The contribution of ammonia to methamphetamine neurotoxicity

Laura E. Halpin

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A Dissertation
entitled
The Contribution of Ammonia to Methamphetamine Neurotoxicity
by
Laura E. Halpin
Submitted to the Graduate Faculty as partial fulfillment of the requirements for the
Doctor of Philosophy Degree in Biomedical Sciences

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August 2013
Methamphetamine neurotoxicity results from multiple interacting mechanisms of damage, yet the contribution of peripheral organ damage and ammonia to the long-term monoaminergic terminal damage seen after drug exposure has not been previously investigated. We hypothesized that methamphetamine caused acute liver damage and increases in ammonia which contribute to the long-term striatal neurotoxicity produced by the drug. These effects were investigated using a binge dosing paradigm of methamphetamine in Male Sprague-Dawley rats. Methamphetamine was shown to produce significant hyperthermia-dependent liver damage that contributed to increases in plasma and brain ammonia concentrations. These increases in ammonia were also shown to contribute significantly to the long term striatal dopamine and serotonin terminal damage produced by the drug. Ammonia was demonstrated to mediate this neurotoxicity via increases in glutamate and subsequent excitotoxicity. These findings identify liver damage and ammonia as novel mediators of the neurotoxicity of methamphetamine and highlight the importance of considering peripheral organ function in the study of neuronal damage.
For my teachers, professors, mentors, and patients. The opportunity to learn from you has been and always will be the greatest of privileges.
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List of Abbreviations

5HT ......................... Serotonin
aCSF ......................... Artificial Cerebrospinal Fluid
AIDS ......................... Acquired Immunodeficiency Syndrome
ALT ......................... Alanine Aminotransferase
AMPA ...................... 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid
AST ......................... Aspartate Aminotransferase
BAS ......................... Bioanalytical Systems Inc.
Bcl ......................... B-cell lymphoma
BD ......................... Becton-Dickinson
BMDS .................. Bio Medic Data Systems
CNS ......................... Central Nervous System
COX ...................... Cyclooxygenase
DAT ......................... Dopamine Transporter
EAAT ...................... Excitatory Amino Acid Transporter
FDA ......................... Food and Drug Administration
GABA ...................... Gamma-aminobutyric Acid
GLT-1 .................. Glutamate Transporter-1
GLAST .................. Glutamate Aspartate Transporter
GS .......................... Glutamine Synthetase
GYKI 52466 .............. 4-(8-Methyl-9H-1,3-dioxolo[4,5-h][2,3]benzodiazepin-5-yl)-benzenamine dihydrochloride
H&E ...................... Hematoxylin and Eosin
HRP ...................... Horseradish Peroxidase
IL ......................... Interleukin
Lac ........................ Lactulose
LDS ...................... Lithium Dodecyl Sulfate
MWCO ................... Molecular Weight Cut-Off
OD ........................ Outer Diameter
PE ........................ Polyethylene
PVDF .................... Polyvinylidene Difluoride
Sal ....................... Saline
SAMSHA ................ Substance Abuse and Mental Health Services Administration
SERT .................... Serotonin Transporter
SEM ...................... Standard Error of the Mean
METH ................... Methamphetamine
MDMA .................. 3,4-methylenedioxymethamphetamine
NMDA .................. N-methyl-D-aspartate
TNF ...................... Tumor Necrosis Factor
TUNEL ................ Terminal deoxynucleotidyl transferase dUTP nick end labeling
UNODC ................ United Nations Office on Drugs and Crime
Veh ...................... Vehicle
Chapter 1:

General Introduction to Methamphetamine Neurotoxicity, Liver Damage and Ammonia

1.1 Methamphetamine Use

Methamphetamine (METH) is a widely abused psychostimulant. The synthetic substituted-amphetamine can be inexpensively manufactured from household ingredients contributing to the significant prevalence of its use. In 2011, the lifetime prevalence of METH use was 4.6% and prevalence of current METH use is 0.2% of the population. Throughout 2011, over 130,000 people tried METH for the first time and the mean age of first-time METH users was 17.8 years old (SAMSHA, 2012b). METH exposure is also a clinically relevant concern as nearly 150,000 people presented to emergency departments in 2010 for complaints associated with amphetamine-type stimulant use (SAMSHA, 2012a). Beyond their highly prevalent use within the United States, amphetamine-type stimulants are the second most-widely used class of drugs worldwide, second to cannabis (UNODC, 2012). The prevalence of METH use is greater than 1% of the population aged 15-64 throughout other North American countries, Asia, and Australia, as well as in
some regions of Europe and Africa, where the prevalence of METH-use is increasing (UNODC, 2012).

Since it was first synthesized in 1919 and made commercially available during the middle of the twentieth century, METH has been used for many indications including asthma treatment, to heighten focus and alertness, and weight loss. The drug has also been used extensively by the military to increase alertness, reduce fatigue and decrease appetite (Vearrier et al., 2012). Soon after METH was commercialized, the addictive and other adverse effects became apparent. The Food and Drug Administration (FDA) first restricted the distribution of METH in 1959, requiring prescriptions for patients to receive METH-containing inhalers. In 1970, the drug was given Schedule II classification under the Comprehensive Drug Abuse Prevention and Control Act of 1970 (Rasmussen, 2008). Today, the drug is manufactured under the name desoxyn and its FDA approved indications include attention deficit hyperactivity disorder and for the short-term, adjunctive treatment of exogenous obesity. Currently, nearly all recreational METH users obtain illicitly manufactured drug that was prepared in homemade laboratories (Gonzales et al., 2010).

1.2 Acute Effects of METH on Neurotransmitter Release

METH causes acute increases in synaptic dopamine, serotonin and norepinephrine. These increases result from the binding of the drug to the dopamine transporter (DAT), serotonin transporter (SERT), and norepinephrine (NET). In addition to binding these transporters, METH is a substrate that is transported into the axon terminal (Rothman and Baumann, 2003). Following intracellular transport, amphetamines contribute to vesicle disruption, causing increased cytoplasmic dopamine and serotonin
(Sulzer and Rayport, 1990). Subsequent to increases in cytoplasmic dopamine and serotonin, reversal of the dopamine and serotonin transporters causes significant, action potential-independent neurotransmitter release (Sulzer et al., 1995). In addition to reversal, acute decreases in neurotransmitter transporter function also contribute to increases in dopamine (Fleckenstein et al., 1997a). Secondary to increases in dopamine, METH causes acute increases in glutamate as a result of D1 dopamine receptor-mediated disinhibition of corticostriatal glutamate release (Nash and Yamamoto, 1992, Mark et al., 2004).

1.3 Acute Behavioral Effects of METH Exposure

The acute behavioral effects of METH exposure result primarily from the effect of the drug on monoamine concentrations. Increases in norepinephrine during drug exposure are associated with arousal, reduced fatigue and appetite, as well as increases in heart rate, respiration rate, blood pressure and pupil dilation (Shappell et al., 1996, Berridge, 2006, Mendelson et al., 2006). METH-induced increases in dopamine contribute to the euphoria, paranoia, auditory and visual hallucinations, improved attention, manic symptoms, and psychomotor activation produced by the drug (Bell, 1973, Perez-Reyes et al., 1991, Shappell et al., 1996, Mendelson et al., 2006). Depending on the receptor subtypes that are activated, acute increases in serotonin can contribute to either relaxation and increased social interaction, or anxiety (Martin et al., 1971, Harris et al., 2003, Mendelson et al., 2006). The drug also produces significant increases in body temperature in the range of 39-42 °C as a result of both central effects within the hypothalamus involving serotonin and norepinephrine, and peripheral vasoconstriction, hyperlocomotion and altered metabolism (Wang et al., 1990, Brown et al., 2007, Sprague
et al., 2007, Benamar et al., 2008). Chronic unpredictable stress, which produces significant increases in corticosterone, has also been shown to potentiate METH-induced hyperthermia in a 5HT_2 receptor dependent manner, further highlighting the role of serotonin in METH-induced hyperthermia (Doyle and Yamamoto, 2010). METH-induced hyperthermia is associated with the neurotoxicity produced by the drug. Blockade of drug induced increases in hyperthermia during METH-treatment protects against the long-term monoaminergic terminal damage produced by the drug, although hyperthermia alone is not adequate to produce this terminal damage (Bowyer et al., 1992, Bowyer et al., 1994, Albers and Sonsalla, 1995). Accordingly, hyperthermia appears to potentiate many of the mechanisms contributing to METH-induced neurotoxicity including action of the drug at the dopamine and serotonin transporters, as well as increases in oxidative stress and brain edema (Fleckenstein et al., 1997b, Haughey et al., 2000, Xie et al., 2000, Kiyatkin and Sharma, 2009). METH-induced hyperthermia may also contribute tissue damage produced by the drug as significant increases in core temperature alone can damage the liver and other peripheral organs.

1.4 Persistent Dopamine and Serotonin Terminal Damage

Subsequent to the acute effects of exposure, METH produces long-term damage to dopaminergic and serotonergic axon terminals in the striatum, hippocampus and prefrontal cortex (Ricaurte et al., 1980, Wagner et al., 1980, Seiden et al., 1988). This damage has been shown to persist for at least 2 years after drug exposure in rodents, non-human primates and humans (Seiden et al., 1988, Woolverton et al., 1989, McCann et al., 1998, Volkow et al., 2001a, McCann et al., 2005) Neurochemical markers of this toxicity include decreases in dopamine and serotonin tissue content, decreases in DAT
and SERT expression coupled with decreases in transporter $V_{\text{max}}$ without changes in $K_m$, and decreases in the expression of tyrosine and tryptophan hydroxylase, the rate limiting enzymes for dopamine and serotonin, respectively (Hotchkiss and Gibb, 1980, Wagner et al., 1980, Ricaurte et al., 1982, Ricaurte et al., 1985, Commins et al., 1987). Beyond changes in tissue content and neurotransmitter specific proteins, morphological changes indicative of axon terminal damage have been reported including the presence of swollen, distorted nerve terminals and positive Fink-Heimer staining, as well as edematous and degenerative changes identified by electron microscopy (Lorez, 1981, Ricaurte et al., 1982, Sharma and Kiyatkin, 2009). Recent METH self-administration studies with longer access paradigms further confirm the terminal damage produced by substituted amphetamine exposure and show decreases in DAT and SERT and tyrosine hydroxylase, as well as decreases in tissue content, all indicative of drug-induced monoaminergic terminal damage (Krasnova et al., 2010, McFadden et al., 2012a, McFadden et al., 2012b).

1.5 Neuronal Cell Death

Though not as extensively studied, there is also supporting evidence that METH may produce cell death, in addition to damaging dopamine and serotonin terminals. Increases in terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), a marker for apoptotic cell death, has been reported after exposure to METH in the prefrontal cortex and striatum (Kadota and Kadota, 2004, Cadet et al., 2005, Zhu et al., 2006b). This cell death has been identified in different subpopulations of GABA-interneurons. Within the hippocampus, cell death of calbindin-containing GABA interneurons has been demonstrated, and in the striatum, METH-contributes to the death
of parvalbumin containing GABA interneurons (Zhu et al., 2006a, Kuczenski et al., 2007). Mitochondrial damage and endoplasmic reticulum stress have been associated with this METH-induced apoptosis (Cadet et al., 2005). Specifically, the drug has been shown to contribute to apoptosis through increases in caspase-3 activity and the Fas/FasL cell death pathways (Jayanthi et al., 2005). The drug also produces DNA damage and alterations in the expression of Bcl-2 related genes, which may contribute to GABA interneuron cell death (Jayanthi et al., 2001, Jeng et al., 2006).

1.6 Long Term Behavioral Consequences of METH Exposure

Repeated METH use contributes to abuse and dependence on the drug. In addition to intense craving, withdrawal from the drug is associated with disturbed sleep, anxiety, depressed mood, agitation and reduced energy (McGregor et al., 2005). Exposure to the drug is also associated with numerous long-term neuropsychological impairments associated with the persistent and significant dopamine and serotonin terminal damage seen after drug exposure. These persistent neuropsychological effects include impaired impulse control, working memory, decision making, attention, and motor coordination (Rogers et al., 1999, Volkow et al., 2001b, Simon et al., 2002, Clark et al., 2006, Johanson et al., 2006). Abstinent patients who were previously dependent on the drug also have a high potential for relapse and impairments in cortex-mediated decision making which may be the result of decreases in dopamine activity (Paulus et al., 2005, Wang et al., 2012). While each of these impairments are exhibited by numerous METH-dependent subjects, it is also worth noting that the presence of these symptoms before METH use is unknown and may precede and contribute to METH abuse and dependence.
The long-term damage produced by METH may also represent a vulnerability factor and contribute to protracted psychosis and psychotic disorders, in addition to the acute psychotic effects of the drugs (Flaum and Schultz, 1996). Abuse of amphetamines is associated with a significant increase in the prevalence of psychosis and this association is especially strong in the context of METH use and amphetamine abuse in the mid-late teen years (Harris and Batki, 2000, McKetin et al., 2006). Psychosis presenting subsequent to amphetamine-type stimulant use or in the context of schizophrenia share many common features and have been suggested to share a common neural substrate (Hermens et al., 2009). Of these, substituted-amphetamine induced alterations to dopamine, GABA and glutamate neurotransmitter systems are likely the most significant contributors to protracted psychosis and vulnerability to psychotic disorders. Hyperactivity of the mesolimbic dopamine pathway is strongly associated with the positive symptoms of schizophrenia and a target of many antipsychotic drugs. At first glance, the hyperactive mesolimbic dopamine activity postulated to contribute to psychosis appears to be paradoxically associated with the dopaminergic depletions produced by METH; however mesolimbic dopamine projections are relatively spared from METH-induced dopamine terminal damage (Granado et al., 2010). It is also worth noting that the mesocortical dopamine depletions produced by METH may contribute to the negative, cognitive and affective symptoms associated with schizophrenia.

Beyond changes in dopamine function, the alteration of glutamate and GABA neurotransmission produced by METH represent mechanisms by which the drug contributes to psychosis. Sensory gating, which is important for modulating the perceptual distortions contributing to psychosis, has been shown to be affected by METH
Hadamitzky et al., 2011). METH-induced decreases in sensorimotor gating have been attributed to alterations in both glutamate and downstream GABA neurotransmission in cortico-striatal-thalamic-cortical sensory filtering circuits (Arai et al., 2008, Mizoguchi et al., 2009).

1.7 Mechanisms of Neurotoxicity

1.7.1 Excitotoxicity

Excitotoxicity is a mechanism of neurotoxicity that has been implicated in the monoaminergic terminal damage produced by METH. Excitotoxicity results from the activation of calcium permeable glutamate receptors and excessive influx of calcium leading to protease activation, mitochondrial damage and nitric oxide formation (Choi, 1994). METH has been demonstrated to produce significant increases in extracellular glutamate within the striatum as a result of dopamine-mediated disinhibition of glutamate release from corticostriatal projections (Nash and Yamamoto, 1992, Mark et al., 2004). Subsequent to increases in glutamate, calcium permeable AMPA receptors located on dopamine and serotonin terminals in the striatum may be activated contributing to calcium influx (Keefe et al., 1993, Betarbet and Greenamyre, 1999, Hernandez et al., 2003). This is supported by findings that METH-induced activation of calpain, a calcium activated protease and marker of excitotoxic terminal damage, is decreased when AMPA receptor activation is prevented in the context of METH treatment (Staszewski and Yamamoto, 2006). NMDA and metabotropic receptor antagonists have also been shown to protect against the damage produced by METH, suggesting that the activation of multiple types of glutamate receptors may contribute to the neurotoxicity of METH (Farfel et al., 1992, Battaglia et al., 2002). In addition to increases in protease activity,
increases in glutamate and excitotoxicity have been linked to free radical and nitric oxide formation (Dawson and Dawson, 1996, Deng and Cadet, 1999, Battaglia et al., 2002) and the resultant oxidative stress represents a mechanism by which METH-induced increases in dopamine and glutamate synergize to cause monoaminergic terminal damage (Tata and Yamamoto, 2007)

1.7.2 Oxidative Stress

Oxidative stress is another mechanism by which METH causes neuronal damage. Reactive oxygen species are formed subsequent to substituted amphetamine exposure through numerous mechanisms. Acute increases in dopamine contribute to oxidatively damaged axon terminals after METH exposure (Schmidt et al., 1985). Direct oxidation of dopamine leading to quinone formation, iron-catalyzed dopamine metabolism via the Fenton Reaction, and metabolism of dopamine by monoamine oxidase-A contribute to superoxide and hydrogen peroxide production (Graham, 1978, Yamamoto and Zhu, 1998, LaVoie and Hastings, 1999). In addition to dopamine, METH treatment leads to oxidative stress via increases in reactive nitrogen species resulting from nitric oxide synthase activity (Imam et al., 2000). Increases in free radical formation and oxidative stress after METH are further supported by depletions in antioxidant enzymes after drug exposure (Jayanthi et al., 1998, Jayanthi et al., 1999). Subsequent to METH exposure, increased oxidative damage including lipid peroxidation and protein carbonyl formation has been shown (Yamamoto and Zhu, 1998, Gluck et al., 2001). Specific nitration and nitrosylation of proteins important for monoamine synthesis and release including VMAT-2 and tyrosine and tryptophan hydroxylase has also been reported (Kuhn et al., 1999, Kuhn and Geddes, 1999, Eyerman and Yamamoto, 2007). The oxidative
modification of these proteins is significant as it disrupts their function and contributes to their degradation, thereby contributing to amphetamine neurotoxicity. Antioxidant treatments have been shown to be neuroprotective for the damage produced by METH, highlighting the significant contribution of oxidative stress to the neurotoxicity of substituted amphetamines (Fukami et al., 2004)

1.7.3 Altered Metabolism and Mitochondrial Damage

METH also contributes to monoaminergic terminal damage though altered energy metabolism, including mitochondrial damage. During exposure to METH, there is a high demand for energy and acute increases in energy metabolism in brain regions where the drugs increase neuronal activity (Pontieri et al., 1990). Soon after the excessive increase in energy metabolism reported during drug exposure, there is evidence of compromised energy metabolism and depleted energy stores (Chan et al., 1994, Huang et al., 1999). Damage to mitochondria and specific complexes of the electron transport chain including complex II-III and IV contribute to these decreases in energy metabolism (Burrows et al., 2000a). Glutamate and nitric oxide-dependent decreases in complex II have also been shown to play a significant role in METH neurotoxicity during psychostimulant exposure, as well as excitotoxically-mediated mitochondrial damage (Burrows et al., 2000b, Brown et al., 2005). The dysfunctional energy metabolism observed subsequent to METH exposure is believed to result from the increased neuronal energy requirements during drug exposure, as well as mitochondrial damage from glutamate-mediated excitotoxicity (Brown et al., 2005). When additional energy sources are supplied to neurons during drug exposure or increases in glutamate are blocked, excitotoxic mitochondria damage, as well as dopamine and serotonin terminal damage is prevented,
supporting a significant role for these effects in axon terminal damage (Stephans et al., 1998).

1.8 Emerging Mechanisms of Toxicity

Numerous, complex interacting mechanisms have been identified as significant contributors to the neurotoxic effects of METH, yet many studies only examine these effects within neurons. Because the drug is always administered systemically and affects other cells within the brain, as well as peripheral organ function, it is important to consider how METH produces non-neuronal damage and how this damage may contribute to the neurotoxicity of the drug. Identification and understanding of these non-neuronal contributors is important because they have the potential to significantly contribute to the excitotoxicity, oxidative stress and metabolic compromise produced by METH. Furthermore, elucidating these non-neuronal mediators of toxicity may reveal novel targets for the treatment of the neurotoxicity produced by METH.

1.8.1 Inflammation

In addition to increasing neuronal activity, METH has been shown to increase microglial activity, which can contribute significantly to neuroinflammation (Escubedo et al., 1998, Thomas et al., 2004a). METH-induced increases in both dopamine and glutamate may contribute to microglial activation and inflammation via the formation of dopamine quinones and/or NMDA receptor activation (Thomas and Kuhn, 2005b, Kuhn et al., 2006). Microglial activation increases pro-inflammatory cytokines and is associated with the neuronal terminal damage produced by METH (Thomas et al., 2004b). In the context of METH treatment, the expression of IL-1β, IL-6, and TNF-α have all been shown to increase in the striatum. Conversely, knockout of IL-6 production
attenuates the damage produced by the drug (Ladenheim et al., 2000, Sriram et al., 2006). Reduction of TNF-α and direct inhibition of microglial activation, however, is not protective, suggesting that inflammation plays a complex role in neurotoxicity and may both contribute to and protect against monoaminergic terminal damage (Nakajima et al., 2004, Sriram et al., 2006). Although METH appears to activate microglia and increase cytokine formation, the degree to which this inflammation contributes to the neurotoxicity of the METH alone is unclear. It has been suggested that increases in cytokine formation may contribute to the neurotoxicity of METH as a result of increases in cyclooxygenase (COX) activity. Furthermore, the peroxidase activity resulting from COX activity may contribute to the oxidative damage produced by the drug (Thomas and Kuhn, 2005a). However, the role of COX activity in the monoaminergic terminal damage produced by METH remains unclear as only knockout, but not pharmacologic inhibition of COX activity is protective (Thomas and Kuhn, 2005a). In fact, selective inhibition of COX-2 has been shown to potentiate METH-induced dopaminergic toxicity (Zhang et al., 2007). Accordingly, further studies are needed to understand the contribution of inflammation to METH neurotoxicity.

1.8.2 Blood-Brain Barrier Damage

Another non-neuronal consequence of METH exposure is damage to the blood brain barrier. The blood brain barrier is responsible for separating the neuronal parenchyma from the arterial blood supply of the brain and is comprised of endothelial cells with tight junctions, pericytes and astrocytic end feet (Saunders et al., 2008). METH alone produces a transient increase in blood-brain barrier permeability, allowing for the influx of molecules into the brain that would otherwise be limited by this
functional barrier (Bowyer and Ali, 2006, Sharma and Ali, 2006, Silva et al., 2010). The mechanism by which the drug contributes to this damage has not been fully elucidated; however hyperthermia, oxidative stress, increases in nitric oxide and protease activity targeting endothelial tight junctions have all been implicated in increases in blood-brain barrier permeability (Bowyer et al., 2008, Ramirez et al., 2009, Martins et al., 2011, Martins et al., 2013). Accordingly, METH may be causing blood-brain barrier damage though many of the same mechanisms by which the drug causes neuronal damage. Increases in blood brain barrier permeability may also contribute to the neurotoxicity of METH. Alterations in blood-brain barrier function have been shown to correlate with hypoxia as well as depletions in tyrosine hydroxylase (Kousik et al., 2011). METH-induced increases in blood-brain barrier permeability may also contribute to increased neurologic involvement in infections including NeuroAIDS, herpes simplex-2, and cryptococcal meningitis (Cisneros and Ghorpade, 2012, Conant et al., 2012, Valencia et al., 2012, Eugenin et al., 2013).

1.8.3 Peripheral Organ Damage

Although studies of METH focus on the central nervous system effects of the drug, METH has been reported to cause significant peripheral organ damage (Smith and Fischer, 1970). Clinical reports suggest that the cardiac, renal, muscular, and hepatic organ systems may be especially susceptible to damage by METH (Jones et al., 1994, Kamijo et al., 2002, Wijetunga et al., 2003, Ago et al., 2006). METH is metabolized in the liver by the cytochrome p450 2D6 system and excreted by the kidneys (Caldwell et al., 1972, Kim et al., 2004). In fact, the highest and longest lasting concentrations of METH after drug exposure have been seen in the liver (Volkow et al., 2010). Further
understanding of the peripheral organ damage produced by METH is important because the brain is highly dependent on peripheral organ function. Therefore, damage to peripheral organs may contribute significantly to the established neurotoxicity of the drug and represents a novel target for interventions to treat the neurotoxicity of METH and its neuropsychiatric consequences.

1.9 METH-Induced Liver damage

Of the organ systems reported to be damaged by METH, we have focused on the liver because of the significant concentrations of METH measured there, as well as the fact that liver damage, alone in other contexts, is associated with neurotoxicity (Felipo and Butterworth, 2002, Volkow et al., 2010). The liver is important for the metabolism and detoxification of chemicals in the body, as well as protein synthesis, bile production, and energy storage. METH has the potential to damage the liver though many direct or indirect mechanisms. The metabolism of METH via the cytochrome p450 system may result in oxidative and mitochondrial damage to hepatocytes (Pourahmad et al., 2010, Letelier et al., 2011). Hepatocytes may also be damaged by extra-hepatic effects of METH, including hyperthermia or changes in cardiovascular function (Kew et al., 1970, Skibba et al., 1989, Wang et al., 1990, Chen, 2007).

1.10 Ammonia and Neurotoxicity

The significance of drug-mediated peripheral organ damage is highlighted by the dependence of the brain on peripheral organ function and that alterations in peripheral physiology can contribute to neuronal pathology. Clinically, the neurologic symptoms that present in the context of liver failure are termed hepatic encephalopathy. A key feature of the pathology that contributes to neuronal damage is ammonia (Al Sibae and
Liver damage is associated with increases in ammonia as the liver metabolizes ammonia to urea via the urea cycle so it can be efficiently excreted via the kidneys (Felipo and Butterworth, 2002). Similar to METH, ammonia alone has been shown to cause neurotoxicity via excitotoxicity, oxidative damage, and metabolic compromise (Hawkins et al., 1973, Kosenko et al., 1995, Kosenko et al., 1997, Saez et al., 1999, Llansola et al., 2007). Accordingly, ammonia could be a peripherally derived mediator of METH neurotoxicity which may contribute synergistically to the neurotoxic mechanisms that were classically thought to result from the direct neuronal effects of the drug.

### 1.11 Summary of Past Findings and Gaps in Knowledge

As reviewed above, METH produces long-term dopamine and serotonin terminal damage and numerous mechanisms by which the drug produces this damage have been identified. These include oxidative stress, mitochondrial damage and excitotoxicity (Figure 1.1). However, it remains to be established if the drug contributes to peripheral organ damage and whether peripheral organ damage contributes to the neurotoxicity of the drug. Furthermore, the potential role played by ammonia in the neurotoxicity of METH has not been examined. Ammonia may represent a novel, peripherally derived mediator of METH-neurotoxicity. Investigation of the role of peripheral organ damage and ammonia in the neurotoxicity of METH is significant because they represent novel targets for the treatment of the damage produced by the drug and more broadly highlights the significant contribution of altered peripheral organ function to neuropsychiatric disorders.
Figure 1.1: Model of METH Neurotoxicity: METH causes increases in dopamine, serotonin, glutamate and hyperthermia which contribute to established mechanisms of neurotoxicity including oxidative stress, metabolic compromise and excitotoxicity. These mechanisms converge to produce the long-term, persistent neurotoxicity associated with drug use (solid arrows). In the current series of studies, we hypothesize that METH causes liver damage and increases ammonia which play a critical role in the established mechanisms of damage and neurotoxicity (arrows and ?). We also examine if liver damage results from hyperthermia and if increases in ammonia contribute to increased glutamate (arrow and ?).
1.12 Hypotheses

The overarching hypothesis of this dissertation research was that the peripheral organ effects of METH contribute to the long-term neurotoxicity of the drug. More specifically, METH produces liver damage and increases ammonia to cause long term striatal dopamine and serotonin terminal damage mediated by established excitotoxic mechanisms. Chapter 2, features experiments that examined the effects of METH on the liver and determined whether METH-induced hyperthermia is a mechanism by which the drug contributes to the hepatic damage. Chapter 3, includes experiments that examined if the liver damage produced by METH affects ammonia metabolism and whether the increases in ammonia contribute to the neurotoxicity of the drug. Chapter 4 highlights experiments that determined if ammonia contributes to the neurotoxicity of METH via increases in extracellular glutamate and subsequent excitotoxic damage. These series of related studies were designed to establish a role for peripheral organ damage and ammonia in the neurotoxicity of METH.
Chapter 2:

Methamphetamine causes Acute Hyperthermia-Dependent Liver Damage

2.1 Introduction

Methamphetamine (METH) is a widely abused psychostimulant that causes neuronal damage (Ricaurte et al., 1980, Seiden et al., 1988), yet few studies have examined peripheral organs for damage caused by the drug. Most studies of METH have focused on the central nervous system effects of the drug, as the drug contributes to altered neuronal function, addiction, and cellular damage. An early report on the clinical presentation of intravenous METH abusers noted hepatitis as the second most common presenting complaint (Smith and Fischer, 1970) but since that finding, little more has been studied along these lines until a clinical report demonstrating that METH causes acute liver failure in the absence of viral hepatitis (Kamijo et al., 2002). Thus, there is a high potential for METH to produce hepatotoxicity (Smith and Fischer, 1970, Kamijo et al., 2002, Wijetunga et al., 2003, Ago et al., 2006). Despite these findings, the hepatocellular damage produced by METH has yet to be extensively characterized and the mechanism by which the drug contributes to this damage is unknown.
METH-induced hepatocellular damage may be the consequence of the direct effects of the drug on the liver, other peripheral effects on cardiac function, or alterations in body temperature. In fact, METH causes considerable hyperthermia which is strongly linked to the neuronal damage produced by the drug (Bowyer et al., 1992, Bowyer et al., 1994, Albers and Sonsalla, 1995, Haughey et al., 2000, Xie et al., 2000, Kiyatkin and Sharma, 2009). The significant hyperthermia (39-42 °C) produced by amphetamine exposure likely results from drug-induced increases in monoamine concentrations and the complex integration of the subsequent hyperlocomotion, altered metabolism, changes in hypothalamic neurotransmission, and vasoconstriction (Wang et al., 1990, Brown et al., 2007, Sprague et al., 2007, Benamar et al., 2008). Also worth noting is that hyperthermia, in the context of heat stroke, produces significant changes in hepatocellular morphology (Kew et al., 1970, Bianchi et al., 1972, Weigand et al., 2007).

Changes in hepatocellular morphology can be detected using hematoxylin and eosin staining for acidic and basic components of the tissue at the light microscopic level. In addition, specific ultrastructural changes to hepatocytes can be characterized using electron microscopy (Katz, 1989, Batt and Ferrari, 1995). Hepatocellular enzyme serum concentrations can also be utilized as selective biomarkers for hepatic tissue damage (Ozer et al., 2008). Therefore, to examine if METH causes liver damage, we used H&E staining and AST and ALT measurements to examine hepatocellular morphology after METH treatment. To further characterize acute liver damage after a binge dose of METH, we used transmission electron microscopy to assess damage at 24 hours after METH. We have also quantified AST and ALT at this same time point to examine if
METH produces persistent increases in these measures. Finally, we examined if METH-induced hyperthermia contributes to drug-induced liver damage.

2.2 Methods

Rat Treatment

Male Sprague Dawley rats were treated with METH (10 mg/kg i.p. every 2 hr x 4) or saline (1 mL/kg i.p. every 2 hr x 4). This dose simulates the concentration and binge dosing paradigm reported in METH-dependent humans and produces the same long-term neuronal damage seen in humans after METH exposure (McCann et al., 1998, Volkow et al., 2001, McKetin et al., 2006, Cruickshank and Dyer, 2009). Rats were killed by rapid decapitation 24 hours after the last injection of drug or were anesthetized for ventricular perfusion and exsanguination for ultrastructural investigation of tissues. Body temperature was remotely monitored during the experiments using transponders that were subcutaneously implanted 2 days before drug treatment (IPTT-300 transponder, BDMS). Implantation of temperature transponders allowed for remote, repeated, and noninvasive monitoring of temperature during drug treatment. In some experiments, METH-induced hyperthermia was blocked by cooling the external environment with ice packs placed around the test cages. Temperature was measured every 10 minutes throughout drug treatment and for 5 hours after the last injection in all groups to ensure that the body temperature of METH-treated normothermic rats was kept at the same temperature as saline treated rats. All treatments were carried out in accordance with the National Institute of Health Guide for Care and Use of Rats. All treatments have also been approved by the University of Toledo Institutional Animal Care and Use Committee.

Transmission Electron Microscopy
Twenty-four hours after drug treatment, rats were anesthetized (5 mg/kg of xylazine and 75 mg/kg of ketamine) and transcardially perfused via the left ventricle with 100 mL of phosphate buffered saline (pH 7.4) subsequently followed by 200 mL of 3% glutaraldehyde buffered with 0.2 M sodium cacodylate (pH 7.2). After perfusion, livers were harvested and processed using standard electron microscopy procedures including fixation with 1% osmium tetroxide followed by an en bloc stain using saturated aqueous uranyl acetate (pH 3.3), and dehydrated using a series of gradient ethanol concentrations (30%, 50%, 70%, 90%, 95% and 100%) and acetone. A combination of acetone and resin Embed It™ Low Viscosity Resin Kit, Polysciences Inc. was used to infiltrate the tissue with resin. Tissue was then embedded in 100% resin and polymerized overnight at 80 °C. Ultra thin sections (60-90 nm) were acquired using an OMU3 C. Reichert Ultramicrotome and stained using saturated uranyl acetate and Reynold’s lead citrate. Images were acquired using a Philips CM 10 transmission electron microscope.

Hematoxylin and Eosin Staining

Liver tissue was postfixed overnight in 10% buffered formalin (Fisher Scientific) and embedded in paraffin. Blocks were sectioned at 4 µm and stained with hematoxylin and eosin using a Leica Autostainer XL.

Asparate Aminotransferase and Alanine Aminotransferase Determination

Trunk blood was collected after rapid decapitation for measuremen of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Serum was prepared by allowing blood to coagulate and then centrifuged for 10 minutes at 3000 x g to separate the serum. AST and ALT levels were determined using a UniCel DxC800 Synchron Clinical System (Beckman Coulter).
Statistical Methods

For samples examined at 2 hours after METH, analyses for AST and ALT levels in saline and METH treated rats and brain concentration of METH in rats treated with lactulose or vehicle and METH were carried out using a t-test. For samples examined at 24 hours after treatment, analysis of AST and ALT levels in the 3 different treatment groups was carried out using a one-way ANOVA with post-hoc Tukey tests to determine differences between groups. These tests were performed using SigmaPlot 11.0 Software (Systat Software, Inc. SigmaPlot for Windows). All data are presented as Mean ±SEM. Sample sizes were determined based on a power of 80% or greater and α level in all studies was 0.05 or less.

Results

METH and liver damage

Hematoxylin and eosin staining were used to examine the effects of METH or saline on the livers of treated rats. Livers were examined at 2 and 24 hours after the last of the 4 injections. At 2 hours, there is moderate vascular and sinusoidal congestion evident throughout the livers of all METH treated rats (Figure 2.1). This vascular and sinusoidal congestion were observed in the livers of all METH treated rats examined at this timepoint and the image in Figure 2.1 is representative of the congestion seen in the livers of all rats. Similarly, at 24 hours, extensive cytoplasmic damage is observed in the livers of 100% of METH treated rats.

To confirm that the morphologic changes depicted in Figure 2.1 represent significant hepatocellular damage, the serum concentration of hepatocyte cytosolic enzymes was measured at 2 hours after the last injection of METH (10 mg/kg i.p. every 2
hrs x 4) or saline (1 mL/kg i.p. every 2 hrs x 4) in the same rats examined in Figure 2.1. When hepatocytes are damaged, cytosolic contents such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are released into the blood and can be measured. At 2 hrs after the last injection of METH, serum AST concentration was 193.4 ± 15.7 IU/L in saline treated rats and significantly elevated to 447.8 ± 74.8 IU/L in METH treated rats (t=215.00, p<0.001). At this time point, ALT concentration was 70.6 ± 3.2 IU/L in saline treated rats and significantly increased to 109.3 ± 9.8 IU/L in METH treated rats (t=95.0, p<0.005). Accordingly, AST and ALT levels are significantly elevated by 141.0 ± 44.1% and 54.9 ± 15.2% respectively, in METH treated rats (Figure 2.2).

**METH and Ultrastructural Liver Damage**

Transmission electron microscopy was used to characterize the significant METH-induced alterations in hepatocellular morphology seen at 24 hours after treatment on an ultrastructural level. Ultrastructural changes seen in hepatocytes of 100% of METH-treated rats include microvesicular fatty change, mild hydropic change, increased cytoplasmic glycogen and mitochondrial aggregation (Figure 2.3).

**Environmental Cooling and Methamphetamine-Induced Hyperthermia**

To examine if METH-induced hyperthermia contributes to the liver damage observed 24 hours after exposure to METH, METH-induced acute hyperthermia was prevented by cooling METH-treated rats to the body temperatures of saline control rats throughout METH treatment and for 5 hours after the end of drug treatment. METH treatment caused significant hyperthermia throughout and after METH treatment, which was fully blocked.
by cooling the external environment of rats (Figure 2.4). Cooling resulted in METH treated rats with normothermic body temperatures.

**Hyperthermia and METH-Induced Changes in Liver Morphology**

Hematoxylin and eosin staining was used to determine if hyperthermia contributes to the alterations in hepatocellular morphology throughout the hepatic lobule occurring 24 hrs after METH in hyperthermic, normothermic, and saline control rats. At 24 hr after METH exposure, there was considerable clearing of cytoplasmic staining in hepatocytes throughout the hepatic lobule. The prevention of METH-induced hyperthermia blocked the changes in cellular morphology seen in the livers of METH treated rats (Figure 2.5). The cellular alterations in hyperthermic METH-treated rats were evident in 100% of the METH treated rats and were prevented in all normothermic METH-treated rats.

**Hyperthermia and METH-Induced Hepatocellular Damage**

To examine if the changes observed in hepatocellular morphology at 24 hrs after drug treatment are indicative of cellular damage and if hyperthermia contributes to METH-induced cellular damage, serum concentrations of the hepatocellular enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (Ozer et al., 2008) were measured 24 hours after treatment with METH in rats that were hyperthermic, cooled to normothermia, or saline control rats (Figure 2.6). Serum AST concentration was 335.5 ± 29.1 IU/L in hyperthermic METH treated rats and 177.4 ±13.3 IU/L in saline treated rats, representing an increase of 89.1% ± 16.4 % in hyperthermic METH-treated rats. In normothermic METH-treated rats, AST levels were 231.9 ± 20.4 IU/L, representing an increase of only 30.7% ± 11.5%, compared to Saline treated rats. A one-way ANOVA revealed significant differences between the 3 groups ($F_{(2, 29)} = 13.395$, $p<0.001$).
hoc Tukey test showed significant differences in serum AST levels in hyperthermic METH versus saline treated rats (q=7.205, p<0.001), and hyperthermic METH versus normothermic METH treated rats (q=4.721, p<0.05); however there was no significance differences in serum AST in saline treated versus normothermic METH treated rats (q=2.484, p=0.203).

ALT concentration was 83.9 ± 6.3 IU/L in hyperthermic METH treated rats and 67.5 ±2.6 IU/L in saline treated rats, representing a increase of 24.3% ± 9.3% in METH-treated rats. In normothermic METH-treated rats, ALT levels were 64.8 ± 2.8 IU/L. A one-way ANOVA revealed significant differences between the 3 groups (F(2, 29) = 10.005, p<0.001). A post hoc Tukey test revealed significant differences in serum ALT levels in hyperthermic METH versus saline treated rats (q=5.122, p<0.05), and hyperthermic METH versus normothermic METH treated rats (q=5.800, p<0.05), however there was no significance difference between saline treated and normothermic METH treated rats (q=0.678, p=0.882).
Figure 2.1: METH causes hepatocellular damage visible by H&E Staining: H&E staining was used to examine the effects of METH (10 mg/kg i.p. every 2 hrs x 4) or saline (1 mL/kg i.p. every 2 hrs x 4) on the livers of treated rats. Livers were examined at 2 and 24 hrs after the last injection of METH or saline. A representative image of a hepatic lobule near the central vein is shown (* denotes central vein). The images in the figure are representative of all rats examined for each treatment and at each time point. At 2 hrs, there is moderate vascular and sinusoidal congestion evident throughout the livers of METH treated rats (white arrows). At 24 hrs, this congestion leads to extensive cytoplasmic disappearance in the livers of all METH treated rats (black arrows). (40x magnification)
Figure 2.2: Serum AST and ALT after METH treatment: Rats were treated with METH (10 mg/kg i.p. every 2 hrs x 4) or saline (1 mL/kg i.p. every 2 hrs x4). At 2 hrs after the last injection of METH or saline, A) ALT and B) AST levels are significantly elevated to 54.9 ± 15.2% and 141.0 ± 44.1% in METH treated rats compared to saline controls, respectively. Values are Mean ± SEM, *p<0.05 (n= 12 rats per group)
Figure 2.3: Ultrastructural Changes in Liver 24 Hours After Methamphetamine Exposure: Representative images from METH or Saline treated groups are shown. Hepatocytes of METH treated rats have increased microvesicular lipid (#), hydropic change (&), increased intracellular glycogen stores (*) and mitochondrial aggregation (arrow) compared to control animals. (5200 x magnification)
Figure 2.4: Effect of Environmental Cooling on Methamphetamine-Induced Hyperthermia:
Rats were treated with METH or saline (Arrows denote injections). The external environment of a group of METH treated rats was cooled throughout drug treatment and for 5 hours after the last injection to prevent METH-induced hyperthermia. Cooling was regulated to ensure that the body temperatures of METH-treated cooled rats were similar to that of saline treated rats. METH produced a significant elevation in body temperature throughout treatment compared to the saline treated and METH cooled groups. Body temperature of METH cooled rats did not differ significantly from saline treated rats. * p<0.05 (n=10 per group)
Figure 2.5: Effect of Hyperthermia on Methamphetamine-Induced Changes in Liver Morphology: Hepatocellular morphology was evaluated using H&E sections from livers collected at 24 hours after the last drug injection. A representative picture from all groups of the hepatic lobule near the central vein is shown (asterisk denotes central vein). The livers of METH treated rats show considerably less hepatocellular cytoplasmic staining (arrows denote cytoplasm) compared to saline treated rats. The cytoplasmic clearing was prevented when METH-induced hyperthermia was blocked by cooling the rats to normothermic temperatures.
Figure 2.6: Effect of Hyperthermia on Methamphetamine-Induced Increases in Serum AST and ALT: Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured 24 hours after treatment with METH or saline. A group of METH treated rats were cooled during and after treatment to prevent METH induced hyperthermia. A) AST was significantly elevated by 89.1% ± 16.4 % (Mean ± SEM) in METH treated rats compared to saline treated rats and this increase was blocked when hyperthermia was prevented in METH-treated rats (METH-Cooled). B) ALT was significantly elevated by 24.3% ± 9.3% (Mean ± SEM) in METH-treated rats compared to saline treated rats. This increase was blocked when METH-induced hyperthermia was prevented (METH-Cooled). * p<0.05 compared to saline treated rats, & p<0.05 compared to METH treated rats (n=10 per group)
**Discussion:**

This study examined if METH caused acute liver damage, characterized METH-induced hepatocellular damage, and examined if METH-induced hyperthermia contributes significantly to this structural liver damage and hepatotoxicity. METH caused changes in hepatocellular morphology as well as increases in AST and ALT. METH also produced changes in hepatocellular ultrastructure indicative of generalized cellular stress and damage at 24 hours after drug exposure. Increases in serum AST and ALT at this same time point confirmed that there was cellular damage concurrent with these structural changes. To determine if METH-induced hyperthermia contributes to this hepatocellular damage, hyperthermia was prevented in rats treated with METH by cooling the external environment. The prevention of hyperthermia in METH treated rats blocked METH-induced changes in hepatocellular morphology, as well as increases in AST and ALT, suggesting that hyperthermia plays a significant role in METH-induced liver damage.

Liver damage after exposure to METH has not been examined in-depth previously. While the effects of METH on the brain have been extensively studied, the possible damage to peripheral organs has received less attention. Consistent with clinical reports that METH causes peripheral organ damage (Smith and Fischer, 1970, Kamijo et al., 2002), the current study is the first to show evidence of hepatotoxicity in a rat model of binge METH exposure. Within 2 hours after METH, there is considerable venous and sinusoidal congestion in the liver (Figure 2.1). This congestion is a typical early marker of hepatotoxicity and may be caused by cardiac dysfunction (Hong et al., 1991, Wijetunga et al., 2003), post-hepatic vascular constriction (Wang et al., 1990,
Chen, 2007), and/or direct drug-induced hepatotoxic damage to the liver. Similarly, serum AST and ALT are well established and sensitive markers for liver damage and were elevated at 2 hrs after METH (Figure 2.2). Although increases in AST or ALT could represent damage to other tissues, the elevations in both along with the hepatic histopathological findings, support the view that METH alone causes acute liver damage (Ozer et al., 2008). At 24 hours after the last METH injection, the damage escalated to severe global, decreases in cytoplasmic staining (Figure 2.1), suggestive of autophagy (Rautou et al., 2008).

Electron microscopy of the liver at 24 hours after METH shows microvesicular fatty and hydropic change, increased intracellular glycogen and mitochondrial aggregation (Figure 2.3). These changes are consistent with the generalized hepatocellular damage reported after exposure to hyperthermia and/or heatstroke (Kew et al., 1970, Weigand et al., 2007) evidenced by increases in electron-lucent vacuoles along the sinusoidal border that are indicative of membrane damage and alterations in mitochondria (Wills et al., 1976, Kew et al., 1978). Aggregation of mitochondria may indicate mitochondrial dysfunction and cellular damage (Haga et al., 2003, 2005). Dysfunction of hepatocellular mitochondria may contribute significantly to increases in ammonia as carbamoylphosphate synthetase-1 and ornithine transcarbamylase, two key enzymes in the urea cycle, are located within mitochondria (Adeva et al., 2012). In addition to these markers of damage, METH also increases intracellular glycogen. This may be representative of increased glycogen formation or decreased glycogen breakdown after METH-exposure. It is unclear if this increase in glycogen is a physiologic or pathologic response to METH treatment. Glycogen is made in the liver as a form of
energy storage, increases postprandially, and is regulated by insulin (Parkes and Grieninger, 1985). Glycogen can also accumulate pathophysiologically in conditions of insulin insensitivity or after exposure to alkaloid compounds which inhibit glycogen breakdown (Saul et al., 1985, Messeri et al., 2012). Regardless, METH-induced increases in liver glycogen may represent significant METH-induced alterations in energy metabolism, hepatic function and warrant further study.

To examine the extent of alterations in hepatocellular morphology, H&E staining was used to visualize if this damage is generalized throughout the hepatic lobule. A considerable decrease in H&E staining of the hepatocellular cytoplasm throughout the lobule was observed at 24 hours after METH exposure (Figure 2.5). This decrease in staining is a likely consequence of increased accumulation of hydrophobic substances in the cytoplasm. METH-induced alterations in hepatocellular ultrastructure (Figure 2.3), including increases in glycogen, microvesicular fat and hydropic change all represent increased accumulation of hydrophobic substances, which do not stain with the acidic and basic H&E staining. This extensive lack of H&E staining throughout the hepatic lobule in METH treated rats demonstrates that the effects of METH on hepatocellular morphology are widespread.

To confirm if these changes in morphology are indicative of damage, we measured serum concentrations of aspartate aminotransferase and alanine aminotransferase (ALT and AST) at 24 hours. Both were found to be significantly elevated at 24 hours after METH treatment (Figure 2.6) and support the conclusion that the changes in cellular morphology observed at this time point represent cellular damage. These findings also support the observation of acute liver damage reported at 2 hrs after
METH exposure (Figure 2.2) and suggest that METH is capable of producing persistent hepatocellular damage.

We examined if METH hepatotoxicity was a result of METH-induced hyperthermia based on the fact that the alterations in hepatocellular morphology can be a response to hyperthermia. Amphetamines produce significant increases in body temperature as a result of alterations in both brain and peripheral physiology (Wang et al., 1990, Brown et al., 2007, Sprague et al., 2007, Benamar et al., 2008). The current findings show that when METH-induced hyperthermia is prevented, the changes and hepatocellular morphology and increases in AST and ALT seen 24 hours after METH treatment are blocked (Figure 2.5 and Figure 2.6). These findings indicate that hyperthermia contributes significantly to the hepatotoxicity of METH and extend previous findings that increases in body temperature also play a significant role in METH-induced brain damage (Bowyer et al., 1993, Bowyer et al., 1994) and hepatocellular damage after the METH analogue, 3,4-methylenedioxymethamphetamine (MDMA) (Carvalho et al., 2001).

The primary mechanism by which hyperthermia is believed to contribute to cellular damage is through oxidative stress, as evidenced by increases in lipid peroxidation and depletions in reduced glutathione after hyperthermia exposure (Skibba et al., 1991, Carvalho et al., 1997). Although hyperthermia appears to be an essential component of METH hepatotoxicity, METH may contribute to hepatocellular damage through other mechanisms. METH has been shown to reach some of the highest and longest lasting concentrations in liver and accordingly, has the potential to be directly hepatotoxic (Volkow et al., 2010). METH is metabolized in the liver by the cytochrome
p450 system (Lin et al., 1997) that can lead to the formation of oxidative byproducts and result in cellular damage (Moon et al., 2008, Pourahmad et al., 2010, Letelier et al., 2011). METH has also been shown to have extensive effects on cardiovascular function, causing cardiac dysfunction and widespread vasospasm (Wang et al., 1990, Wijetunga et al., 2003, Chen, 2007), both of which may impede hepatic blood flow, leading to ischemia and contributing to hepatotoxicity.

In conclusion, these studies established that METH does produce acute liver damage, characterized the toxic effects of METH on the liver, and demonstrated that these effects can persist for several hours after drug exposure. Furthermore, METH-induced hyperthermia was identified as a key mechanism for METH-induced hepatotoxicity. These findings highlight the potential role of peripheral organ damage in the neuronal effects and toxicity of drugs of abuse and the importance of addressing hyperthermia in the clinical management of acute METH exposure. To address the consequences of the liver damage identified in this chapter, the measurements of ammonia and how it relates to the neurotoxicity of METH is examined in the following chapter (Chapter 3).
Chapter 3:

Peripheral Ammonia Mediates Methamphetamine Neurotoxicity

3.1 Introduction:

Much research has been directed towards understanding the fundamental processes of addiction to methamphetamine (METH) and the mechanistic underpinnings of its neurotoxicity. The causes of the neurotoxicity are conventionally thought to originate in the brain; however, due to the metabolic dependence of the brain on peripheral organ function and the high susceptibility of the brain to alterations in systemic physiology, the liver damage produced by METH that was identified in Chapter 2 may contribute significantly to the neurological damage caused by the drug.

METH produces relatively specific damage to dopamine and 5-HT terminals in the striatum, hippocampus and prefrontal cortex (Ricaurte et al., 1980, Wagner et al., 1980, Seiden et al., 1988). Established mechanisms include excitotoxicity, oxidative damage, inflammation, and metabolic compromise (Yamamoto and Raudensky, 2008), all of which are assumed to be initiated by factors in the CNS. However, little consideration is given to peripheral organ damage and it is unknown if increases in brain ammonia mediate the neurobiological actions of METH despite knowledge that it can
compromise CNS function as evidenced in hepatic encephalopathy. Because the liver is essential for ammonia metabolism and elimination, hyperammonemia is a key pathological feature of hepatic encephalopathy and is responsible for the neurological sequelae depicted by motor and cognitive deficits (Felipo and Butterworth, 2002).

Beyond understanding the mechanism of METH-induced structural hepatocellular damage, it is also important to determine if there is a correlation between morphologic alteration and diminished liver function which can affect METH-induced neurotoxicity. A key function of the liver is the metabolism of ammonia to urea via the urea cycle. When liver function is compromised, ammonia accumulates and has neurotoxic consequences (Felipo and Butterworth, 2002). Accordingly, it is crucial to examine if METH increases plasma ammonia concentrations and if the prevention of METH-induced liver damage also blocks the increases in peripheral ammonia. In Chapter 2, we found that preventing METH-induced hyperthermia protected against the liver damage caused by the drug. Here, we will examine if preventing liver damage via blocking drug-induced hyperthermia also protects against METH-induced increases in plasma ammonia.

Ammonia, much like METH, causes neuronal damage via excitotoxicity, oxidative stress, and inflammation. Ammonia alone has been shown to decrease the expression of the astrocytic glutamate transporter, EAAT-1, thereby increasing extracellular glutamate, depolarizing glutamate receptors, and producing excitotoxicity (Fan and Szerb, 1993, Chan and Butterworth, 1999, Chan et al., 2000). Ammonia also increases glutamate release from astrocytes (Gorg et al., 2010) and ammonia neurotoxicity is attenuated by glutamate receptor antagonists (Saez et al., 1999, Kosenko et al., 2003a). Ammonia also causes oxidative damage through alterations in oxidative
metabolism (Hawkins et al., 1973, McCandless and Schenker, 1981), increases superoxide radicals, and elevates glutathione peroxidase, superoxide dismutase and catalase activities (Kosenko et al., 1997). Moreover, inflammation mediates ammonia neurotoxicity as ibuprofen attenuates the behavioral consequences of the neuronal damage caused by ammonia (Cauli et al., 2007).

The parallels between the mechanisms underlying ammonia and METH neurotoxicities prompted our hypothesis that METH increases plasma ammonia concentrations, in a liver damage-dependent manner. We have also examined if METH increases brain ammonia concentrations, which converge with the neuropharmacological effects of METH to produce neuronal damage. To this end, we assessed plasma and brain ammonia, and the direct effects of METH and/or ammonia on striatal dopamine and 5HT content. To further substantiate a causative role for ammonia, peripheral ammonia excretion was enhanced with lactulose and the long term effects on dopamine and 5HT content were examined after METH.

3.2 Methods:

Rat Treatment:

Male Sprague Dawley rats received METH (10mg/kg i.p. every 2 hr X 4,) or saline (1 mL/kg i.p. every 2 hr X 4,) (Sigma-Aldrich). This dose of METH was chosen because it produces long term neuronal damage similar to what is seen in studies of human METH users (McCann et al., 1998). In lactulose experiments, rats received lactulose (Pharmaceutical Associates Inc., Greenville, SC) (5.3 g/kg) or vehicle (100 mg/mL galactose and 80 mg/mL lactose) (Fisher Scientific) via oral gavage every 12 hours for 2 days before METH treatment to the day before rats were killed. Lactulose enhances
ammonia excretion and attenuates increases in plasma ammonia and the neurological symptoms of hepatic encephalopathy (Jia and Zhang, 2005, Nicaise et al., 2008, Al Sibae and McGuire, 2009). In some experiments, METH-induced hyperthermia was blocked by cooling the external environment with ice packs placed around the test cages. Temperature was measured every 10 minutes throughout drug treatment and for 5 hours after the last injection in all groups to ensure that the body temperature of METH-treated normothermic rats was kept at the same temperature as saline treated rats. All rats were killed by rapid decapitation 2 hrs after the end of treatment or 24 hrs or 7 days after the start of the METH or saline treatment. Temperature transponders (IPTT-300 transponder, BMDS Inc.) were subcutaneously implanted into the rats 2 days before the beginning of each experiment to allow for equilibration. This method of remote temperature monitoring minimizes stress to the rats produced by repeated rectal recordings. All treatments were carried out in accordance with the National Institute of Health Guide for Care and Use of Rats. All treatments have also been approved by the University of Toledo Institutional Animal Care and Use Committee.

**Plasma Ammonia Determination**

Trunk blood was collected after rapid decapitation for measurement plasma ammonia. Plasma was prepared by collecting blood in BD Microtainer Plasma Separation Tubes (Becton, Dickenson and Company) and centrifuging for 2 minutes at 10,000 x g. Ammonia concentration was determined using a UniCel DxC800 Synchron Clinical System (Beckman Coulter).

**In-Vivo Microdialysis:**
Intracranial probes for use in microdialysis were constructed using PE 20 tubing (Becton Dickinson), silica tubing (OD of 150 µM, Polymicro Technologies), 26 ga stainless steel hypodermic tubing (Small Parts), Hollow Fiber Microdialysis Membrane (4 mm of active membrane, MWCO 13,000, 216 µm, Spectrum Labs), 2 ton waterproof epoxy and tygon microbore tubing (Yamamoto and Pehek, 1990). Surgeries were performed on the morning of the day before drug treatment. Rats were anesthetized using xylazine (5 mg/kg) and ketamine (75 mg/kg) and the probe was stereotaxically lowered into the striatum. For brain ammonia measurements, one day later and after a 1 hr. equilibration period at a flow rate of 1.5 µL/min, baseline samples were collected every hour for 3 hrs. Samples were collected every hour during drug treatment until 2 hrs after the last drug injection. For reverse dialysis experiments, METH (100 µM), and/or ammonium chloride (570µM) were reverse dialyzed for 8 hrs. Probe placement was verified histologically. For reverse dialysis experiments, 450 µM thick sections containing the probe tract cut using a cryostat and tissue adjacent to the tract (1mm) was dissected while the tissue was kept frozen.

**Brain Ammonia Determination:**

Ammonia concentrations in microdialysate were determined using the Sigma-Aldrich Ammonia Assay Kit. This assay uses L-glutamate dehydrogenase to measure ammonia concentration as a stoichiometric product of NADPH levels via the reaction: \( \alpha\)-ketoglutarate + NH\(_4^+\) + NADPH \(\rightarrow\) L-Glu + NADP\(^+\) + H\(_2\)O. NADPH levels were monitored by measuring absorbance at 340 nm using a Molecular Devices Spectramax microplate spectrophotometer. An internal standard made of 10 µg/mL of ammonium
sulfate was used in each determination and only assays in which the concentration of the internal standard was within 5% of the true concentration of ammonia were used.

**HPLC Analysis of Monoamine Tissue Content:**

Tissue was sonicated in 1.0 mL of 0.25 N perchloric acid and centrifuged at 14000 x g for 20 mins at 4°C. For reverse dialysis experiments, 300 µL of perchloric acid was used. Supernatant was then analyzed for 5HT and dopamine using HPLC (Breier et al., 2006). Supernatant was injected on a C18 column (100 x 2 mm, 3µm particle diameter, Phenomenex) and eluted with a mobile phase containing 32 mM citric acid, 54.3 mM sodium phosphate, 0.215 mM octyl sodium sulfate, and 11% methanol (pH 4.4). Concentration of monoamines was determined with a LC-4C amperometric detector (Bioanalytical Systems, Inc.) and data were recorded using EZ Chrom Software. Monoamine levels were normalized to protein content of samples. The pellet from the centrifugation was neutralized using 1.0 mL of 1 M NaOH and protein amount was determined using a Bradford Assay. For reverse dialysis experiments, 100 µL of NaOH was used.

**Western Blot for DAT Immunoreactivity:**

To prepare synaptosomes, striatal tissue was homogenized in 0.32M sucrose. Homogenate was then centrifuged at 800 x g for 24 minutes. The pellet was discarded (P1) Supernatant (S1) was decanted and centrifuged at 22,000 x g for 17 minutes. Supernatant was discarded (S2) and the pellet (P2) was resuspended in cold Millipore water. A Bradford assay was then used to determine protein concentration and samples were diluted (1:4) with LDS sample buffer (Invitrogen). 10 µg of each protein was loaded onto a NuPAGE 4-12%, 1.5 mm x 10 wells, Bis-Tris Gel (Invitrogen). Proteins
were then run at 150V for 80 minutes and transferred to polyvinylidene difluoride membranes at 28 V for 2 hours. Membranes were then washed with TBS-T (20 mM Tris, 137 mM NaCl and 0.5% Tween 20) and blocked for 1 hour at room temperature using TBS-T with 5% powered milk. Membranes were incubated overnight at 4°C with primary antibody to dopamine transporter (DAT, C-20 Santa Cruz Biotechnology). Membranes were then washed with TBS-T incubated with the appropriate horseradish peroxidase secondary antibodies (1:2500 dilution) at room temperature for 1 hour, and developed using the HyGLO-enhanced chemiluminescence (Denville Scientific). All membranes were imaged using the Fuji LAS-4000 mini system and software. The band at 70 kDa was quantified and all bands were normalized to an α-tubulin internal loading control.

**Mass Spectroscopy:**

Mass spectroscopy was used to determine METH concentrations in striata. Tissue was prepared as in the 5HT and dopamine tissue content assays. Supernatant was then injected on to a MetaChem MetaSil Basic column (3.0×100 mm; 3 μM pore size; Varian, Lake Forest, CA) and measured using electrospray ionization as the ionization source.

**Statistical Analysis:**

Analysis of plasma ammonia, hyperthermia, and tissue content of dopamine and 5HT in the striatum was performed using a two-way ANOVA followed by post hoc Tukey multiple comparison tests or t-tests. These tests were performed using SigmaPlot 11.0 Software (Systat Software, Inc. SigmaPlot for Windows). Statistical analysis of brain ammonia levels was carried out using a repeated measures ANOVA test, with each time point as a within-subjects factor and lactulose and METH treatment as between-subjects...
factor. These tests were performed using SPSS Statistics 17.0 Software (IBM Corporation). All data are presented as Mean±Standard Error of the Mean (SEM). α level in all studies is 0.05 or less and sample sizes were chosen to result in a power of 80% or greater.

3.3 Results:

METH and plasma and ammonia

To determine if METH affected ammonia metabolism, plasma ammonia was measured at 2 hrs after the end of METH treatment (10 mg/kg i.p. every 2 hrs x 4) or Saline (1 mL/kg i.p. every 2 hrs x 4). A two-way ANOVA indicated a significant effect of METH treatment ($F(1,31) = 18.25, p<0.001$), lactulose treatment ($F(1,31) = 13.60, p<0.001$), and a significant interaction between METH and lactulose treatment ($F(1,31) = 22.98, p<0.001$). At 2 hrs after the last injection of METH, plasma ammonia levels were significantly elevated by 64.04±9.98%, and this increase was blocked with lactulose treatment (Figure 3.1).

Effect of Hyperthermia on METH-Induced Increases in Plasma Ammonia

To examine if METH-induced increases in plasma ammonia persist for 24 hours and if hyperthermia contributes to those increases, plasma ammonia was measured 24 hours after treatment with METH in hyperthermic and normothermic rats, and saline controls (Figure 3.2). Plasma ammonia concentration was $154.17 \pm 7.51$ µM in hyperthermic METH treated rats and $71.78 \pm 6.51$ µM in saline treated rats, representing an increase of 114.8 ± 10.5%. In normothermic METH-treated rats, plasma ammonia concentration was $80.50 \pm 6.02$ µM, representing only a 12.2 ± 8.3% increase, compared with saline treated rats. A one-way ANOVA revealed significant differences between the
3 groups ($F_{(2, 24)} = 38.45, p<0.001$). A post hoc Tukey test examining group differences revealed significant differences in plasma ammonia in hyperthermic METH versus saline treated rats ($t=9.198, p<0.001$) and hyperthermic METH versus normothermic METH rats ($t=7.481, p<0.001$), however there was no significant difference in plasma ammonia in saline treated versus METH-normothermic rats ($t=0.996, p=0.330$).

**METH and Brain Ammonia**

To examine if elevations in plasma ammonia translate to elevations in brain ammonia, the brain concentration of ammonia was measured using in-vivo microdialysis during METH or saline treatment. Rats were also treated with lactulose or vehicle every 12 hrs for 2 days before and on the morning of drug treatment. Baseline brain ammonia levels in all groups were 29.34±5.33 µM. A repeated measures ANOVA indicated a significant effect of METH treatment ($F_{(7,279)}=2.43, p<0.050$) and a significant interaction between METH and lactulose treatment ($F_{(7,279)}=2.79, p<0.050$) on brain ammonia indicating that METH increased brain ammonia and that this increase was blocked by the peripheral administration of lactulose. Brain ammonia levels in METH treated rats are significantly elevated beginning at the 5 hr time point ($F_{(3,31)}=4.22, p<0.05$). At the 8 hr time point (Figure 3.3), brain concentration of ammonia in saline treated rats was 34.5±8.0 µM and was 67.9±8.7 µM in METH treated rats representing a 196.8±18.0% increase in ammonia in the striata of METH treated rats. Based on the observation that the *in vivo* recovery of ammonia by the microdialysis probe is 35% (data not shown), brain ammonia at 8 hrs after the first METH injection is approximately 200 µM.

**METH and Increased Peripheral Ammonia Excretion**
Rats were given lactulose or vehicle every 12 hrs, p.o. for 2 days before and 6 days after METH or saline treatment to ensure that ammonia excretion was consistently increased throughout the presumed time course over which METH causes neuronal damage.

During drug treatment, the effects of METH and lactulose on body temperature were measured one hour after each METH injection. METH treatment caused significant hyperthermia in both vehicle+METH and lactulose+METH treated rats. Lactulose had no significant effect on hyperthermia (Figure 3.4) indicating that the effects of lactulose are not dependent on alterations in hyperthermia.

To examine if lactulose affected the brain concentration of METH, the concentration of METH in the striatum was measured at 2 hrs after the last injection of METH using LC-MS-MS. Each of the 4 injections were within the half-life of METH and at this time point the drug has presumably reached an approximate steady-state concentration in the brain, thus optimizing the detection of any effect that lactulose may have on the brain concentration of METH. Striatal concentration of METH in vehicle+METH treated rats was $2.11 \times 10^{-2} \pm 0.28 \times 10^{-2}$ ppm/mg protein and $1.96 \times 10^{-2} \pm 0.13 \times 10^{-2}$ ppm/mg protein in lactulose+METH treated rats. The concentrations of METH in the brain did not differ significantly between the vehicle+METH and lactulose+METH groups supporting the view that lactulose does not alter the concentration of METH in the brain.

To examine the effects of METH and lactulose on striatal tissue content of dopamine and 5-HT, rats were killed at 7 days after METH or saline treatment and tissue content of dopamine and 5HT was measured. A two-way ANOVA indicated a
significant effect of METH treatment \( (F_{(1,23)}=30.51, \ p<0.001) \) and a significant interaction between METH and lactulose treatment \( (F_{(1,23)}=11.03 \ p<0.005) \) on dopamine tissue content indicating that the effects of METH on dopamine content varied according to whether the rats were treated with lactulose. Tukey post-hoc analysis revealed that there was no effect of lactulose treatment within saline treated groups \( (q=2.5; \ p=0.093) \); however lactulose attenuated the dopamine depletion caused by METH \( (q=8.08; \ p<0.001) \). Treatment with lactulose significantly attenuated the striatal dopamine depletions caused by METH as METH depleted striatal dopamine by only \( 34.5 \pm 7.9\% \) in lactulose treated rats (Figure 3.5A), suggesting that ammonia may play a role in the dopaminergic toxicity produced by METH.

Likewise, a two way ANOVA showed a significant effect of METH treatment on striatal 5-HT content \( (F_{(1,23)}=27.59, \ p<0.001) \) and a significant interaction between METH treatment and lactulose treatment on striatal 5-HT \( (F_{(1,23)}=2.28 \ p<0.050) \). Tukey post-hoc tests indicated that lactulose had no effect within saline treated rats \( (q=0.98; \ p=0.34) \); however, lactulose attenuated the 5-HT depletion caused by METH \( (q=3.80; \ p<0.001) \). METH treatment produced a depletion of 5-HT by only \( 15.2 \pm 8.2\% \) in lactulose treated rats, which was not significantly different compared to saline treated rats (Figure 3.5B), indicating that ammonia may play a key role in METH-induced serotonergic damage.

Both pre- and post-treatment with lactulose were required for attenuation of METH-induced neuronal terminal damage (data not shown), indicating that elevations in brain ammonia both during and in the days subsequent to METH treatment may play a role in monoaminergic terminal damage. Pre-treatment with lactulose was required as
the drug takes 24 to 48 hours of dosing to be effective (Jia and Zhang, 2005, Nicaise et al., 2008, Al Sibae and McGuire, 2009).

To confirm the effects of lactulose on dopamine tissue content, DAT immunoreactivity was measured in the striatum at 7 days after METH treatment. A two-way ANOVA indicated a significant effect of METH treatment ($F_{(1,23)}=4.708, p<0.05$) and a significant interaction between METH and lactulose treatment ($F_{(1,23)}=4.708, p<0.05$). Post-hoc tests reveal no effect of lactulose within saline treatment groups ($q=2.129, p=0.311$) and a significant effect of lactulose within METH treatment groups ($q=5.729, p<0.05$). METH treatment produced a $50.5\pm19.5\%$ depletion in Vehicle+METH treated rats and a non-statistically significant $19.5\pm9.8\%$ depletion in Lactulose+METH treated rats (Figure 3.6).

**Local Administration of METH and Ammonia**

To examine if ammonia directly contributes to the neuronal damage caused by METH, METH (100 µM) and/or ammonia (570 µM) was reverse dialyzed into the striatum for 8 hours. Dopamine and 5-HT tissue content of the tissue adjacent to the microdialysis probe was dissected 7 days later. Our previous studies showed that the local administration of 100 µM METH into the striatum increased striatal dopamine release to the same levels caused by the systemic administrations of 10 mg/kg, every 2 hrs x 4 injections (Burrows et al., 2000). Ammonia (570 µM) was reverse dialysed to approximate the same brain concentration of ammonia previously measured after systemic METH treatment (Figure 3.7), considering the in-vivo recovery of ammonia by the microdialysis probe is 35%. The effects of reverse dialysis of METH and ammonia on dopamine and 5-HT tissue content in the striatum were determined (Figure 3.5). A
two-way ANOVA showed a significant interaction between METH and ammonia on dopamine tissue content \( (F_{(1,32)}=4.5, p<0.050) \) and post-hoc Tukey tests revealed a significant difference between the combination of METH and ammonia compared to METH alone \( (q=4.8, p<0.005) \) or ammonia alone \( (q=4.9, p<0.005) \) (Figure 3.7A). Similarly, a two-way ANOVA showed a significant interaction between METH and ammonia on 5HT tissue content \( (F_{(1,32)}=5.6, p<0.050) \). Tukey post hoc tests revealed a significant difference between the combination of METH and ammonia compared to METH alone \( (q=4.4, p<0.005) \) or ammonia alone \( (q=6.0, p<0.001) \) (Figure 3.7B).

Specifically, the combination of ammonia and METH produced a 51.5±12.5% depletion of dopamine and 41.8±7.0% depletion of 5-HT, when compared with artificial CSF controls, demonstrating that ammonia synergizes with METH to directly contribute to the neuronal damage caused by METH.
**Figure 3.1:** METH and plasma ammonia: Rats were treated with METH (10 mg/kg i.p. every 2 hrs x 4) or saline (1 mL/kg i.p. every 2 hrs x 4) and Lactulose (5.3 g/kg p.o. every 12 hrs) or vehicle (8 mL/kg, every 12 hrs). At 2 hrs after the last injection of METH or saline, plasma ammonia levels are significantly elevated by 64.04±9.98% (Mean±SEM) and this increase is blocked by lactulose treatment (*= p<0.001 compared to Lac Sal, &= p<0.001 compared to Veh METH). (n=8 rats per group) Veh=Vehicle, Lac=Lactulose, Sal=Saline
Figure 3.2: Effect of Hyperthermia on Methamphetamine-Induced Persistent Increases in Plasma Ammonia: Plasma ammonia was measured 24 hours after treatment with METH or saline. A group of METH treated rats were cooled to prevent METH-induced hyperthermia. METH treatment significantly elevated plasma ammonia concentrations by $114.8 \pm 10.5\%$. This increase was blocked by preventing METH-induced hyperthermia (METH-Cooled) * $p<0.05$ compared to saline treated rats, & $p<0.05$ compared to METH treated rats (n=10 per group)
Figure 3.3: METH and brain ammonia: Rats were treated with METH (10 mg/kg x 4 i.p. every 2 hrs) or saline (1 mL/kg x 4 i.p. every 2 hrs) and lactulose (5.3 g/kg p.o. every 12 hrs) or vehicle (8 mL/kg, every 12 hrs). During drug treatment (arrows denote injections), striatal ammonia concentration was measured using in-vivo microdialysis. Vehicle+METH treatment significantly increases brain ammonia concentrations starting at timepoint 5 and Lactulose pretreatment attenuates this increase (*=p<0.05). Baseline=29.34±5.33 µM. (n=8-10 rats per group) Veh=Vehicle, Lac=Lactulose, Sal=Saline
Figure 3.4: Hyperthermia after METH Treatment: Rats were treated with METH (10 mg/kg i.p. every 2 hrs x 4) or saline (1 mL/kg i.p. every 2 hrs x 4) and lactulose (5.3 g/kg p.o. every 12 hrs) or vehicle (8 mL/kg, every 12 hrs). During drug treatment, temperature was measured one hour after each METH injection (denoted by arrows). METH treatment caused significant hyperthermia in both vehicle+METH and lactulose+METH treated rats, and lactulose had no significant effect on hyperthermia. (*=p<0.001 compared with Veh Sal) (n=6-9 per group) Veh=Vehicle, Lac=Lactulose, Sal=Saline
Figure 3.5: Effect of Lactulose on METH-induced monoamine depletions: Rats were treated with METH (10 mg/kg i.p. every 2 hrs x 4) or saline (1 mL/kg i.p. every 2 hrs x 4) and lactulose (5.3 g/kg p.o. every 12 hrs) or vehicle and tissue content of dopamine or 5-HT in the striatum was measured 7 days later. A) Treatment with lactulose significantly attenuated the striatal dopamine depletions caused by METH as METH depletes dopamine by only 34.5 ± 7.9% (Mean±SEM) in lactulose treated rats. B) METH depletes 5-HT by 15.2 ± 8.2% (Mean±SEM) in lactulose treated rats, which was not statistically significant compared to saline treated rats. (* p<0.001 compared to Lac Sal. & p<0.001 compared to Veh METH). (n=6-9 rats per group) Veh=Vehicle, Lac=Lactulose, Sal=Saline
Figure 3.6: Effect of Lactulose on METH-induced reductions in DAT Immunoreactivity: Rats were treated with METH (10 mg/kg i.p. every 2 hrs x 4) or saline (1 mL/kg i.p. every 2 hrs x 4) and lactulose (5.3 g/kg p.o. every 12 hrs) or vehicle and tissue content of dopamine or 5-HT in the striatum were measured 7 days later. A) Treatment with lactulose significantly blocked the decrease in DAT immunoreactivity as METH produced only a 19.5±9.8% depletion in Lactulose+METH treated rats. B) Representative western blot for DAT immunoreactivity and α-tubulin internal control (* p<0.05 compared to Lac Sal. & p<0.05 compared to Veh METH). (n=6 rats per group) Veh=Vehicle, Lac=Lactulose, Sal=Saline
Figure 3.7: Effects of local administration of ammonia and METH into the striatum: Rats were reverse dialyzed with artificial cerebrospinal fluid, METH(100 µM) and/or Ammonia (570 µM), and/or GKY1 52466 (100 µM) in the striatum for 8 hrs and dopamine and 5HT tissue content of the striatal tissue around the probe membrane (1 mm) was measured 7 days later. A) Treatment with the combination of METH and ammonia depletes dopamine in the tissue around the probe by 51.5±12.5% (Mean±SEM). B) Treatment with the combination of METH and Ammonia depletes 5-HT in the tissue around the probe by 41.8±7.0% (Mean±SEM) compared to aCSF.
3.4 Discussion:

This study examined the relationship between changes in ammonia in periphery and the brain and the long term depletions of dopamine and 5HT in the brain typically associated with a high dose binge regimen of METH. METH caused increases in plasma ammonia that were paralleled by increases in brain ammonia. These increases were decreased by the enhancement of peripheral ammonia excretion that in turn, attenuated the long-term depletions of brain dopamine and 5HT produced by systemic exposure to METH. Furthermore, the local administration of ammonia with METH reproduced the depletions of striatal dopamine and 5HT produced by the systemic administrations of METH alone.

Peripheral ammonia levels were also measured at 2 and 24 hours after METH exposure to determine if the hepatotoxicity at this time point had functional consequences. The liver is the primary organ of ammonia metabolism via the urea cycle. When liver function is compromised, ammonia accumulates and leads to neuronal damage (Felipo and Butterworth, 2002). Plasma ammonia levels were significantly elevated at 2 and 24 hours after METH exposure. The finding that increases seen at 24 hours after METH were blocked by preventing METH-induced hyperthermia (Figure 3.2) and is consistent with the blockade of METH-induced liver damage described in Chapter 2. Interestingly, the increases in ammonia seen at 24 hours after METH treatment were comparable to those reported at just 2 hours after METH treatment (Figure 3.1). These findings demonstrate that the hepatocellular damage produced by METH is sufficient to cause persistent increases in plasma ammonia. Furthermore, the findings that both METH-induced liver damage and increases in ammonia were blocked by the prevention
of hyperthermia further strengthen the association between METH-induced liver damage and increases in ammonia to levels that can damage the brain. This also suggests that increases in ammonia may be a mechanism by which hyperthermia contributes to METH neurotoxicity. It is worth noting that while the liver is the primary ammonia metabolizing organ, damage to other organ systems which have been reported to be altered by METH, namely the cardiac, renal and musculoskeletal systems, also may contribute directly or indirectly to increases in ammonia (Smith and Fischer, 1970, Kamijo et al., 2002, Wijetunga et al., 2003).

No previous studies have examined if and how liver damage and/or ammonia contribute to the long-term neuronal damage produced by METH. The current findings show that METH produced elevations of ammonia in the plasma and striatum that were temporally and physiologically related to the liver damage produced by the drug. In parallel with the liver pathology at 2 hrs after the last METH injection, brain ammonia increased to about 70 µM in the striatal microdialysate (Figure 3.3). This translates to a brain ammonia concentration of approximately 200 µM, considering the recovery of ammonia from microdialysis is approximately 35 %. In cases of hepatic encephalopathy, ammonia concentrations in brain range from 300-1000 µM (Swain et al., 1992, Clemmesen et al., 1999). Although the concentrations of ammonia observed in the brain after METH are less than those in hepatic encephalopathy, lower concentrations of ammonia in combination with METH exposure appear to cause neurotoxic effects. Thus, liver damage may be an unrecognized consequence of METH exposure and a cause of neuronal damage mediated by peripherally derived ammonia even in the absence of hepatic encephalopathy per se. Also worth noting is that METH-induced damage to other
Peripheral organ systems may contribute to increases in ammonia either directly, as in the case of renal damage and rhabdomyolysis, or indirectly via influence on hepatic function, as in the case cardiac dysfunction and especially, right-sided heart failure (Smith and Fischer, 1970, Kamijo et al., 2002, Wijetunga et al., 2003, Ago et al., 2006). Thus, studies of the possible contributions by these organ systems and ammonia to METH-induced neurotoxicity are warranted.

We also investigated if ammonia derived from the periphery contributes to the elevations in brain ammonia and the neuronal damage after METH exposure. Peripheral ammonia excretion was increased using lactulose. Lactulose is a synthetic disaccharide that is not absorbed systemically but remains in the gut. It is degraded by bacteria in the colon thereby acidifying the colonic contents, converting ammonia to the ammonium ion ($\text{NH}_4^+$), thus trapping ammonia and preventing its absorption. Lactulose also functions as a laxative to reduce time for absorption and metabolism of protein, which is a source of ammonia. Lactulose prevented the acute increases in brain ammonia and the long term decreases in dopamine and 5-HT content, without affecting METH-induced hyperthermia (Figure 3.4, 3.5) and the brain concentrations of METH. This supports the view that the effects of lactulose are likely due to its effects on peripheral ammonia and not alterations in the direct pharmacological actions of METH in the brain.

Excretion of ammonia partially protected against dopamine, but blocked the depletions of 5-HT tissue content produced by METH treatment. The partial protection of dopamine terminals can be explained by the fact that the established causes of dopaminergic terminal damage, such as the interaction of METH with the dopamine transporter (DAT) and vesicular monoamine transporter-2 (VMAT-2), (Fleckenstein et
al., 1997, Hogan et al., 2000, Brown et al., 2002) might be relatively less affected by ammonia. Therefore, METH-induced increases in intra- and extracellular dopamine can produce ROS and dopamine-quinones and oxidatively damage dopamine terminals regardless of the presence of ammonia (Schmidt et al., 1985, Fornstedt et al., 1989, LaVoie and Hastings, 1999). In contrast, there may be causes of serotonergic terminal damage that are especially ammonia-dependent. Specifically, hyperammoniemia increases the uptake of L-tryptophan (Grippon et al., 1986), the expression and activity of monoamine oxidase (Mousseau et al., 1997), and production of 5-HIAA (Batshaw et al., 1986). Accordingly, ammonia-induced increases in 5-HT turnover may increase the production of oxidative intermediates. Furthermore, hyperammonemia increases the production of the excitotoxin and L-tryptophan metabolite, quinolinic acid (Moroni et al., 1986).

The central application of METH with ammonia recapitulated the neuronal damage produced by systemic METH exposure, even though administration of METH or ammonia alone did not produce neuronal damage (Figure 3.7). The apparent synergy between ammonia and METH indicates both are necessary, but alone are not sufficient to produce toxicity. Ammonia could be toxic through excitotoxic and oxidative damage (Fan and Szerb, 1993, Kosenko et al., 1995, Kosenko et al., 1997, Chan and Butterworth, 1999) while METH confers selectivity to dopamine and 5HT terminals through its effects on dopamine and 5HT transporters and the dopamine-dependent activation of the striatonigral pathway resulting in increases in extracellular glutamate in the striatum (Mark et al., 2004).
In conclusion, these studies identify a small molecule derived from the periphery that mediates the neuronal damage produced by METH and note the importance of considering the periphery in the context of METH-induced neurological dysfunction and damage. These findings suggest that co-morbid conditions affecting liver function or ammonia metabolism may negatively contribute to the neurotoxic consequences of METH exposure. In particular, co-morbid alcoholic cirrhosis or viral hepatitis with METH use may exacerbate acute METH-mediated liver damage and subsequent neurotoxicity. Furthermore, these findings highlight acute peripheral organ damage as a possible mediator of damage in other neurological disorders.
Chapter 4:

Contribution of Ammonia to Methamphetamine-Induced Increases in Extracellular Glutamate and Excitotoxicity

4.1 Introduction

Ammonia may contribute to METH-neurotoxicity by mediating drug-induced increases in glutamate and excitotoxicity. Excitotoxicity is a mechanism of neuronal damage resulting from increases in extracellular glutamate and glutamate receptor activation, which leads to increases in intracellular calcium (Choi, 1994). Excitotoxicity has been identified as a significant mechanism of neuronal damage in many disorders including Alzheimer’s’ disease, Huntington’s disease, ischemic stroke, and seizures (Choi, 1988). Glutamate is the primary excitatory neurotransmitter in the central nervous system and is released in a calcium-dependent manner from both pre-synaptic terminals in response to depolarization, as well as from astrocytes (Magistretti, 2006, Santello and Volterra, 2009). Subsequent to vesicular release, glutamate interacts with both pre- and post-synaptic receptors including the AMPA, NMDA, and Kainate receptors. Glutamate released into the synapse is removed by excitatory amino acid transporters located primarily on astrocytes (Danbolt, 2001).
Of particular relevance to excitotoxicity are the calcium permeable glutamate receptors which include the NMDA receptor and non-GluR2 subunit containing AMPA receptors (Hollmann et al., 1991). Physiologically, these calcium-permeable glutamate receptors are important for the development of synaptic plasticity, but are overactivated in the context of significantly elevated extracellular glutamate leading to significant increases in intracellular calcium (Liu and Zukin, 2007). Within the striatum, the expression of calcium permeable AMPA receptors is increased compared to NMDA receptor expression (Fan et al., 1999, Dunah et al., 2000). In particular, non-Glu-R2 subunit containing, calcium permeable AMPA receptors have been identified on dopamine and serotonin terminals within the striatum, making these terminals susceptible to the excitotoxic terminal damage (Keefe et al., 1993, Betarbet and Greenamyre, 1999, Hernandez et al., 2003). Non-GluR2 containing AMPA receptors may also be located on GABA interneurons, also increasing the vulnerability of those cells to excitotoxic damage (Segovia et al., 1997).

Increases in intracellular calcium contribute to cellular damage through numerous mechanisms including the activation of proteases and production of free radicals. The activity of the calcium activated protease, calpain, increases in response to increased intracellular calcium. Conversely, when calpain activity is inhibited, protection against excitotoxicity is provided (Siman et al., 1989, Lee et al., 1991, Squier et al., 1994). Calpain contributes to neuronal damage through the degradation of essential cytoskeletal proteins, including spectrin, actin, MAP2, neurofilament and Tau (Schlaepfer et al., 1985, Billger et al., 1988, Harris and Morrow, 1988, Johnson et al., 1989, Villa et al., 1998). Free radicals are produced in response to increases in intracellular calcium through
increases in phospholipase A₂ activity, mitochondrial damage, and nitric oxide production (Chan et al., 1985, Dumuis et al., 1988, Dugan et al., 1995, Gow et al., 1997). Increases in free radicals leads to the oxidative damage of membranes and proteins (Stamler and Hausladen, 1998). Oxidative modification of specific proteins can reduce the activity of those proteins and further perpetuate cellular damage (Gow et al., 1996).

Subsequent to release, excess glutamate in the synapse is then removed via excitatory amino acid transporters, located primarily on astrocytes, and metabolized intracellularly. Decreased astrocytic uptake or intracellular metabolism of glutamate can contribute to increases in extracellular glutamate and excitotoxicity. Excitatory amino acid transporters (EAATs) are responsible for the uptake of glutamate and include EAAT1/glutamate aspartate transporter (GLAST), EAAT2/glutamate transporter-1 (GLT1), and EAATs 3-5 (Had-Aissouni, 2012). The primary glutamate transporters on astrocytes include EAAT-1/GLAST and EAAT-2/GLT-1 (Lehre et al., 1995). In addition to these glutamate transporters, the cystine-glutamate antiporter system is also important for astrocytic glutamate uptake (Sato et al., 1999). Typically, these transporters move extracellular glutamate into astrocytes from the synapse, however the directionality of these transporters has been shown to be dependent on extracellular potassium levels, such that an increase in extracellular potassium reverses the directionality of these transporters preventing uptake and producing non-vesicular release of glutamate from astrocytes (Barbour et al., 1988, Szatkowski et al., 1990, Grewer et al., 2008). Once taken up into cells, glutamate, along with ammonia, is metabolized to glutamine via glutamine synthetase (Haberle et al., 2006). Glutamine produced from this reaction is then transported into presynaptic terminals and used for the synthesis of glutamate as part of
the glutamate-glutamine cycle. The glutamine produced from this reaction also serves as a nitrogen source for the biosynthesis of many other important compounds including tryptophan, glucosamine-6-phosphate, histadine and carbamoyl phosphate (Woolfolk et al., 1966). Interestingly, glutamine synthetase activity is reduced by nitration subsequent to glutamate receptor activation and increases in nitric oxide (McBean et al., 1995, Gorg et al., 2007).

Increases in extracellular glutamate and excitotoxicity contribute significantly to the neuronal damage produced by METH. METH produces significant increases in extracellular glutamate levels and inhibition of glutamate receptor activation, or blocking increases in extracellular glutamate is neuroprotective for the damage produced by the drug (Farfel et al., 1992, Nash and Yamamoto, 1992, Mark et al., 2004, Tata and Yamamoto, 2007). METH has also been show to increase calpain-mediated spectrin proteolysis, a marker of excitotoxicity in the striatum, in an AMPA receptor-activation manner (Staszewski and Yamamoto, 2006). Increases in extracellular glutamate are observed acutely during treatment with METH, and result from dopamine D1-receptor mediated disinhibition of corticostriatal glutamate release (Mark et al., 2004). In-vitro, high doses of METH have also been shown to differentially affect glutamine synthetase expression (Stadlin et al., 1998).

As with METH, the neuronal damage produced by ammonia may result from increases in glutamate and excitotoxicity as this damage is also attenuated with glutamate receptor antagonist treatment (Saez et al., 1999, Kosenko et al., 2003). Ammonia induced increases in extracellular glutamate result from alteration of the local uptake, metabolism and release of glutamate from astrocytes. Ammonia has been shown to decrease the
expression of both GLAST and GLT-1 (Chan and Butterworth, 1999, Chan et al., 2000). High concentrations of ammonia have also been shown to increase calcium-dependent exocytotic glutamate release from astrocytes *in-vitro* (Gorg et al., 2010). More acutely, the ammonium ion, which has a similar ionic radius to the potassium ion, may contribute to increases in glutamate by reversing potassium-gradient dependent glutamate transporters (Rose, 2002). The metabolism of glutamate within astrocytes is also affected by ammonia. Glutamine synthetase is the key glutamate and ammonia metabolizing enzyme in the brain, is decreased after ammonia exposure and is modified by nitration or nitrosylation (Kosenko et al., 2003, Gorg et al., 2007). Inhibition of glutamine synthetase results in the accumulation of both glutamate and ammonia and therefore, represents a feed-forward mechanism by which ammonia may mediate METH-induced neurotoxicity. Beyond affecting extracellular glutamate levels, ammonia has been shown to increase NMDA receptor activation via membrane depolarization and removal of the Mg$^{2+}$ block of NMDA receptors (Fan and Szerb, 1993).

Accordingly, the contribution of ammonia to METH-induced elevations in extracellular glutamate was examined. It was hypothesized that ammonia contributes directly to the increases in extracellular glutamate seen during METH treatment as a result of alteration of local glutamate processing, and that this altered glutamate processing, in addition to the previously identified increases in corticostriatal glutamate release, is required for METH-induced increases in glutamate. In addition to examining the contribution of ammonia to METH-induced increases in extracellular glutamate, we have determined if ammonia has a net, downstream effect on METH-induced excitotoxicity by examining calpain-mediated spectrin proteolysis. Finally, we have
examined if ammonia and METH contribute to decreased expression or activity of glutamine synthetase and as potential mechanism by which the drug and/or ammonia can contribute to feed-forward mechanisms of neurotoxicity, thereby amplifying the damage caused by both METH and ammonia.

4.2 Methods:

Rat Treatment:
Male Sprague Dawley rats received a high dose of METH (10mg/kg q 2 hr X 4, i.p.) or saline (1 mL/kg q 2 hr X 4, i.p.). In lactulose experiments, rats received lactulose (5.3 g/kg) or vehicle via oral gavage every 12 hours for 2 days before METH treatment until the day before rats were killed. All rats were killed by live decapitation 2 hrs, 5 days or 7 days after the start of the Meth or Saline treatment. Subcutaneous temperature transponders were implanted into the rats 2 days before the beginning of each experiment to allow for accurate monitoring of temperature and to reduce animal stress when temperature was taken. The transponders used were 2.2x14 mm and weigh 120 mg (IPTT-300 transponder, BMDS Inc.). All treatments were carried out in accordance with the National Institute of Health Guide for Care and Use of rats. All treatments are approved by the University of Toledo Institutional Animal Care and Use Committee.

HPLC Analysis of Monoamine Tissue Content:
Tissue within 1 mm of probe track was dissected and sonicated in 300 µL of 0.25 N Perchloric Acid and centrifuged at 14000 x g for 20 minutes at 4°C. Supernatant was injected on a C18 column (100x2 mm, 3µm particle diameter, Phenomenex) and eluted with a mobile phase containing 32 mM citric acid, 54.3 mM sodium phosphate, 0.215
mM octyl sodium sulfate, and 11% methanol (pH 4.4). Concentration of monoamines was determined with a LC-4C amperometric detector (BAS Bioanalytical) and data was recorded using EZ Chrom Software. Monoamine levels were normalized to protein content of samples. The pellet from the centrifugation was neutralized using 100 µL of 1 M NaOH and protein amount was determined using a Bradford Assay.

**HPLC Analysis of Glutamate:**
Dialysate (20 µL) was injected on a C18 column (150x2 mm, 3µm particle diameter, Phenomenex) and eluted with a mobile phase containing 0.1 M Na2HPO4, 0.1 mM EDTA, and 7.5% Methanol  (pH 3.2). Glutamate was derivatized for electrochemical detection with o-phtaldialdehyde. Concentration of glutamate was determined with a LC-4C amperometric detector (BAS Bioanalytical) and data was recorded using EZ Chrom Software. Glutamate levels were normalized to baseline levels where appropriate.

**In-Vivo Microdialysis:**
Intracranial probes were constructed using PE 20 tubing (Becton Dickinson), silica tubing (OD of 150 µM, Polymicro Technologies), 26 ga stainless steel hypodermic tubing (Small Parts), Hollow Fiber Microdialysis Membrane ( 4 mm of active membrane, MWCO 13,000, 216 µm, Spectrum Labs), 2-ton waterproof epoxy and tygon microbore tubing. Surgeries were performed the day before drug treatment. Rats were anesthetized using xylazine (5 mg/kg) and ketamine (75 mg/kg) and the probe was stereotaxically lowered into the striatum (AP+1.2, ML+3.0, DV-6.5 mm). One day later, and after a 1 hour equilibration period, baseline samples were collected every hour for 3 hrs at a flow rate of 1.5 µL/min. Samples were collected every hour during drug treatment until 2 hrs after the last drug injection. Probe placement was verified histologically.
Spectrin Breakdown Product Analysis:
5 days after METH or saline and lactulose or vehicle treatment, rats were be killed by rapid decapitation and striata were dissected. Tissue was homogenized in a buffer containing 10mM Tris, 10 mM EGTA, 250 mM sucrose, and Halt protease inhibitor cocktail (Thermo Scientific). 30 µg of protein of each sample was separated via electrophoresis, and transferred to a PVDF membrane. Spectrin breakdown products were examined using an antibody for non-erythroid α-Spectrin (MAB1622; Chemicon) and a HRP-conjugated secondary antibody. The spectrin breakdown product specific to calpain mediated proteolysis (145 kDa) was analyzed using the Fuji LAS-400 Mini System. Immunoreactivity of calpain-specific spectrin breakdown product was normalized to α-tubulin to as a loading and transfer control. Data are expressed as a percentage of vehicle-saline treated animals.

Glutamine Synthetase Protein Level Measurement:
2 hours after METH or saline treatment rats were killed by rapid decapitation and striata were dissected. Tissue was homogenized in RIPA buffer (1% Igepal CA-630, 0.5% Sodium Deoxycholate, and 0.1% Sodium Dodecyl Sulfate) containing Halt Protease Inhibitor Cocktail (Thermo Scientific). A Bradford assay was used to determine protein content and samples were diluted (1:4) with LDS sample buffer. 5 µg of protein was separated via electrophoresis and transferred to a PVDF membrane. Glutamine Synthetase levels were examined using a primary antibody to Glutamine Synthetase (MAB302, Chemicon) and a HRP-conjugated secondary antibody. The single band present at 45 kDa was analyzed using the Fuji LAS-400 Mini System. Immunoreactivity
of glutamine synthetase was normalized to α-tubulin to as a loading and transfer control. Data are expressed as a percentage of vehicle-saline treated animals.

**Glutamine Synthetase Activity Assay:**

Glutamine synthetase activity was measured using the glutamyltransferase activity of the enzyme which catalyzes the reaction producing γ-glutamylhydroxymate from glutamine and hydroxylamine (Webb and Brown, 1976). 2 hours after METH or saline treatment rats were killed by rapid decapitation and striata were dissected. The striatum from a single hemisphere was homogenized in 400 μL of ice cold RIPA buffer (1% Igepal CA-630, 0.5% Sodium Deoxycholate, and 0.1% Sodium Dodecyl Sulfate). Protease inhibitor was not used because it alone produced detectable product that was not dependent on glutamine synthetase activity. RIPA buffer alone does not produce any product. 50 μL of tissue homogenate was added to the reaction mixture which contained 60 mM L-glutamine, 15 mM hydroxylamine-HCl, 20 mM Sodium Arsenite, 0.4 mM ADP, 3 mM Manganese Chloride, and 60 mM Imidazole-HCl (pH 6.8) to a total volume of 500 μL. After combining tissue with the reaction mixture, the reaction ran for 30 minutes at 37 °C. After 30 minutes, the reaction was stopped by addition of 0.37 mM Iron Chloride, 0.67 mM HCl and 0.2 mM trichloracetic acid. The solution was then centrifuged at 14,000 x g for 10 minutes to separate protein. Product formation in the supernatant was measured and quantified by measuring the absorbance at 500 nm and using a standard curve of γ-glutamylhydroxymate (Sigma-Aldrich). To determine the amount of product formation that was specific to glutamine synthetase activity the reaction was run for each sample with and without the arsenite cofactor. Product formation in the absence of arsenite for each sample was subtracted from the product formation with arsenite to
determine the glutamine synthetase-dependent product formation. On average, about 20% of the product formation from tissue was not glutamine synthetase dependent. Product formation was also normalized to protein concentration of each sample, which was determined using a Bradford assay. Data are expressed as a percentage of vehicle-saline treated controls.

Statistical Analysis:

Analysis of tissue content of dopamine and 5HT in the striatum, and spectrin proteolysis was performed using a two-way ANOVA followed by post hoc Tukey multiple comparison tests or t-tests. These tests were performed using SigmaPlot 11.0 Software (Systat Software, Inc. SigmaPlot for Windows). Statistical analysis of brain glutamate levels was carried out using a repeated measures ANOVA test, with each time point as a within-subjects factor and ammonia, lactulose, or METH treatment as between-subjects factors. These tests were performed using SPSS Statistics 17.0 Software (IBM Corporation). All data are presented as Mean±Standard Error of the Mean (SEM). α level in all studies is 0.05 or less and sample sizes were chosen to result in a power of 80% or greater.

4.3 Results:

Effect of AMPA Receptor Antagonism on Local Administration of METH and Ammonia

To identify a mechanism by which ammonia and METH may contribute to striatal damage, ammonia (570 µM)+ METH (100 µM), and/or GYKI 52466 (100 µM) was reverse dialyzed for 8 hrs and the tissue adjacent to the microdialysis probe was dissected 7 days later for measurement of dopamine and 5-HT tissue content. GYKI 52466 is an
AMPA receptor antagonist that has been shown to prevent METH-induced increases in calpain-mediated spectrin proteolysis (Staszewski and Yamamoto, 2006). The effect of GYKI 52466 on METH- and ammonia-induced decreases in dopamine and 5HT tissue content was determined. A two-way ANOVA revealed a significant interaction between the combination of METH +ammonia, and GYKI 52466 treatment on dopamine tissue content \((F(1,39)=3.392, p<0.05)\). A post hoc t-test revealed that there is a significant effect of 52466 GYKI treatment on the METH+ammonia-induced depletions in dopamine tissue content \((t=3.298, p<0.05)\) (Figure 4.1A). Similarly, when the effects of GYKI 52466 on METH+ammonia-induced depletions in 5HT were examined, a two-way ANOVA revealed a significant interaction between the combination of METH+ammonia, and GYKI 52466 treatment \((F(1,39)=3.624, p<0.05)\). A post-hoc Tukey test revealed a significant effect of GYKI 52466 treatment on METH and ammonia-induced depletions in 5HT content \((q=5.541, p<0.05)\) (Figure 4.1B). Thus, GYKI 52466 blocked the decreases in DA and 5HT content produced by ammonia+METH.

**Effect of Locally Administered Ammonia on Extracellular Glutamate**

To examine if the increases in brain ammonia seen during systemic METH treatment were capable of altering extracellular glutamate levels, 200 µM of ammonia, which is the striatal concentration measured during systemic METH treatment, was administered into the striatum for 8 hours using reverse dialysis and changes in extracellular glutamate were measured (Figure 4.2). A repeated measures ANOVA shows that ammonia produced significant elevations in extracellular glutamate, compared to animals reverse dialysed with artificial cerebrospinal fluid throughout all time points \((F_{(1,13)}= 13.531, p<0.05)\).
These increases represent an approximate 2-3-fold increase in extracellular glutamate.

Effect of Ammonia on Systemic-METH-Induced Increases in Extracellular Glutamate

To determine to what degree ammonia contributes to the increases in extracellular glutamate produced during systemic METH treatment, rats were treated with lactulose (5.3 g/kg p.o. every 12 hours) or vehicle (8mL/kg) and treated with METH (10 mg/kg i.p. every 2 hours x 4 injections) or saline (1 mL/kg i.p. every 2 hrs x 4). Extracellular glutamate was measured during treatment with METH or Saline using in-vivo microdialysis (Figure 4.3). Baseline glutamate levels in all groups were 1365.24±183.69 pg of glutamate. A repeated measures ANOVA shows a main effect of METH treatment ($F_{(7,167)}=5.033$, $p<0.050$) and a significant interaction between METH and lactulose treatment ($F_{(7,167)}=3.295$ $p<0.050$). Striatal glutamate levels were significantly elevated in METH treated animals at the 6 hour time point ($F_{(3,20)}=4.768$ $p<0.050$). These data demonstrate that increasing peripheral ammonia excretion with lactulose fully attenuates METH-induced elevations in glutamate.

Effect of Ammonia on Calpain-Mediated Spectrin Proteolysis

Calpain is a calcium activated protease that produces a specific spectrin breakdown product at 145 kDa and is a marker for excitotoxic damage (Harris and Morrow, 1988, Staszewski and Yamamoto, 2006). To examine the contribution of ammonia to METH-induced excitotoxic terminal damage, the spectrin breakdown product at 145 kDa was measured in the striatum of rats treated with lactulose (5.3 g/kg p.o. every 12 hours) or vehicle (8mL/kg) and treated with METH (10 mg/kg i.p. every 2 hours x 4 injections) or
saline (1 mL/kg i.p. every 2 hrs x 4) 5 days after treatment with METH or Saline. METH produced 123.4 ± 4.9% increase in calpain-specific spectrin breakdown, which was completed blocked by lactulose treatment. A two-way ANOVA revealed a significant interaction between METH and lactulose treatment ($F_{(3,31)} = 4.436, p<0.05$). Post-hoc tukey tests indicate that METH has a significant effect on calpain-specific spectrin breakdown ($q= 4.927, p<0.005$) and this effect is blocked by lactulose treatment ($q=4.364, p<0/05$) (Figure 4.4A). A representative western blot for spectrin and α-tubulin immunoreactivity is also shown (Figure 4.4B)

**Effect of METH on Glutamine Synthetase Protein Levels**

To examine if METH produces acute decreases in glutamine synthetase expression, rats were treated with METH (10 mg/kg i.p. every 2 hours x 4 injections) or saline (1 mL/kg i.p. every 2 hrs x 4) and glutamate synthetase immunoreactivty was quantified in the striatum at 2 hours after drug treatment. Glutamine synthetase immunoreactivity did not significantly differ between groups treated with METH or saline (Figure 4.5A). A representative western blot for glutamine synthetase and α-tubulin immunoreactivity (Figure 4.5B) is also shown.

**Effect of METH on Glutamine Synthetase Activity**

To determine if METH-induced produces acute decreases in glutamine synthetase activity, rats were treated with METH (10 mg/kg i.p. every 2 hours x 4 injections) or saline (1 mL/kg i.p. every 2 hrs x 4). At 2 hours after drug treatment, glutamate synthetase activity was quantified in the striatum using an assay that measures glutamine synthetase mediated γ-glutamylhydroxmate ($γ$-GH) formation. Both total $γ$-GH (Figure
4.6A) and γ-GH specific to glutamine synthetase activity (Figure 4.6B) were quantified and METH did not have a significant effect on glutamine synthetase activity.
Figure 4.1: The Dopamine and Serotonin Terminal Damage Produced by the Combination of NH$_3$ and METH Requires AMPA Receptor Activation. Rats were reverse dialyzed with METH (100 µM), Ammonia (570 µM), and/or GKYI 52466 (100 µM) in the striatum for 8 hrs and dopamine and 5HT tissue content of the striatal tissue around the probe membrane (1 mm) was measured 7 days later. A) Treatment with GYKI 52466 significantly attenuates depletions in dopamine tissue content produced by METH and ammonia. B) Treatment with GKYI 52466 significantly attenuates depletions in 5HT tissue content produced by the combination of METH and ammonia. (* p<0.05 compared to aCSF, & p<0.05 compared to Ammonia METH) (n=10 rats per group) aCSF=artificial cerebrospinal fluid, GYKI=GYKI 52466
Figure 4.2: Effect of Local Ammonia Administration on Extracellular Glutamate Levels: Artificial cerebrospinal fluid (aCSF) or NH₃ (570 µM) was reverse dialysed into the striatum for 8 hours and extracellular glutamate was measured. Reverse dialysis of NH₃ significantly elevates striatal glutamate levels (*p<0.05). (n=6-8 rats per group)
Figure 4.3: Effect of Ammonia on Systemic METH-induced Increases in Extracellular Glutamate: Rats were treated with METH (10 mg/kg x 4 every 2 hrs) or saline (1 mL/kg x4 every 2 hrs) and Lactulose (5.3 g/kg every 12 hrs) or vehicle (8 mL/kg every 12 hrs). During METH treatment (arrows denote injections), striatal glutamate concentration was monitored using in-vivo microdialysis. Vehicle+METH treatment significantly increases brain glutamate concentrations starting at timepoint 6. Lactulose pretreatment blocks the increase. Baseline=1345.05 ± 162.55 pg/20 µL dialysate (*p<0.05). (n=8-10 rats per group).
Figure 4.4: Effect of Ammonia on METH-induced Increases in Calpain-Mediated Spectrin Proteolysis: Rats were treated METH (10 mg/kg x 4 every 2 hrs) or saline (1 mL/kg x 4 every 2 hrs) and Lactulose (5.3 g/kg every 12 hrs) or vehicle (8 mL/kg every 12 hours) and killed 5 days after drug treatment. A) Lactulose prevents METH induced increases in the spectrin breakdown product (SBP) which is specific for calpain proteolysis (* p< 0.05 compared to Veh Sal) (n=6-9 per group) B) Representative western blot for spectrin breakdown products and α-tubulin loading control.
Figure 4.5: Effect of METH on Glutamine Synthetase Expression: Rats were treated with METH (10 mg/kg x 4 every 2 hrs) or saline (1 mL/kg x 4 every 2 hrs) and glutamine synthetase immunoreactivity was measured at 2 hours after treatment. A) METH treatment had no significant effect on glutamine synthetase immunoreactivity. (n=8 per group) B) Representative western blot for glutamine synthetase and α-tubulin loading control. (GS=glutamine synthetase)
Figure 4.6: Effect of METH on Glutamine Synthetase Activity: Rats were treated with METH (10 mg/kg x 4 every 2 hrs) or saline (1 mL/kg x4 every 2 hrs) and glutamine synthetase activity was measured using an assay for γ glutamylhydroxymate formation. METH treatment had no significant effect on total γ-glutamyltransferase activity from tissue or γ-glutamyltransferase activity specific to glutamine synthetase. (n= 8 per group) (γ-GH= γ-glutamylhydroxymate)
4.4 Discussion:

These findings demonstrate that ammonia mediates METH neurotoxicity via increases in extracellular glutamate and excitotoxicity. Activation of the AMPA glutamate receptor was found to be required for the long term dopamine and serotonin terminal damage produced by the direct administration of ammonia and METH. Ammonia, at the brain concentrations measured during systemic METH exposure, was also found to be significant enough to alter local extracellular glutamate levels in the striatum. To examine to what degree ammonia affected increases in extracellular glutamate seen during systemic METH treatment, rats were treated with lactulose to block increases in brain ammonia during METH treatment. Blocking increases in brain ammonia during METH treatment also blocked METH-induced increases in extracellular glutamate, suggesting that increases in brain ammonia are required for increases in glutamate. Consistent with these findings, ammonia was also found to be required for METH-induced excitotoxicity, as measured by calpain-mediated spectrin proteolysis. To determine a mechanism by which ammonia may contribute to METH-induced increases in extracellular glutamate, we examined if METH treatment affected glutamine synthetase protein levels and activity, yet found that neither was affected by METH treatment. This suggests that the ammonia contributes to METH-induced increases in glutamate through a mechanism other than effects on glutamine synthetase.

The neuronal damage produced by the local administration of ammonia with METH into the striatum was blocked by the local administration of the AMPA antagonist, GYKI52466 (Figure 4.1). This suggests that the combination of ammonia and METH could activate presynaptic AMPA receptors through elevations in
extracellular glutamate. Systemic but not the central administration of METH treatment has been shown to increase extracellular glutamate (Tata and Yamamoto, 2007). Increased extracellular glutamate can then activate calcium-permeable AMPA receptors on striatal dopamine and 5HT terminals (Hollmann et al., 1991, Lai et al., 2003) resulting in both increased dopamine and 5HT release (Ohta et al., 1994), and excitotoxic terminal damage (Staszewski and Yamamoto, 2006). Ammonia has been shown to increase glutamate release from astrocytes (Gorg et al., 2010) and increase extracellular glutamate by decreasing glutamate transporter expression (Chan et al., 2000) and glutamine synthetase activity (Kosenko et al., 2003), all of which can combine with the effects of METH on dopamine and 5HT terminals to produce long term 5HT and dopamine depletions.

To determine if ammonia was directly affecting extracellular glutamate levels, we reversed dialysed ammonia, at the same brain concentrations measured during systemic METH treatment, directly into the striatum and measured extracellular glutamate. High concentrations of ammonia affect the processing of glutamate by astrocytes in vitro (Gorg et al., 2010), however it was unknown if ammonia alone, at the concentrations produced by systemic METH treatment, was capable of producing similar effects on extracellular glutamate. We found that reverse dialyzing ammonia into the brain for 8 hours produced a significant and consistent 2-3 fold increase in brain glutamate (Figure 4.2). This increase is comparable to the 4-5 fold increase in brain glutamate seen with systemic METH treatment, yet suggests that systemic METH treatment contributes to increases extracellular glutamate through other mechanisms other than those mediated by ammonia alone. In addition to the effect of ammonia reported in the present study, METH-induced
increases in dopamine have been shown to contribute to increased presynaptic glutamate release from corticostriatal projections as a result of D1-receptor mediated disinhibition (Mark et al., 2004). Accordingly, it appears that METH-induced increases in brain ammonia are capable of contributing to drug-induced elevations in extracellular glutamate.

Interestingly, local administration of ammonia alone is not capable of producing the long-term terminal dopamine and serotonin damage seen after systemic METH treatment although we show that ammonia alone is capable of increasing extracellular glutamate (Figure 3.7). This finding is consistent with previous reports that the direct administration of glutamate alone into the striatum does not produce long-term depletions in dopamine tissue content (Tata and Yamamoto, 2007). The direct administration of either ammonia or glutamate into the striatum only produces monoaminergic terminal damage when combined with METH. Accordingly, it appears that increases in ammonia, via increases extracellular glutamate, as well as other effects of METH, especially increases in dopamine, interact synergistically to produce the long-term monoaminergic terminal damage caused by the drug (Abekawa et al., 1994, Stephans and Yamamoto, 1994). This synergistic interaction explains why the damage produced by METH, which appears to be significantly mediated by global increases in ammonia, is selective for brain regions that contain the typical targets for METH, such as dopamine and serotonin transporters as well as glutamatergic projections.

To examine to what degree ammonia-induced elevations in glutamate are required for the increases in extracellular glutamate seen during systemic METH treatment, we blocked METH-induced increases in brain ammonia and found that this also fully
blocked METH-induced increases in extracellular glutamate (Figure 4.3). These findings suggest that increases in ammonia are required for METH-induced increases in extracellular glutamate. Ammonia has been shown to increase glutamate through alteration of astrocytic uptake, metabolism, and release (Chan and Butterworth, 1999, Kosenko et al., 2003, Gorg et al., 2010). This is important for extracellular glutamate levels because astrocytic reuptake and metabolism is the primary mechanism by which glutamate is removed from the synapse subsequent to presynaptic release (Benjamin and Quastel, 1975, Danbolt, 2001). Although increased corticostriatal glutamate release subsequent to METH-induced increases in dopamine has been identified as a mechanism by which the drug increases glutamate (Stephans and Yamamoto, 1994), the current findings have identified another significant mechanism by which the drug contributes to increases in glutamate in a potentially dopamine-independent manner. Accordingly, these findings suggest that both dopamine-mediated increases in glutamate release from corticostriatal projections, as well an ammonia-dependent metabolism of glutamate in astrocytes, are required for METH-induced increases in extracellular glutamate.

To confirm if ammonia also significantly contributes to METH-induced excitotoxicity, we measured whether increases in brain ammonia were also required for increases in calpain-mediated spectrin proteolysis. When increases in brain ammonia were blocked during and after METH treatment with lactulose, this prevented METH induced increases in calpain-mediated spectrin proteolysis (Figure 4.4). Calpain is protease that targets essential cytoskeletal proteins, including spectrin, which is activated subsequent to calcium influx in the context of excitotoxicity (Siman et al., 1989). METH has previously been shown to increase calpain-mediated spectrin proteolysis in an AMPA
receptor activation dependent manner (Staszewski and Yamamoto, 2006). The finding that ammonia is also required for METH-induced calpain-mediated spectrin proteolysis suggests that it is important for both increases in glutamate as well as excitotoxicity.

We also examined if systemic METH treatment affects glutamine synthetase protein levels or activity. Glutamine synthetase is an astrocytic enzyme responsible for metabolizing both ammonia and glutamate to produce glutamine. This enzyme has been shown to be decreased long-term after ammonia exposure, as well as after METH treatment in vitro (Stadlin et al., 1998, Kosenko et al., 2003). We examined glutamine synthetase protein levels and activity at 2 hours after METH treatment because altered enzyme activity at this time point would represent a mechanism by which altered astrocytic metabolism of glutamate may be contributing to acute increases in glutamate. We found that neither glutamine synthetase protein levels (Figure 4.5) nor activity (Figure 4.6) was altered. This supports the conclusion that the acute ammonia-induced increases in glutamate observed subsequent to METH are not due to decreased glutamine synthetase protein levels or activity. Glutamine synthetase activity has been shown to decrease in response to nitration subsequent to oxidative damage (Gorg et al., 2007). METH-induced oxidative damage affecting protein function has been reported at time points as early as 1 hour after the binge dosing regimen, however these increases in oxidative damage are reported in neurons and are likely the result of increases in intracellular dopamine and nitric oxide formation (LaVoie and Hastings, 1999a, b, Eyerman and Yamamoto, 2007). As glutamine synthetase is an astrocytic enzyme the lack of an effect on this protein suggests that METH-induced oxidative damage, nitric oxide formation, and possibly increases in intracellular dopamine may be specific to
neuron terminals at early time points whereas the effects of ammonia on non-neuronal cells such as astrocytes are more protracted based on evidence that increases in glial fibrillary acidic protein (GFAP) are only observed days after drug exposure (Hess et al., 1990, O'Callaghan and Miller, 1994, Northrop and Yamamoto, 2012). This finding also suggests a different mechanism is responsible for the contribution ammonia to METH induced increases in glutamate. Ammonia may be affecting astrocytic glutamate reuptake, upstream of glutamate synthase at the level of the excitatory amino acid transporters. Although ammonia has been shown to affect transporter expression at later time points after exposure, it may also affect the function of the transporter more acutely by altering the ionic gradients that are responsible for determining the directionality of the transporter (Barbour et al., 1988, Szatkowski et al., 1990, Chan and Butterworth, 1999, Grewer et al., 2008). Alteration of directionality of the transporter would prevent the removal of glutamate from the synapse, allow glutamate within astrocytes to be released, and contribute to increases in extracellular glutamate.

Overall, these findings identify ammonia as a key mediator for METH-induced increases in glutamate and excitotoxicity. These findings have broader significance as they demonstrate that acute increases in brain ammonia contribute to increases in extracellular glutamate, suggesting ammonia may function as small molecule modulator of excitatory neurotransmission, as well as a significant contributor to excitotoxicity. These findings may also have significance in the context of other disorders where excitotoxicity is believed to play a role including Alzheimer’s disease, ischemic stroke, Huntington’s chorea and others (Choi, 1988).
Chapter 5:

General Discussion of Findings

5.1 Summary of Findings:

We hypothesized that the effects of METH on peripheral organ function contribute to the established striatal dopamine and serotonin terminal damage produced by the drug. The findings in this dissertation support the conclusion that METH-induced liver damage and increases in ammonia play a significant role in the neurotoxicity of the drug. We demonstrate that METH produces significant acute, liver damage that persists for at least 24 hours after drug exposure. This liver damage appears to result from METH-induced hyperthermia. Concurrent with this liver damage, the drug also produces significant increases in both plasma and brain ammonia. These increases in ammonia synergize with METH to contribute to the long term striatal dopamine and serotonin terminal damage produced by the drug. Ammonia appears to contribute significantly to this neuronal damage through increases in extracellular glutamate and excitotoxic damage. These findings identify ammonia as a novel, peripherally-derived mediator of the neurotoxicity of METH (Figure 5.1).
Figure 5.1: Summary of Findings: We have examined the role that METH-induced peripheral organ damage plays in the neurotoxicity of the drug. METH produced acute, hyperthermia-dependent liver damage which contributes to increases in plasma ammonia. Increases in brain ammonia synergize with METH to produce long-term dopamine and serotonin terminal damage though increases in glutamate and subsequent excitotoxicity.
5.2 General Discussion

In chapter 2, we established that METH produces alterations in hepatocellular morphology and increases in AST and ALT that persist for at least 24 hours after a binge drug exposure. These findings support that METH exposure causes acute liver damage (Ozer et al., 2008). METH-induced hyperthermia was also shown to play a significant role in this liver damage as the changes in morphology and increases in AST and ALT were prevented when drug-induced hyperthermia was blocked. Prior to these studies, liver damage after METH had been noted in clinical reports, but the liver damage produced by the drug had yet to be extensively studied (Smith and Fischer, 1970, Kamijo et al., 2002). The findings in this dissertation illustrate for the first time that the drug produces significant and persistent damage to the liver. Because liver function is critical for many aspects of physiology and metabolism, it prompted us to hypothesize liver damage in mediating the neurotoxicity of the drug. Beyond the liver, METH-induced hyperthermia may contribute to the damage of other peripheral organs, warranting the study of the contribution of METH-induced hyperthermia to the damage of other peripheral organs reported to be affected by METH, including the renal, cardiovascular and muscular systems (Kamijo et al., 2002, Wijetunga et al., 2003, Ago et al., 2006, Yarmolenko et al., 2011).

The liver is essential in the metabolism of ammonia and drove us to hypothesize that the METH-induced liver damage reported in Chapter 2 may affect ammonia metabolism (Felipo and Butterworth, 2002). Therefore, we examined if plasma and brain ammonia levels were elevated at the same time points as the liver damage and
found that METH also produces significant elevations in ammonia (Chapter 3). We also found that when METH-induced liver damage was prevented by blocking hyperthermia, this also prevented increases in ammonia and further strengthened the link between METH-induced liver damage and increases in ammonia. To examine if these increases in ammonia contributed to the dopamine and serotonin terminal damage produced by METH, we blocked drug-induced increases in ammonia using lactulose and found that this also attenuated the long-term neurotoxicity of the drug. To determine if ammonia was contributing directly to the neurotoxicity of METH, we directly administered ammonia alone or the combination of ammonia and METH to the striatum in concentrations similar to those measured during systemic METH treatment. We found that only the combination of METH and ammonia was able to produce the long-term dopamine and serotonin terminal damage seen after systemic METH treatment, suggesting that ammonia acts as a peripherally derived mediator of METH-neurotoxicity and identifying ammonia as a novel mediator of the neurotoxicity of METH. This suggests that ammonia alone is not capable of producing neuronal damage but can synergize with other toxic compounds to contribute to neurotoxicity. This highlights the potentially important role of acute liver damage and previously assumed subclinical increases in ammonia in mediating other neuropsychiatric disorders (Swain et al., 1992, Clemmesen et al., 1999). In the context of METH-neurotoxicity, these findings also suggest that disorders which compromise liver function, including cirrhosis and hepatitis, which are prevalent in poly-substance and intravenous drug abusers, may exacerbate the neurotoxic consequences of METH (Bao et al., 2010, Gao and Bataller, 2011, Parikh et al., 2012).
We have also examined how increases in brain ammonia contribute to the neurotoxicity of METH (Chapter 4). We examined if the activation of calcium permeable AMPA receptors contributed to the dopamine and serotonin terminal damage produced by the direct administration of METH and ammonia into the striatum and found that when these receptors were blocked, the damage produced by the combination of ammonia and METH was prevented. We also found that ammonia, at the brain concentrations produced by systemic METH treatment, was adequate to increase local glutamate levels, and that during systemic METH treatment, ammonia was required for METH-induced increases in extracellular glutamate and the subsequent excitotoxic terminal damage produced by the drug. These findings suggest that ammonia may synergize with METH to increase extracellular glutamate and cause excitotoxicity to dopamine and serotonin terminals. These findings are significant because they determine a mechanism by which ammonia plays a significant role in METH neurotoxicity. Beyond this, identification of ammonia as a significant mediator of excitotoxic terminal damage suggests that it may play a role in other disorders where excitotoxicity has been identified as a mechanism of neuronal damage including stroke, seizures, Alzheimer’s disease and Huntington’s disease (Choi, 1988). Beyond suggesting a role for ammonia in pathologic conditions, these findings also suggest a physiological role for ammonia in excitatory neurotransmission. In addition to being a metabolic intermediate, these findings support that ammonia may significantly modulate glutamate levels and neurotransmission, even at moderate concentrations in the brain. Although we have yet to determine how ammonia is affecting glutamate concentrations in the brain, we hypothesize that ammonia may be affecting the local reuptake or release of glutamate
within astrocytes (Barbour et al., 1988, Szatkowski et al., 1990, Chan and Butterworth, 1999, Grewer et al., 2008, Gorg et al., 2010).

These current findings also have important implications for the acute treatment of METH exposure. Recommendations for the acute management of METH exposure focus on reducing patient agitation, tachycardia, tachypnea, and hypertension (Richards et al., 1997). The findings in this dissertation support the proposition that hyperthermia, liver damage, and increases in ammonia should also be addressed during acute drug exposure to reduce the neurotoxicity of the drug. Reducing the dopamine and serotonin terminal damage produced by the drug is important because this damage has been associated with cognitive deficits as well as likelihood of relapse in patients (Volkow et al., 2001, Wang et al., 2012). Our current findings determined that METH-induced hyperthermia contributes significantly to the liver damage and increases in ammonia seen after drug exposure. Accordingly, reducing acute METH-induced hyperthermia may be important for reducing the hepatotoxicity of the drug. We also found that treatment with lactulose prevented drug induced increases in ammonia and striatal glutamate, as well as the long term dopamine and serotonin terminal damage produced by METH. Lactulose is currently used clinically for the treatment of hepatic encephalopathy in both in- and outpatient settings (Al Sibae and McGuire, 2009). These findings suggest that treating patients with lactulose during acute METH exposure may reduce the neurotoxicity of the drug. Lactulose also has the potential for use in METH-dependent patients to reduce the neuronal damage caused by repeated drug use. Treatment with lactulose may reduce the dopamine and serotonin terminal damage caused by the drug and potentially reduce the neuropsychiatric consequences of drug exposure including impaired decision making,
reduced impulse control, and addiction (Rogers et al., 1999, Simon et al., 2002, Paulus et al., 2005, Clark et al., 2006, Wang et al., 2012). While our findings support the clinical use of lactulose to reduce the neurotoxicity of METH, one limitation with this treatment is that lactulose, in our studies, as well as clinically, requires 24-48 hours of treatment to reduce ammonia levels and neuronal damage (Al Sibae and McGuire, 2009). In addition to lactulose, there are other fast-acting formulations which are intravenously administered to rapidly reduce ammonia, such as ornithine phenylacetate (Jalan et al., 2007). This may represent an even more effective inpatient intervention to reduce METH-induced increases in ammonia and neurotoxicity.

Finally, these findings also broadly highlight the importance of examining the connection between peripheral organ and neuronal damage. In contrast to the traditional line of thinking that the neurotoxicity of METH is a result of the drug’s direct action on the brain, these findings illustrate that liver damage and increases in peripheral ammonia contribute significantly to the neurotoxicity of METH. In addition to identifying a peripherally-derived mediator of toxicity, we have also established a peripherally-acting intervention (i.e. lactulose) for this neuronal damage. These findings support the importance of considering the periphery in the further understanding and development of novel interventions for neuronal damage. Beyond considering the role of peripherally derived ammonia in glutamate-mediated neuronal damage, changes in the peripheral organ function may also contribute significantly to neuronal inflammation, metabolism, blood flow and signaling via both hormones and neurotransmitters. Accordingly, altered peripheral physiology may represent a significant contributing factor to many neuronal disorders, as well as a target for the treatment of these disorders.
Taken together, these findings support that peripherally-derived ammonia contributes to the neurotoxicity of METH through increases in extracellular glutamate and excitotoxicity. Overall, these findings identify a role for ammonia and peripheral organ damage in the neurotoxicity of METH and provide further evidence that peripheral organ damage can play a significant role in neuropsychiatric disorders.
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