standard ^{125}I microscale slides (American Radiolabeled Chemicals, St. Louis, MO) were placed on one cassette and exposed to same Kodak MR film (Eastman Kodak

Co., Rochester, NY, USA) for 24 h to keep variables constant.

Synaptosomal [^{3}H]5-HT uptake. Hippocampal [^{3}H]5-HT uptake was determined using rat hippocampal synaptosomes prepared as previously described (McFadden, Hunt, et al., 2012). Briefly, synaptosomes were prepared by homogenizing freshly dissected hippocampal tissue in ice-cold 0.32 M sucrose buffer (pH 7.4) and centrifuged (800 g, 12 min; 4 °C). The supernatants were centrifuged (22 000 g, 15 min; 4 °C) and the resulting pellets were resuspended in ice-cold assay buffer (in mM: 126 NaCl, 4.8 KCl, 1.3 CaCl2, 16 sodium phosphate, 1.4 MgSO4, 11 glucose and 1 ascorbic acid; pH 7.4) and 1 μM pargyline. Samples were incubated for 10 min at 37 °C, and the assays were initiated by the addition of [^{3}H]5-HT (5 nM final concentration). Following incubation for 3 min, samples were placed on ice to stop the reaction. Samples were then filtered through GF/B filters (Whatman, USA) soaked previously in 0.05% polyethylenimine. Filters were rapidly washed three times with 3 ml of ice-cold 0.32 M sucrose buffer using a filtering manifold (Brandel, USA). Nonspecific values were determined in the presence of 10 μM fluoxetine. Radioactivity trapped in filters was counted using a liquid scintillation counter. Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories Inc., USA).

Data quantitation and statistical analyses. The recognition index (i.e., the ratio of the time rats spend exploring the novel object divided by the time rats spend exploring both objects) was used as the dependent variable for episodic memory. Two independent, blinded raters scored each behavioral test with a

reliability correlation of ≥0.94 for the recognition index. For autoradiography, optical densities from four replicate slices per rat were quantified using ImageJ software (National Institutes of Health, USA). Specific binding was obtained by subtracting film background from mean density values and converting to fmol/mg using the standard curve generated from ^{125}I standards. The optical densities of the samples were within the linear range of the standards. Statistical analyses were conducted using GraphPad Prism 5.01 software (La Jolla, CA). The recognition index was first compared to the chance exploration value of 0.5 in each group. Values above 0.5 indicate higher preference for the novel object; thus, confirming occurrence of episodic memory. Mean concentrations ± standard error of the mean (SEM) were analyzed using one-way analysis of variance followed by Newman-Keuls post hoc test for determination of significance among groups. Correlation analysis of recognition index and either nAChR or SERT density was achieved by using Pearson correlation coefficient. Differences among groups were considered significant if the probability of error was less than 5% ($p < 0.05$).

Results

NIC administration via drinking water from adolescence to adulthood (i.e., PND 40–96) attenuated the NOR deficits caused by repeated high-dose METH injections when administered at PND 89 and with NOR testing performed on PND 94 (Figure 5.1A) ($F_{3,34} = 10.01$, $p < 0.0001$). Similarly, *ad libitum* exposure to an escalating-dose regimen of NIC attenuated METH-induced deficits in NOR when NIC was administered from PND 61 to 100 and METH at PND 93, with

NOR testing on PND 98 (Figure 5.1B) ($F_{3,30} = 3.698$, $p = 0.0224$). METH *per se* did not induce deficits in NOR when administered at PND 54 (Figure 5.1C) ($F_{3,31} = 1.273$, $p = 0.3009$).

Results presented in Figure 5.2A demonstrate that NIC pretreatment via drinking water from adolescence to adulthood (i.e., PND 40–89), but discontinued during and after METH, attenuated the NOR deficits caused by METH when METH was administered 2 h after NIC removal at PND 89 and NOR testing performed at PND 94 ($F_{5,38} = 2.342$, $p < 0.05$). NIC pretreatment from PND 40–88, and discontinued during and after METH, also attenuated METH-induced NOR deficits when METH was given 24 h after NIC removal on PND 89 and NOR testing performed at PND 94 ($F_{5,38} = 2.342$, $p < 0.05$). Furthermore, data presented in Figure 5.2B demonstrate that NIC posttreatment given from PND 89–96 initiated 2 h after the last METH injection also attenuated the NOR deficits caused by METH when assessed at PND 94 ($F_{3,35} = 3.296$, $p = 0.0316$)

Using the same tissues described in Figure 5.1A, data presented in Figure 5.3 indicate that NIC administration from PND 40–96 does not protect against the persistent (i.e., 7 d) METH-induced deficits in SERT density as assessed by [^{125}I]RTI-55 binding to hippocampal CA1 (panel A, $F_{3,34} = 59.53$), CA3 (panel B, $F_{3,34} = 35.51$), DG (panel C, $F_{3,34} = 61.90$) and PRh (panel D, $F_{3,33} = 56.32$) slices. Similarly, using tissues from the same animals described in Figures 5.1 and 5.2, data presented in Table 5.1 indicate that oral NIC administration does not protect against the persistent (i.e., 7 d) METH-induced deficits in SERT function as assessed by [^{3}H]5-HT uptake from hippocampal synaptosomes when NIC was

given from PND 40–96 ($F_{3,32}$ = 87.68), PND 61–100 ($F_{3,35}$ = 15.08), PND 40–88/89/96 ($F_{5,42}$ = 14.43), or PND 89–96 ($F_{3,34}$ = 40.04) and METH administered to adult rats (≥PND 89). When METH was administered to young adults at PND 54, NIC treatment from PND 40–61 attenuated the persistent (i.e., 7 d) METH-induced SERT function deficits ($F_{3,31}$ = 20.82) (Table 5.1).

Again, using the same tissues described in Figure 5.1A, data presented in Figure 5.4 reveal that binge METH administration *per se* causes long-lasting (i.e., 7 d) reduction in α4β2 nAChR density as assessed by [^{125}I]-epibatidine binding to CA1 (panel A) but has no effect on [^{125}I]-epibatidine binding in CA3 (panel B), DG (panel C), or PRh (panel D). NIC *per se* increased [^{125}I]-epibatidine binding to CA1 ($F_{3,35}$ = 97.71, $p < 0.0001$), CA3 ($F_{3,35}$ = 80.58, $p < 0.0001$), DG ($F_{3,35}$ = 18.57, $p < 0.0001$), and PRh ($F_{3,34}$ = 38.46, $p < 0.0001$) in both METH- and saline-treated rats (Figure 5.4).

Correlation analysis of data presented in Figures 5.3 and 5.4 was performed in order to evaluate possible association of NOR and SERT density (right y-axis) or NOR and α4β2 nAChR density (left y-axis). These data are presented in Figure 5.5 and demonstrate that NOR does not correlate with SERT density in the CA1 ($r(10) = 0.04$, $p = 0.556$, panel A), CA3 ($r(10) = 0.02$, $p = 0.648$, panel B), DG ($r(10) = 0.05$, $p = 0.507$, panel C) or PRh ($r(10) = 0.03$, $p = 0.590$, panel D) in rats treated with oral NIC from PND 40–96 and METH administrations at PND 89. Conversely, NOR and α4β2 nAChR density were positively correlated in the CA1 ($r(10) = 0.66$, $p = 0.002$), CA3 ($r(10) = 0.59$, $p = 0.006$), DG ($r(10) = 0.63$, $p = 0.006$), and PRh ($r(10) = 0.55$, $p = 0.009$) in these

same animals. No correlation between NOR and SERT density or NOR and α4β2 nAChR was found in METH-naïve rats or in NIC-naïve METH-treated rats (data not shown).

Representative autoradiograms of [^{125}I]RTI-55 and [^{125}I]-epibatidine binding are presented in Figure 5.6.

Discussion

To date, extensive literature has demonstrated that NIC administration improves memory function in patients with schizophrenia or dementia (Jubelt et al., 2008; Newhouse et al., 2012) or attenuates memory deficits in laboratory animals induced by sleep deprivation, chronic stress, beta-amyloid infusion, cholinergic lesion, or METH administrations (Yamazaki et al., 2002; Aleisa et al., 2011; Alkadhi, 2011; Mizoguchi et al., 2011; Kruk-Slomka et al., 2014). However, despite evidence indicating that METH abuse is associated with memory impairment (Kalechstein et al., 2003; Scott et al., 2007; Kalechstein et al., 2009; Casaletto et al., 2014) and that many METH addicts are exposed to NIC via cigarette smoking (McCann et al., 2008), few studies have investigated the effects of NIC on the METH-associated memory deficits (Mizoguchi et al., 2011). The present study reveals that long-term oral NIC treatment beginning during either adolescence or adulthood attenuates METH-induced episodic memory deficits as assessed by NOR, suggesting that NIC affords cognitive neuroprotection. This neuroprotective effect of NIC persisted even when NIC was removed 2 or 24 h prior to METH administrations. Furthermore, oral NIC treatment also attenuated NOR deficits when administered after METH

treatment. It is unlikely that the NIC effects on NOR are mediated by serotonergic neurons in the hippocampus and/or PRh because NIC did not attenuate METH-induced serotonergic deficits. In contrast, NIC increased the density of α4β2 nAChRs in CA1, CA3, DG and PRh in both, saline- and METH-treated rats.

Many studies have demonstrated that NIC prevents memory deficits when administered before a lesion (Yamazaki et al., 2002; Aleisa et al., 2011; Alkadhi, 2011; Srivareerat et al., 2011) or improved memory when administered after a lesion (Jubelt et al., 2008; Mizoguchi et al., 2011; Newhouse et al., 2012). Similarly, the current data indicate that both pre- and/or posttreatment with NIC attenuates METH-induced episodic memory deficits. These data suggest that the neuroprotective effect of NIC achieved via pretreatment might occur by neuroadaptations caused by NIC in a nondamaged system that can ultimately mitigate injury-induced memory loss. This hypothesis is based on findings that chronic NIC administration to healthy individuals (Perry et al., 1999) or nonlesioned rodents (Melichercik et al., 2012; Kruk-Slomka et al., 2014) increases nAChRs binding, leading to increases in long-term potentiation (LTP), a widely accepted process of memory formation (Fujii et al., 1999; Fujii et al., 2000; Welsby et al., 2006, 2009), and improves object recognition memory in normal rats (Melichercik et al., 2012; Kruk-Slomka et al., 2014). Additionally, these positive effects of NIC on memory seem to last several days after NIC removal (Levin and Torry, 1996) perhaps due to long-lasting increases in LTP (Yamazaki et al., 2006; Huang et al., 2008). In previous studies, *in vivo* NIC pretreatment prevented LTP deficits in area CA1 of the hippocampus in parallel

with attenuation of memory deficits induced by cholinergic lesion, chronic stress or beta-amyloid infusion (Yamazaki et al., 2002; Alkadhi, 2011; Srivareerat et al., 2011).

The neuroprotective effect of NIC achieved via posttreatment might occur via effects of NIC on brain derived nuclear factor (BDNF) as well as LTP, which ameliorate memory deficits in a damaged system (Yamazaki et al., 2002; Srivareerat et al., 2011). In support of this hypothesis, in laboratory animals, NIC reversed memory deficits induced by cholinergic lesion by augmenting NMDA receptors function and LTP in the CA1 region (Yamazaki et al., 2002). Some clinical studies have shown that NIC treatment improves episodic memory and attention in patients with schizophrenia or dementia, which demonstrates that NIC can ameliorate memory deficits in damaged systems (Levin et al., 1996; Jubelt et al., 2008; Newhouse et al., 2012).

To explore a possible mechanism underlying NIC-induced cognitive neuroprotection, we examined α4β2 nAChRs density in hippocampal and PRh slices. Previous studies have shown that the increases in hippocampal LTP by NIC administration are mediated by α4β2 nAChRs (Fujii et al., 2000; Jia et al., 2010; Nakauchi and Sumikawa, 2012). In fact, patients with dementia display significant reductions in α4β2 nAChRs binding in neocortical and hippocampal regions, and these seem to correlate with progressive cognitive declines (Perry et al., 2000). Particularly, in patients with Alzheimer's disease, there is a loss of α4β2 nAChRs binding, but not of α3 or α7 nAChR subtypes (Perry et al., 2000). The present findings revealed that chronic oral NIC administration attenuates

METH-induced deficits in α4β2 nAChRs binding to CA1 region of the hippocampus and augments α4β2 nAChRs binding to CA3, DG, and PRh. Furthermore, α4β2 nAChR density correlated with NOR performance in animals treated with NIC and METH. In other words, rat performance at the NOR test was directly proportional to α4β2 nAChR density. This specific subtype of nAChR was selected for study because despite evidence that synaptic plasticity can also be mediated by α7 nAChRs (Halff et al., 2014), studies have demonstrated that α4β2 nAChRs mediate excitatory postsynaptic potentials in the CA1 hippocampus (Bliss and Collingridge, 1993; Bell et al., 2011; Nakauchi and Sumikawa, 2012). Furthermore, *in vitro* studies by Swant et al. (2010) demonstrated that METH reduces LTP in the CA1 hippocampus, suggesting that, in combination with present data, METH reduces α4β2 nAChRs, whereby LTP is reduced in the CA1 region. The mechanism by which METH causes deficits in α4β2 nAChRs is unknown, but evidence indicates that METH damages hippocampal and cortical neurons integrity, including serotonergic neurons (McFadden, Hunt, et al., 2012; Reichel et al., 2012), where α4β2 nAChRs are expressed (Seth et al., 2002; Cucchiaro and Commons, 2003), which might lead to reductions in nAChRs density. Overall, these data suggest that NIC protection to METH-induced memory deficits might be mediated by upregulation of α4β2 nAChRs.

The effects of NIC abstinence on METH-induced NOR deficits were also evaluated in order to investigate how long NIC neuroprotection persists. As demonstrated in the NIC/saline group in Figure 5.2A, 5-d abstinence from NIC

had no effect on NOR as reported previously (Kenney et al., 2011). Nevertheless, NIC neuroprotection against METH-induced NOR deficits remained even 5–6 d after the cessation of NIC exposure (e.g., NM(2) and NM(3) Figure 5.2A) indicating that the neuroprotective effect of NIC lasts for at least 6 d of NIC abstinence. Others have demonstrated that NIC-induced augmentation in α4β2 nAChRs in the hippocampus remains after 6 d of NIC removal (Gould et al., 2012), suggesting that the potential neuroprotective mechanism of NIC parallels NOR protection. Furthermore, previous studies revealed that chronic NIC pretreatment improved working memory in rats even after 2 weeks of NIC abstinence (Levin and Torry, 1996). In the amygdala, 7 d oral NIC to mice facilitated LTP induced by high-frequency stimulation, and this facilitation of LTP lasted for at least 72 h after NIC was stopped (Huang et al., 2008). Similarly, increased NMDA receptors function induced by 10 d NIC in rats lasted for 8 d after NIC removal (Yamazaki et al., 2006). Others have suggested that the longer the NIC exposure, the longer synaptic facilitation lasts (Huang et al., 2008).

Another principal finding of current experiments is that long-term NIC treatment does not attenuate METH-induced serotonergic deficits in the hippocampus and PRh, despite affording protection against memory deficits. Previous studies have indicated that METH administrations to rats cause deficits in SERT density in the hippocampus and PRh as well as deficits in the NOR test, suggesting a possible relationship between hippocampal/PRh SERT neurons and NOR (Belcher et al., 2005; Belcher et al., 2008; Reichel et al., 2012). However, the present data suggest that such a relationship is unlikely as

indicated by data presented in Figure 5.1C and Table 5.1, in which METH administration caused significant whole hippocampal SERT deficits, but not NOR deficits when given to PND 54 rats. Of note, NOR is strongly mediated by PRh functions, and thus it is possible that these animals had no SERT deficits in the PRh, which could explain the lack of NOR deficits. Secondly, several NIC treatment paradigms attenuated METH-induced NOR deficits independently of attenuation of SERT density deficits. Lastly, our correlation analysis demonstrates that performance in the NOR test is not correlated with SERT density in hippocampal or PRh regions. In agreement with these data, previous studies have indicated that SERT loss correlates with either depression or anxiety in individuals with memory dysfunction, such Alzheimer's disease or METH dependence (Chen et al., 1996; Sekine et al., 2006; Ouchi et al., 2009). For example, NIC increases 5-HT release, which activates 5-HT(1A) receptors and induces anxiogenic effects (see review by Seth et al., 2002). Thus, current data suggesting that NIC neuroprotection to METH-induced memory deficits is not mediated by protection of serotonergic neurons in the hippocampus or PRh cortex are in agreement with previous findings demonstrating that the serotonergic effects of NIC in the hippocampus and cortex are associated with anxiety or aggression, as opposed to memory deficits.

Notably, current data also revealed that NIC administration beginning in adolescence attenuates METH-induced hippocampal SERT function deficits when METH was administered to young rats (i.e., PND 54), but not older rats ($\geq$PND 89) (Table 5.1). A possible mechanism by which NIC attenuates METH-

induced deficits in SERT function in young, but not older, rats might involve nAChRs. The α4β2 and α7 nAChRs play a role in 5-HT release in the hippocampus, as demonstrated by findings that they are expressed either in the nucleus raphe or in serotonergic terminals in the hippocampus (Cucchiaro and Commons, 2003; Aznar et al., 2005). Furthermore, age-differences in nAChRs density in the hippocampus and cortex have been reported (Doura et al., 2008). Specifically, these studies found that adolescent rats have a higher density of nAChRs than adult rats. Thus, NIC binding to nAChRs potentially may lead to a 5-HT release in the hippocampus/cortex that might differ between adolescents and adults.

Clinical studies with adolescent METH abusers have demonstrated that executive function is only mildly compromised and verbal memory is not affected, despite several domains of cognitive function being impaired (psychomotor speed, fine motor speed, verbal intelligence, and spatial organization) (King et al., 2010). In line with these findings, previous preclinical studies with adolescent mice showed that 7 or 14 d of noncontingent METH administrations did not impact NOR and synaptic plasticity (North et al., 2012). Of note, METH administrations to PND 51–60 do not induce long-term deficits in spatial memory in the Morris water maze (Vorhees et al., 2005). These previous findings are in agreement with current data shown in Figure 5.1C in which METH administration *per se* given to PND 54 rats does not impact episodic memory as assessed by NOR.

In summary, NIC has cognitive neuroprotection and cognitive enhancing

properties, and several mechanisms underlying this phenomenon have been suggested including increases in LTP and BDNF levels and reduction in oxidative stress (Soto-Otero et al., 2002; Srivareerat et al., 2011). METH abuse is associated with significant cognitive impairment and, despite the fact that many METH abusers smoke cigarettes and are thus exposed to NIC (McCann et al., 2008), few studies have evaluated the effects of NIC on METH-induced cognitive deficits (Mizoguchi et al., 2011). The findings of the present studies demonstrate that NIC pretreatment as well as posttreatment attenuate METH-induced episodic memory deficits as assessed by NOR in rats. Furthermore, NIC did not attenuate the serotonergic deficits caused by METH, but augmented α4β2 nAChRs density in CA1, CA3, and DG hippocampal regions as well as in PRh.

Figure 5.1 Chronic NIC administration via drinking water initiated during adolescence or adulthood attenuates NOR deficits caused by METH. A. Rats received oral NIC (N) at increasing concentrations via drinking water at doses delineated in figure inset or tap water (S) from PND 40 to 96 and either saline (S) or METH (M) administrations (4 x 7.5 mg/kg/injection, s.c., 2 h apart) at PND 89. B. Rats received oral NIC (N) at increasing concentrations via drinking water at doses delineated in figure inset or tap water (S) from PND 61 to 100 and either saline (S) or METH (M) administrations (4 x 7.5 mg/kg/injection, s.c., 2 h apart) at PND 93. C. Rats received oral NIC (N) at increasing concentrations via drinking water at doses delineated in figure inset or tap water (S) from PND 40 to 61 and either saline (S) or METH (M) administrations (4 x 7.5 mg/kg/injection, s.c., 2 h apart) at PND 54. NOR was assessed 5 days after METH. Data are expressed as mean $\pm$ S.E.M. of (A) n = 8–10, (B) n = 8–11 or (C) n = 7–10 determinations. *Represent values statistically different from saline-controls ($p < 0.05$) as well as values not different from the chance exploration of 0.5 illustrated by the dashed line ($p < 0.05$). Legend: SS = tap water/saline injections; SM = tap water/METH injections; NS = NIC water/saline injections; NM = NIC water/METH injections.

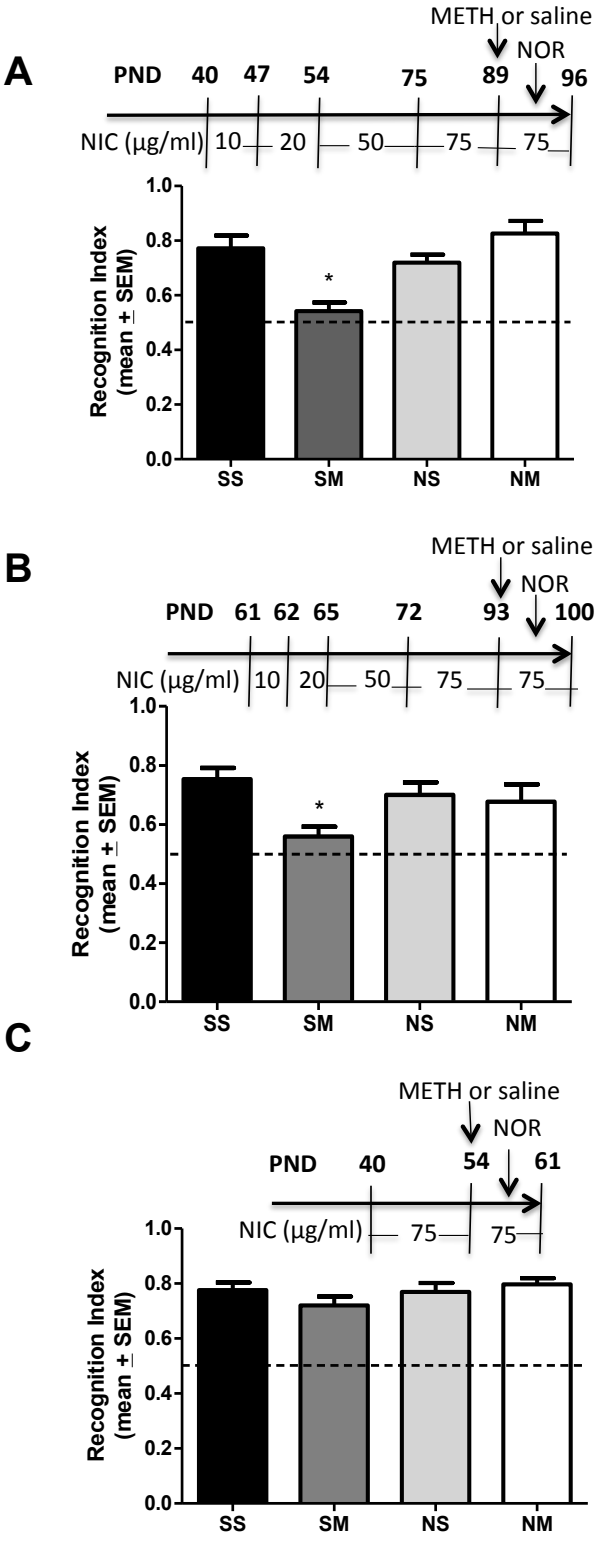
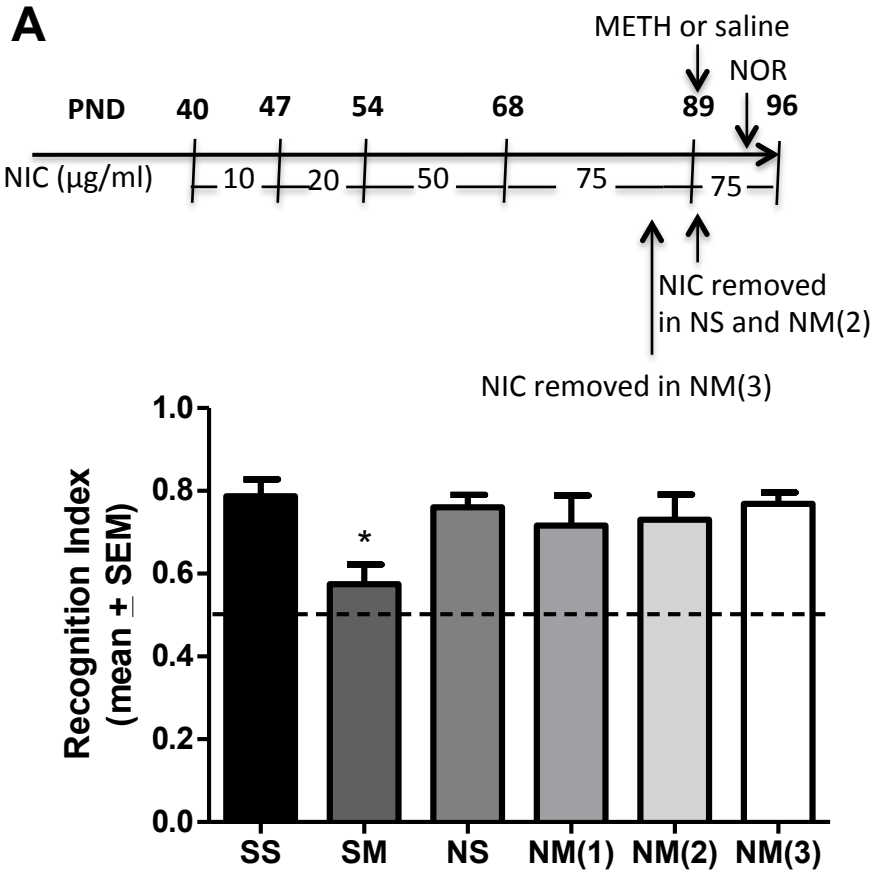

A
METH or saline
NOR
PND 40 47 54 75 89 96
NIC (µg/ml) 10 20 50 75 75
Recognition Index (mean ± SEM)
1.0
0.8
0.6
0.4
0.2
0.0
*
SS SM NS NM
B
METH or saline
NOR
PND 61 62 65 72 93 100
NIC (µg/ml) 10 20 50 75 75
Recognition Index (mean ± SEM)
1.0
0.8
0.6
0.4
0.2
0.0
*
SS SM NS NM
C
METH or saline
NOR
PND 40 54 61
NIC (µg/ml) 75 75
Recognition Index (mean ± SEM)
1.0
0.8
0.6
0.4
0.2
0.0
SS SM NS NM

Figure 5.2 Chronic NIC administration via drinking water given either as pretreatment or posttreatment attenuates METH-induced deficits in NOR. A. PND 40 rats received either tap water (SS and SM groups) or NIC water (10–75 µg/ml) until PND 88 (NM(3) group), PND 89 (NS and NM(2) group) or PND 96 (NM(1) group). METH (4 x 7.5 mg/kg/injection, s.c., 2 h apart) or saline (1ml/kg/injection) was given at PND 89. In NM(3) group NIC water was replaced by tap water 24 h prior to the first METH injection. In NS and NM(2) groups NIC water was replaced by tap water 2 h prior to the first METH or saline injection. B. PND 89 rats received either METH (4 x 7.5 mg/kg/injection, s.c., 2 h apart) or saline (1ml/kg/injection) injections, and 2 h after the last injection they received either tap water (SS and SM groups) or NIC water (75 µg/ml) until PND 96 (NS and NM groups). NOR testing was initiated 3 d after METH or saline injections. Data are expressed as mean $\pm$ S.E.M. of n = 6–11 determinations. *Represent values statistically different from saline-controls ($p < 0.05$) as well as values that are not statistically different from the chance exploration of 0.5 illustrated by the dashed line ($p < 0.05$). Legend: SS = tap water/saline injections; SM = tap water/METH injections; NS = NIC water/saline injections; NM = NIC water/METH injections.


A
METH or saline
NOR
PND
40
47
54
68
89
96
NIC (µg/ml)
10
20
50
75
75
NIC removed
in NS and NM(2)
NIC removed in NM(3)
Recognition Index
(mean ± SEM)
1.0
0.8
0.6
0.4
0.2
0.0
*
SS
SM
NS
NM(1)
NM(2)
NM(3)

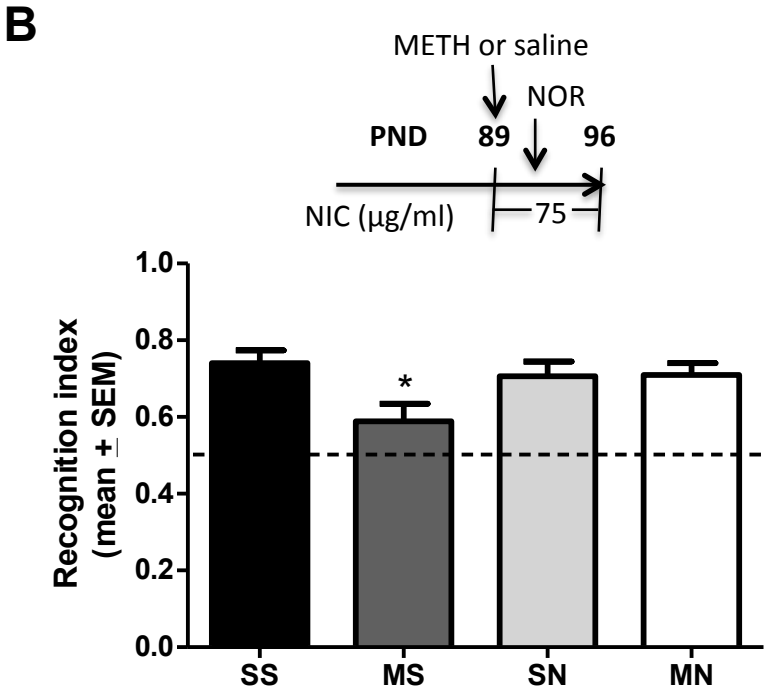

B
METH or saline
NOR
PND
89
96
NIC (µg/ml)
75
Recognition index
(mean ± SEM)
1.0
0.8
0.6
0.4
0.2
0.0
*
SS
MS
SN
MN

Figure 5.3 NIC neuroprotection of METH-induced memory deficits is not mediated by attenuation of serotonergic deficits. Rats were treated as described in Figure 5.1. panel A. Brains were harvested 7 d after METH or saline injections and SERT binding to A. Hippocampal CA1 region B. Hippocampal CA3 region C. Hippocampal DG region and C. PRh was assessed via [^{125}I]RTI-55 autoradiography. Data are expressed as mean values $\pm$ S.E.M. of n = 8–12 determinations. *Represent values that are statistically different from saline-controls ($p < 0.05$). Legend: SS = tap water/saline injections; SM = tap water/METH injections; NS = NIC water/saline injections; NM = NIC water/METH injections.

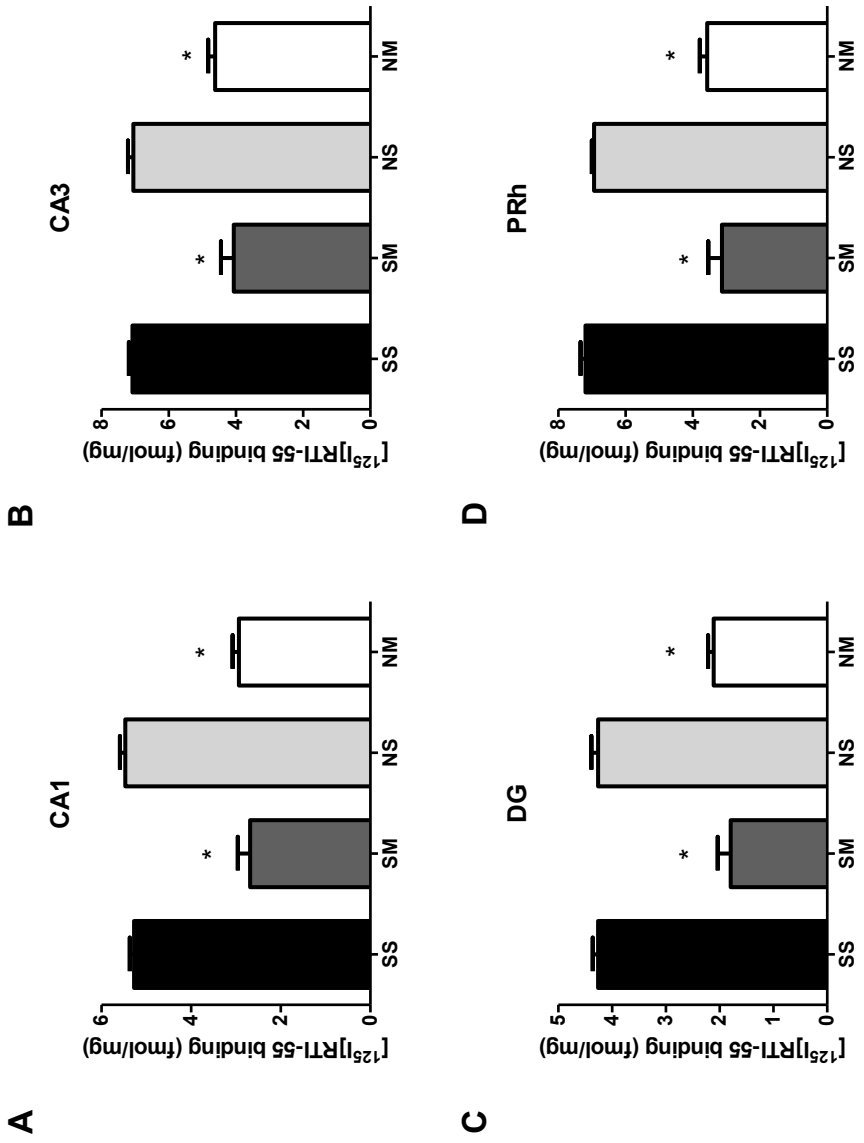

A
CA1
[125I]RTI-55 binding (fmol/mg)
0
2
4
6
SS
SM
NS
NM
*
*
B
CA3
[125I]RTI-55 binding (fmol/mg)
0
2
4
6
8
SS
SM
NS
NM
*
*
C
DG
[125I]RTI-55 binding (fmol/mg)
0
1
2
3
4
5
SS
SM
NS
NM
*
*
D
PRh
[125I]RTI-55 binding (fmol/mg)
0
2
4
6
8
SS
SM
NS
NM
*
*

Table 5.1 Long-term NIC administration does not attenuate METH-induced deficits in [^{3}H]5-HT uptake from hippocampal synaptosomes.

[^{3}H]5-HT uptake from hippocampal synaptosomes (fmol/mg) *(SEM)*

Experiment	Treatment Group					
	SS	SM	NS	NM(1)	NM(2)	NM(3)
PND 40–96 *(Figure 5.1A)*	1.467 *(0.061)*	0.296* *(0.061)*	1.393 *(0.101)*	0.308* *(0.055)*		
PND 61–100 *(Figure 5.1B)*	0.547 *(0.045)*	0.251* *(0.048)*	0.611 *(0.050)*	0.291* *(0.042)*		
PND 40–61 *(Figure 5.1C)*	1.048 *(0.097)*	0.286* *(0.057)*	1.293 *(0.145)*	0.611*# *(0.079)*		
PND 40–88/89/96 *(Figure 5.2A)*	0.611 *(0.026)*	0.160* *(0.024)*	0.599 *(0.041)*	0.286* *(0.063)*	0.313* *(0.065)*	0.336* *(0.047)*
PND 89–96 *(Figure 5.2B)*	1.455 *(0.058)*	0.542* *(0.070)*	1.528 *(0.094)*	0.653* *(0.095)*		

*Rats received oral NIC (N) via drinking water or tap water (S) from PND40–96, PND61–100, PND40–61, PND40–88 (NM(3)), 89 (NM(2)), or 96 (NM(1)) or from PND89–96 and either saline (S) or METH (M) injections at doses and ages delineated in figures 5.1 and 5.2 insets and methods. Hippocampal tissues were harvested 7 d after last METH or saline injection. [^{3}H]5-HT uptake was performed as described in methods. Data are expressed as mean values ± S.E.M. *Values significant different from METH-naïve controls ($p < 0.05$). #Values significant different from SM.*

Figure 5.4 Long-term NIC administration increases hippocampal and PRh α4β2 nAChRs density in METH-treated rats. Rats were treated as described in Figure 5.1 panel A. Brains were harvested 7 d after METH or saline injections and α4β2 density to A. Hippocampal CA1 region B. Hippocampal CA3 region C. Hippocampal DG region and D. PRh was assessed via [^{125}I]-epibatidine autoradiography. Data are expressed as mean values $\pm$ S.E.M. of n = 8–12 determinations. *Represent values that are statistically different from SS (p < 0.05). #Represent values that are statistically different from SM (p < 0.05). Legend: SS = tap water/saline injections; SM = tap water/METH injections; NS = NIC water/saline injections; NM = NIC water/METH injections.

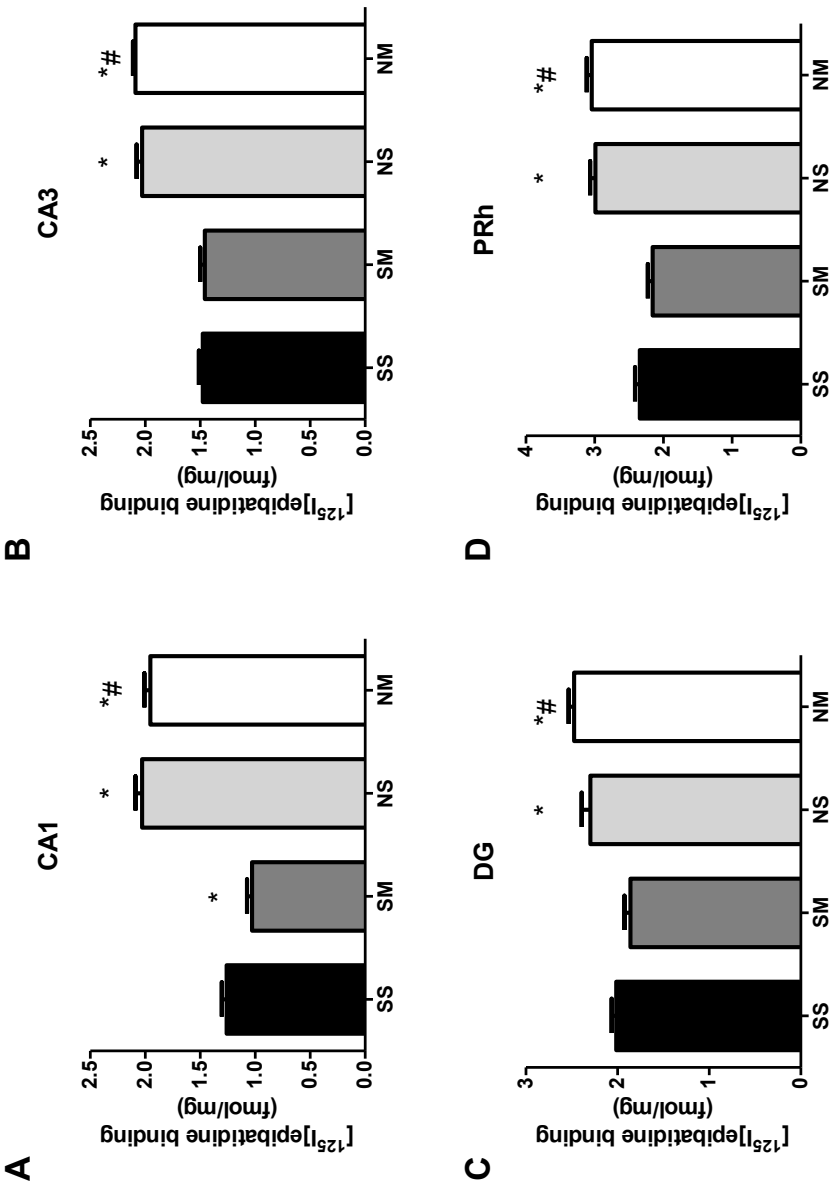

A
CA1
B
CA3
C
DG
D
PRh
[125I]epibatidine binding
(fmol/mg)
SS
SM
NS
NM
*
*#
#*

Figure 5.5 Performance in NOR test correlates with α4β2 nAChRs density, but not with SERT density, in rats treated with NIC and METH. Data from NM group presented in Figures 5.3 and 5.4 were used for this correlation analysis.

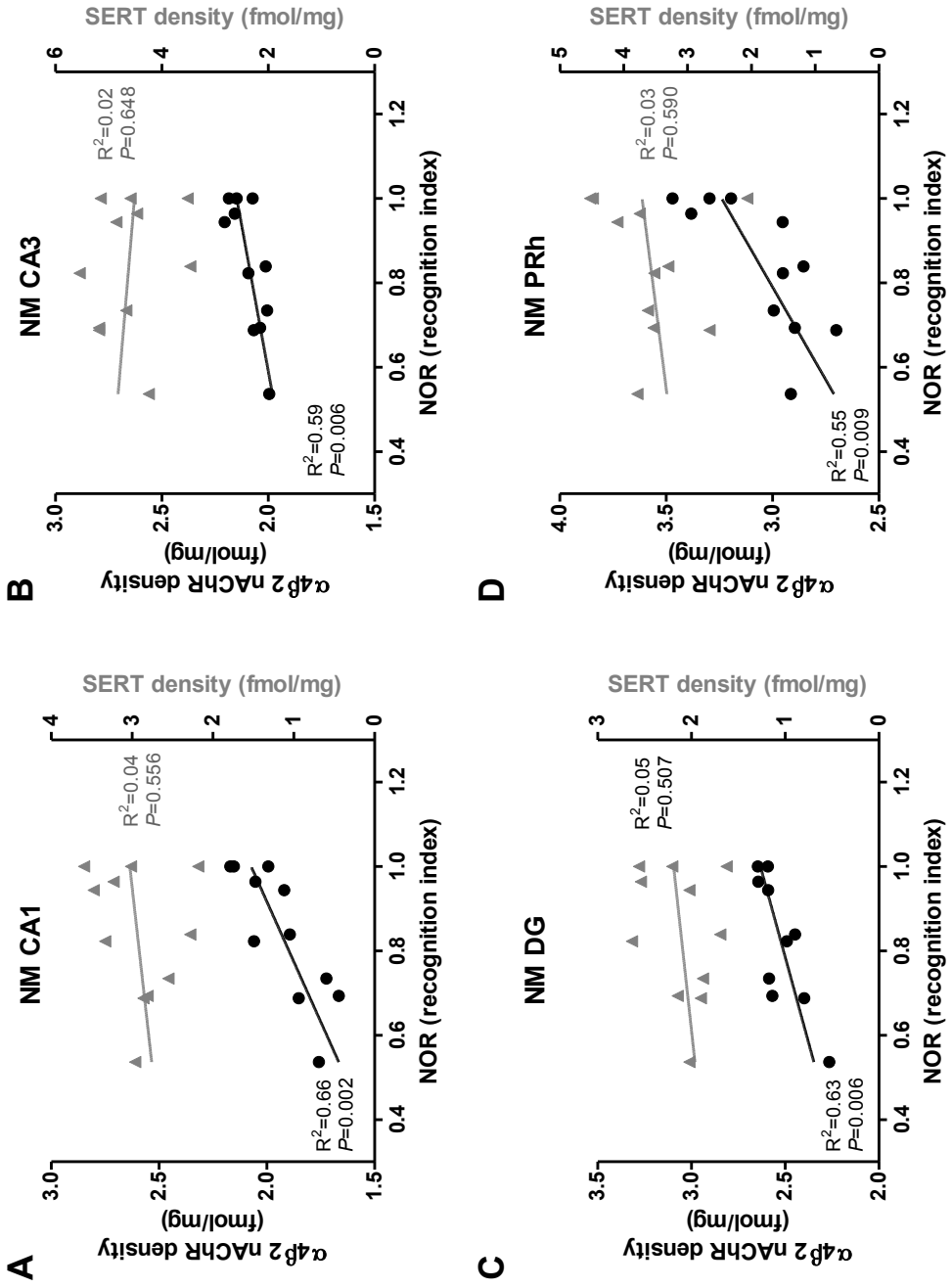
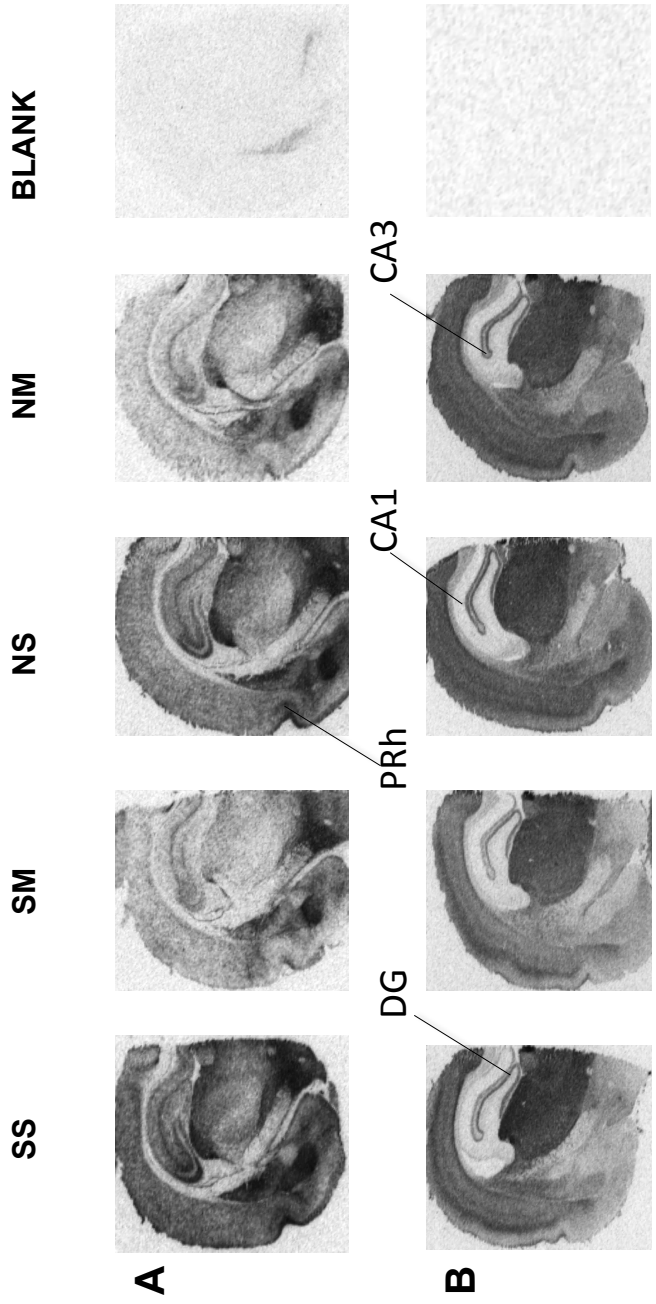

A
NM CA1
α4β2 nAChR density (fmol/mg)
SERT density (fmol/mg)
NOR (recognition index)
R2=0.04
P=0.556
R2=0.66
P=0.002
B
NM CA3
α4β2 nAChR density (fmol/mg)
SERT density (fmol/mg)
NOR (recognition index)
R2=0.02
P=0.648
R2=0.59
P=0.006
C
NM DG
α4β2 nAChR density (fmol/mg)
SERT density (fmol/mg)
NOR (recognition index)
R2=0.05
P=0.507
R2=0.63
P=0.006
D
NM PRh
α4β2 nAChR density (fmol/mg)
SERT density (fmol/mg)
NOR (recognition index)
R2=0.03
P=0.590
R2=0.55
P=0.009

Figure 5.6 Representative autoradiographs depicting the effects of NIC and METH treatments on hippocampus and PRh. A. SERT ([^{125}I]RTI-55 binding), B. α4β2 nAChR ([^{125}I]-epibatidine binding) Legend: SS = tap water/saline injections; SM = tap water/METH injections; NS = NIC water/saline injections; NM = NIC water/METH injections; Blank = nonspecific binding.


BLANK
NM
NS
SM
SS
A
B
CA3
CA1
PRh
DG

CHAPTER 6

CONCLUDING REMARKS AND FUTURE DIRECTIONS

This dissertation consisted of three aims. The first aim was to investigate whether nicotine (NIC) administration affords neuroprotection to the dopaminergic, serotonergic, and episodic memory deficits caused by methamphetamine (METH). The second aim was to determine the impact of age of NIC onset, duration of NIC treatment, and NIC withdrawal on METH-induced dopaminergic, serotonergic, and memory deficits. Lastly, the third aim consisted of investigating the involvement of β2-containing nicotinic receptors in the neuroprotection afforded by NIC. The overall hypothesis tested in this dissertation was that long-term NIC treatment protects against METH-induced dopaminergic and memory deficits through actions involving β2-nicotinic receptors-associated systems.

Results presented in this dissertation demonstrated that chronic NIC pre plus postadministration or preadministration only to rats, given intermittently via drinking water as to mimic the human smoking condition, attenuated the persistent (i.e., 7-d) METH-induced dopaminergic deficits in striatal and nucleus accumbens core when NIC was initiated either during adolescence (postnatal date (PND) 40) or young adulthood (PND 61) and METH administered during adulthood (PND 89–100). However, a longer duration of NIC administration was

necessary for dopaminergic protection to occur when NIC was initiated during young adulthood (PND 61). Notably, dopaminergic neuroprotection remained even when NIC was removed 2h or 24h before METH administrations (i.e., when NIC was absent during and the 7 d after METH). These data indicated that chronic NIC preadministration affords neuroprotection to the dopaminergic deficits caused by METH and that both, age of onset and duration of NIC treatment are important for neuroprotection, but 24 h NIC withdrawal does not interfere with neuroprotection.

Data presented in this dissertation also revealed that chronic NIC pre- plus postadministration, preadministration only, or postadministration only to rats, also via drinking water, attenuated METH-induced episodic memory deficits when NIC was initiated either during adolescence (PND 40) or young adulthood (PND 61) and METH administered either during youth (PND 54) or adulthood (89–100). Furthermore, memory neuroprotection remained even when NIC was removed 2 h or 24 h before METH administrations. In other words, in every experiment tested, NIC afforded memory neuroprotection indicating that none, age of NIC onset, duration of NIC treatment, or NIC withdrawal interfere with memory neuroprotection. However, oral NIC treatment only afforded serotonergic neuroprotection when given from PND 40 to 61 with METH administrations at PND 54. These data indicated that NIC memory neuroprotection is not mediated by protecting the integrity of serotonergic neurons in the hippocampus or perirhinal cortex (PRh).

Lastly, this dissertation presented several findings on the involvement of

β2-nicotinic receptors in NIC dopaminergic and memory neuroprotection. First, METH administrations *per se* caused persistent major deficits in α6β2 nicotinic acetylcholine receptors (nAChRs) (~25%) and minor deficits in α4β2 nAChRs (~10%) density in the striatum and nucleus accumbens core. Second, in METH-treated rats pre-exposed to NIC, α4β2 nAChR density was increased. These effects persisted even when NIC was removed 7–8 d prior to assay. However, exposure to NIC did not attenuate METH-induced deficits in α6β2 nAChRs, suggesting that dopaminergic terminals expressing α6β2 receptors are more vulnerable to METH neurotoxicity. Lastly, chronic NIC administration *per se* caused upregulation of α4β2 and downregulation in α6β2 nAChRs density. Previous evidence demonstrated that chronic NIC administration leads to upregulation of α4β2 with concomitant downregulation of α6β2 because of exchange in α6 for α4 subunits. Thus, data suggest that by the time rats received METH administrations, those rats pre-exposed to chronic NIC had more terminals expressing α4β2 and fewer terminals expressing α6β2 nAChRs. These findings lead to the speculation that replacing α6β2 by α4β2 nAChRs in dopaminergic terminals is neuroprotective. In the hippocampus, METH administrations *per se* caused persistent deficits in α4β2 nAChR density in the CA1 region, and chronic NIC exposure not only attenuated these deficits, but also increased α4β2 density in the CA3, dentate gyrus and PRh. Furthermore, α4β2 nAChRs density was positively correlated with episodic memory in METH-treated rats pre-exposed to NIC, suggesting that NIC memory neuroprotection is mediated by increases in α4β2 receptors in these regions.

As noted above and presented in this dissertation, high-dose METH administration *per se* caused persistent deficits in nAChRs density in the striatum, nucleus accumbens core and hippocampus. However, the mechanisms by which METH causes deficits in nAChRs in these regions are unknown. Previous *in vitro* studies demonstrated that METH directly binds to nAChRs and alters their trafficking (Garcia-Rates et al., 2007; Chipana et al., 2008). Data from these studies further indicated that METH binding to nAChRs causes calcium release and oxidative stress. However, it is unclear whether these phenomena occur *in vivo,* as well as whether these potential METH-induced alterations in nAChRs are persistent and contribute to neurotoxicity. These present findings will provide further insights into the mechanism of NIC neuroprotection as well as potential targets for remedies for the neurotoxic consequences of METH since, as presented in this dissertation, nAChRs might be involved in neuroprotection.

As shown in this dissertation, METH reduced α4β2 nAChRs density in the striatum and hippocampus. These receptors are located throughout these regions, i.e., α4β2 nAChRs are found in dopaminergic or serotonergic, glutamatergic, GABAergic and cholinergic neurons in the striatum and hippocampus. It is unknown whether only a specific population of these neurons expressing α4β2 nAChRs is susceptible to METH neurotoxicity. In other words, is METH leading to α4β2 nAChRs reductions in dopaminergic, glutamatergic, GABAergic, and cholinergic neurons or only in dopaminergic neurons? Future studies with double-labeling techniques investigating the colocalization of α4β2 nAChRs, and either dopamine (DA) transporter (DAT) or serotonin transporter

(SERT) will provide answers to which neurons are being affected by METH and consequently which neurons are being protected by NIC. Data presented herein showed that NIC administration did not protect against METH-induced SERT deficits, but still attenuated both memory deficits and α4β2 nAChRs density deficits. These data suggest that NIC-induced upregulation of α4β2 nAChRs located in serotonergic neurons are not mediating episodic memory protection afforded by NIC. Data suggest that the NIC-induced upregulation in α4β2 nAChRs that mediate memory neuroprotection are located in neurons other than serotonergic.

Another important consideration from studies presented in this dissertation is that observations on nAChRs, DAT, and NIC/metabolites levels were evaluated 7 d after METH administrations. In order to further study the mechanism of NIC neuroprotection, these outcome measures (nAChRs, DAT, NIC/metabolites) should be assessed shortly after METH or NIC administrations. For example, is NIC altering the acute effects of METH on DAT and SERT function? Do METH administrations acutely cause alterations in nAChRs density and is NIC affecting this mechanism? Are NIC/metabolites absent 2 or 24 h after NIC removal from drinking water?

As discussed above, the proposed model of NIC dopaminergic neuroprotection is that NIC has increased α4β2 nAChR and decreased α6β2 nAChR expression by the time rats receive METH administrations, as seen in data from rats treated chronically with NIC via drinking water and saline injections. These effects potentially lead to reduction in phasic (vesicular) DA

release and increase in tonic (nonvesicular/DAT reversal) DA release, which potentially leads to reduction in cytosolic reactive species, increased vesicular DA sequestration, and reduction in postsynaptic DA receptor activation. If pre-exposure to NIC decreases METH-induced vesicular DA release, both vesicular DA content and potassium-stimulated DA release should be greater in METH-treated rats pre-exposed to NIC in comparison to METH-treated rats naïve to NIC. Postsynaptic events could be assessed by the downstream effect of DA receptor activation, such as neurotensin levels. For example, high-dose METH administration acutely increases neurotensin tissue levels in the striatum, and this effect is blocked by D1, but not D2, antagonist administration (Hanson et al., 1992; Wagstaff et al., 1996). These findings indicate that METH causes DA release, which activates D1 receptors and leads to increased neurotensin tissue levels. If NIC pre-exposure acutely reduces METH-induced vesicular DA release and hence DA receptor activation, METH-treated rats pre-exposed to NIC should have a lower neurotensin tissue level than METH-treated rats naïve to NIC 1-h after the last METH administration. In fact, our unpublished data not included in this dissertation revealed that 7 d after METH administrations pre-exposure to chronic oral NIC blocked METH-induced increases in neurotensin content.

The function of nAChRs was not assessed in this dissertation. In order to investigate whether α4β2 and α6β2 receptors mediate NIC neuroprotection, pharmacological studies with activation or inhibition of these receptors prior to METH administrations are needed. For example, data presented in this dissertation suggest that upregulation of α4β2 nAChRs might mediate the

dopaminergic and memory NIC neuroprotective effect in METH abuse models. However, it is not clear whether selectively increasing the function of α4β2 nAChRs during METH administrations is the mechanism of NIC neuroprotection, which in this case, could be achieved by administering a α4β2 agonist acutely prior to METH. The most selective α4β2 agonist, A85380, is also an agonist to α6β2 nAChRs. Thus, dopaminergic neuroprotection studies should be designed to evaluate administration of A85380 (i.p.) alone or in combination with the α6β2 antagonist α-conotoxin-MII (i.c.v.). Of note, α6β2 nAChRs are not expressed in the hippocampus and PRh; thus, A85380 administration alone would be sufficient to test the hypothesis that α4β2 nAChRs activation protects against METH-induced novel object recognition (NOR) deficits. Another way to test this hypothesis is to treat rats chronically with NIC (via drinking water as performed in this dissertation) and administer the α4β2 antagonist dihydro-beta-erythroidine just prior to METH administrations. In case NIC is affording protection by increasing α4β2 function, then the α4β2 antagonist should inhibit NIC neuroprotection against the persistent METH-induced DAT and NOR deficits. On the other hand, this dissertation also suggests that NIC is affording dopaminergic neuroprotection by reducing α6β2 nAChRs function. In this case, it is expected that a selective α6β2 antagonist given just prior to METH should afford neuroprotection as well.

Other nAChRs might be involved in NIC neuroprotection against METH-induced dopaminergic deficits. The α7 nAChR subtype mediates glutamate release in the striatum, and its antagonism protects against METH-induced

striatal dopaminergic deficits (Escubedo et al., 2009; Northrop et al., 2011). However, it is not known whether METH administrations cause acute and/or persistent deficits in α7 nAChR density. Furthermore, it is unclear whether α7 nAChRs play a role in NIC dopaminergic neuroprotection. For example, chronic NIC administration to rodents either upregulates or has no effect on α7 nAChR expression (Buisson and Bertrand, 2002; Slotkin et al., 2004), suggesting that either activation of α7 mediates neuroprotection or that α7 does not play a role in neuroprotection. Thus, assessing striatal α7 receptors expression in METH-treated rats, as well as assessing DAT function/density in METH-treated rats pre-exposed acutely to α7 agonist (PNU 282987; i.p.) or antagonist (methyllycaconitine (MLA); 5 mg/kg, i.p.) would provide insights into the role of this receptor subtype in neurotoxicity and neuroprotection.

The α7 nAChR subtype is also involved in memory function, and its activation has been shown to attenuate memory deficits in models other than METH. This dissertation did not assess the role of α7 receptors in METH-induced episodic memory deficits, nor assessed the potential involvement of this receptor in NIC memory neuroprotection. Furthermore, it is not known whether METH administrations acutely and/or persistently affect α7 density in the hippocampus and PRh. Future studies should compare α7 expression in the hippocampus and cortex of METH-treated rats naïve to NIC and METH-treated rats chronically pretreated with NIC, then possibly test whether selective activation of α7 (with PNU 282987; i.p.) attenuates METH-induced NOR deficits as well as whether selective antagonism of α7 (with α-bungarotoxin; i.c.v.) during METH

administrations inhibits memory neuroprotection afforded by chronic NIC pre-exposure.

Lastly, other future studies could investigate the potential role of nAChRs among other neuroprotective agents, as well as whether other agents known to modulate nAChRs are also neuroprotective. In other words, is NIC the substance that affords neuroprotection, or are the changes in nAChRs induced by NIC that afford neuroprotection? For example, physical activity protects against the persistent METH neurotoxicity in rats as shown by a running wheel exercise (O'Dell et al., 2012). Several studies have shown that physical activity modulates nAChRs (Desaulniers et al., 1998). For example, exercise training altered nAChRs in the skeletal muscle improving recovery from spasticity in rats (Tsai et al., 2013). Thus, in this case whether running wheel exercise modulated nAChRs in the striatum of METH–treated rats to afford dopaminergic neuroprotection is unknown.

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