Voluntary Oral Methamphetamine Reveals Susceptibilities to Spatial Memory Deficits, Decreased Dopamine Marker Expression and Increased Neuroinflammation in the Hippocampus of Male and Female Mice

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VOLUNTARY ORAL METHAMPHETAMINE REVEALS SUSCEPTIBILITIES TO
SPATIAL MEMORY DEFICITS, DECREASED DOPAMINE MARKER EXPRESSION
AND INCREASED NEUROINFLAMMATION IN THE HIPPOCAMPUS OF MALE
AND FEMALE MICE

by

JORGE ANDRES AVILA

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

The City University of New York

2019
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This manuscript has been read and accepted for the Graduate Faculty in Psychology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

Voluntary Oral Methamphetamine reveals susceptibilities to spatial memory deficits, decreased dopamine marker expression and increased neuroinflammation in the hippocampus of male and female mice

By

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Advisor: Peter A. Serrano

Methamphetamine is an addictive illicit psychostimulant that produces lasting neurochemical and behavioral changes. The mechanisms underlying these deficits have been characterized in animal models using extremely high doses. Currently, better translational models are needed to understand the onset and progression of these deficits that more accurately reflect the gradual and voluntary dosing parameters as chosen by an abuser. To that end, a new model of methamphetamine administration, labeled Voluntary Oral Methamphetamine Administration (VOMA), offers a means to examine the progression of neurotoxicity, behavioral deficits, and the addiction process through a voluntary consumption framework.

Female populations show consistent vulnerabilities to methamphetamine, including greater severity of abuse, and greater shifts in psychological health. Females also show increased neurochemical susceptibilities during abstinence from methamphetamine, exhibiting increased grey-matter loss compared to controls. The mechanisms underlying these female-specific susceptibilities to methamphetamine are unknown.
The overall GOAL of these studies was to establish a voluntary methamphetamine administration model in mice that recapitulates the cognitive and neurochemical deficits shown with previous models to further our understanding of the long-term susceptibilities of at-risk populations, including adolescent and female subjects.

Rodents can exhibit drug-preference and drug-seeking behaviors, and methamphetamine challenges can reproduce the behavioral and neurochemical deficits that human addicts show. However, it is unclear if mice would voluntarily consume methamphetamine, and what effects this administration design would produce in this mice. Thus, the studies presented in this dissertation characterized the utility of the VOMA model to produce 1. naturalistic methamphetamine consumption 2. behavioral deficits and 3. neurochemical changes. This was done in an effort to understand the 4. mechanisms underlying adolescent- and female-specific vulnerabilities to the drug.

To achieve our GOAL, we carried out three (3) specific aims:

**Specific Aim 1: Determine the utility of Voluntary Oral Methamphetamine Administration (VOMA) to produce behavioral and neurochemical deficits as seen in previous models [Chapter 2]**

In modeling MA abuse with rodents, researchers have developed paradigms that inject neurotoxic or binge doses in mice that are 10 times higher than the lower limits we have characterized in our models \(^1\) (reviewed in\(^2\)). Previous work in this line of research has found that two (2) 30mg/kg MA doses can acutely increase spatial working-memory performance but also increase spatial working-memory errors in the long-term. These effects were correlated to decreased DA and synaptic plasticity maker expression in the hippocampus \(^1\). These prior studies
have contributed significantly to our understanding of the mechanisms underlying MA toxicity 2, but offered very little in understanding the voluntary nature of methamphetamine administration.

In order to characterize the utility of VOMA in producing MA consumption that produced cognitive and neurochemical deficits, we combined a spatial cognition design with a drug administration design as follows: mice were randomly assigned to either a water/control group or MA for 28 consecutive days. To carry out voluntary oral MA administration (VOMA) in mice, MA was mixed into a palatable sweetened oatmeal flake that mice were drawn to and ingested orally. Mice were allowed to consume MA throughout a 3-hour administration period, within 15 min intervals. Following 28-days of VOMA, mice were sacrificed, and tissues were collected.

We found that VOMA:

1. Decreased working-memory and reference-memory performance of male mice on the radial arm maze. These behavioral deficits were observed after the 28-day drug administration period over a 2-week abstinence period.

2. Increased neurotoxicity in the hippocampus, as observed through lower dopamine marker expression, increased neuroinflammation, and lower synaptic-plasticity marker expression.

Overall, these results indicate that VOMA can produce the behavioral and the neurochemical deficits observed in previous animal models as well as in human methamphetamine addicts. Additionally, that the molecular deficits were observed in the hippocampus highlights the utility of the model to easily characterize correlative cognitive behaviors and the underlying molecular shifts.
Specific Aim 2: Characterize the role of abstinence in perpetuating methamphetamine-induced deficits using VOMA [Chapter 3]

MA abuse can produce long-term cognitive impairments in abstinent individuals but other studies have shown that long periods of abstinence can improve the recovery of decision making-skills and emotional symptoms. This may help individuals to avert relapse and yet, other reports indicate that relapse can occur after abstinence, as a result of exposure to specific environmental cues. The neurochemical pathways affected by abstinence from MA are also unclear. An understanding of the progression of behavioral and neurochemical changes produced by MA abuse and by abstinence would provide pharmacological targets to aid in recovery from addiction.

In order to characterize the role of abstinence in producing MA-induced behavioral and molecular deficits, we used an acute VOMA design to do the following: put adolescent mice through cognitive assessments directly after 14 days of MA exposure and after a prolonged abstinence period. Tissue was collected directly after VOMA and after a 4-week abstinence period.

We found that VOMA:

1. Produced a transient sensitization to MA as seen through behavior analyses in the drug-context
2. Produced an acute spatial working-memory deficit on the RAM directly after VOMA

We found that abstinence:

1. Did not produce long-term working-memory, reference learning, nor retention deficits on the RAM
2. Increased expression of neuroinflammatory markers in the hippocampus
3. Modulated expression of monoamine proteins in the hippocampus
Overall, these results indicate that abstinence from VOMA acutely affects behavioral performance that is ameliorated by abstinence. Further, abstinence produces neurochemical changes not observed directly following VOMA. This demonstrates that even acute MA exposure is sufficient to produce molecular changes in the brain despite not manifesting in behavior. This highlights the susceptibility of the adolescent brain to acute MA exposure and provides new avenues for pharmacological studies aimed at preventing MA-induced deficits.

Specific Aim 3: Characterize the sex-differences in behavior and neurochemistry following escalating dose of methamphetamine using VOMA [Chapter 4]

Previous work has shown that females exhibit susceptibilities to psychostimulant-addiction. It has been reported that methamphetamine use in female populations can be attributed to factors including weight loss, desire for increased energy and work output, and decreased exhaustion. Data on drug-rehabilitation admissions show that young female participants are admitted at higher rates compared to males. One hypothesis for sex differences in psychostimulant addiction highlights estrogen’s facilitation of neuronal and behavioral development of drug addiction processes in the brain. However, this idea remains untested.

Additionally, previous work has shown that escalating doses of methamphetamine have intrinsic neuroprotective qualities that protect the brain and behavior from a chronic administration of the drug. The nature and source of this neuroprotection is unclear, but previous work has shown that this phenomenon occurs in binge injection models, associated with decreases in methamphetamine binding to DAT and decreases in the resulting DA availability in the synapse. However, the underlying mechanisms that produce this change in DA sensitivity remain unknown.
To test how female subjects respond differentially to VOMA, we modified our administration design to include a 10-day escalation period, wherein mice would receive gradually escalating doses of and access to methamphetamine prior to receiving the maximum access and dose. We hypothesized that this administration design would produce the maximal voluntary consumption over a shorter (18-day) period and produce the maximal behavioral and molecular deficits that could be observed.

We found that Escalation-VOMA:

1. Decreased working-memory of female mice on the radial arm maze shortly after the conclusion of VOMA. This deficit was not observed in male mice that were placed into VOMA.

2. Discrete shifts in neuroprotective signaling (D1, ERα and Akt/GSK3β pathway) in the hippocampus and nucleus accumbens of female mice after a 2-week abstinence period. Male mice that underwent VOMA did not exhibit any molecular changes.

Overall, this study showed that through VOMA, and with the aid of an escalating-dose design, we are able to observe sex-differences in the behavioral and neurochemical changes that methamphetamine can produce. Interestingly, we also observed a previously reported neuroprotective effect of escalating doses in male subjects, but not in females. Our molecular investigations will provide future direction in basic research and clinical endeavors to target methamphetamine addiction.

**Conclusion:** The novel VOMA design has provided a more naturalistic approach to investigating the behavioral and molecular deficits produce by methamphetamine and abstinence during adolescence. With this model, we have successfully characterized longer-term
susceptibilities in mice than previous studies have shown. Furthermore, it has provided new insights into the sex-differences surrounding meth-abuse, including the role of neuroprotective pathway signaling as markers for susceptibilities/resilience to methamphetamine abuse.
ACKNOWLEDGEMENTS

First of all, I would like to thank those directly involved in my PhD training. Dr. Peter Serrano has been instrumental in my training for the last 7 years. He has been a gracious advisor, and I cannot imagine a more successful training phase without him. Although I may not have learned all that he has to teach, I am grateful for his patience and faith in me. My training would not have been complete without the guidance of other advisors. Dr. Maria Figueiredo-Pereira, Dr. Victoria Luine, Dr. Patricia Rockwell, and Dr. Amber Alliger, each experts in their fields, were pivotal in helping me explore various avenues in my research. Their patience, vision, and guidance throughout the years is appreciated. The work and dedication of my committee members, which include Dr. Thomas Preuss, and Dr. Wayne Harding, are appreciated. I would also like to thank Dr. Jesus Angulo for his help in providing pivotal reagents for my research.

Through countless hours carrying out research, I count myself lucky to have shared space with my graduate peers. The guidance, companionship, and help from Veronica Sebastian, Roseanna Zanca, Nicoletta Memos, Denis Shor, Allen Pan, Santiago Uribe-Cano, Chuhyon Corwin, Teneka Jean-Louis, Magdalena Kiprowska has benefited me tremendously, and I thank you for it. Finally, this work would not have been possible without tireless and dedicated undergraduate assistants who trusted my direction and helped us develop some great research. The assistance of Tytus Andrejewski, Fabienne Tavernier, Brigett Carvajal, Diego Alvarado-Matteo, and many others is greatly appreciated. I would like to thank the support systems that helped me get my research done. Thank you to all the staff in the animal facility, specifically Dr. Glennon, Barbara Wolin, and Sonia Acevedo. Thank you to the RISE program, and especially Dr. Victoria Luine, Dr. Regina Miranda and Janerie Rodriguez for your support of my training.
Finally, I would like to acknowledge and thank everyone that has had a significant impact on my life and training, including educators, family, and friends. Thank you for your conversations; for the mentorship; for your help; for your words of encouragement.
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compared to controls (A). However, DP1 levels increase in female mice but not in male mice (B). Hippocampal DP2 levels do not show significant changes in male or female mice, with or without VOMA (C). (**p<.01). Figure 23. Escalation VOMA produces sex differences in hippocampal PKMζ. Escalation VOMA produces no differences in PKCι/λ across male or female mice (A). Escalation VOMA produces sex differences in hippocampal PKMζ levels, with females showing a significant reduction compared to males following VOMA (B). (*p<.05). Figure 24. Escalation VOMA produces increased NAc κOR pathway activity. In response to Escalation VOMA, Hippocampal κOR levels are significantly decreased in male mice but not in female mice (A). Accumbal D1 levels are significantly decreases in female mice, with and without VOMA compared to males (B). Accumbal κOR levels are significantly increased in female mice after VOMA (C) indicating that differences in D1 signaling can be explained partly by κOR-dependent signaling that affects the dopamine system in this region. (*p<.05; **p<.01). Figure 25. Escalation VOMA produces distinct behavioral and molecular responses between male and female mice. In response to Escalation VOMA, female mice exhibit compromised molecular signatures, including deficits in synaptic marker expression, increased neuroinflammatory activity, and decreased neuroprotective pathway activity that may underlie the cognitive deficits evidenced on the radial arm maze. Conversely, male mice that underwent Escalation VOMA exhibit increased neuroprotective activity, and no significant deficits in synaptic marker expression or neuroinflammatory activity, that result in no change in cognitive performance. This model has revealed a specific role for escalating methamphetamine doses to produce neuroprotection but only in male subjects. Female subjects are revealed to be susceptible to MA’s negative effects on cognition and the neurochemistry underlying behavioral performance. Figure 26. Total MA consumed in 28d VOMA (Chapter 2) vs Escalation VOMA (Chapter 3) in Male mice. There was no significant difference between the two groups.
Figure 27. Molecular Mechanisms of D1 and ERα-dependent neuroprotective signaling. In the context of methamphetamine, specific synaptic pathways initiate signaling to promote neuroprotection or neurotoxicity. Chapter 4 explores the contribution of these pathways in the context of neurochemical and behavioral effects in male and female adolescent mice. .................145

Figure 28. 5-HT1b Knockout mice show different rates of consumption across sexes. Mice aged 8-16 weeks at the start of VOMA were put through Escalation VOMA. Tracking of MA consumption revealed the female KO mice consumed significantly less over the 28 days of VOMA [Two-way repeated measures ANOVA: across gender: F_{1,9}=3.685, p=0.08; across days: F_{27,243}=9.67, p<0.001; interaction of gender and days: F_{27,243}=2.23, p<0.001].................................................................153

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LIST OF ABBREVIATIONS

5-HT: serotonin
AMPAr: α-amino-5-hydroxy-3-methyl-4-isoxazol propionic acid receptor
AKT: Protein Kinase B
Bcl-2: B-cell lymphoma 2 protein
Ca2+: calcium ion
cAMP: cyclic adeno-monophosphate
CNS: central nervous system
D1: dopamine 1 receptor
D2: dopamine 2 receptor
DA: dopamine
DAT: dopamine transporter
DP1: Prostaglandin D-prostanoid receptor 1
DP2/CRTH2: Prostaglandin D-prostanoid receptor 2
ERα: Estrogen receptor alpha
GluA: glutamate receptor
GFAP: glial fibrillary acid protein
GSK3β: Glycogen-synthase kinase 3 beta
Iba1: ionized calcium-binding adapter molecule 1
IGF-1R: Insulin-like growth factor 1 receptor
IP: Intraperitoneal
KO: knockout
κOR: kappa-opioid receptor
L-dopa: L-3,4-dihydroxyphenylalanine/levadopa
MDMA: 3,4-methylenedioxyamphetamine
MA: methamphetamine
Mg/kg: milligrams per kilogram
NAC: nucleus accumbens
NMDA: N-methyl-D-aspartate
PD: Parkinson's disease
PFC: prefrontal cortex
PG: prostaglandin
PGD2: prostaglandin D2
PGJ2: prostaglandin J2
PI3K: phosphoinositide kinase-3
PKCι/λ: atypical protein kinase c iota/lambda
PKMζ: atypical protein kinase m zeta
RAM: radial arm maze

ROS: reactive oxygen species

SAMSHA: Substance Abuse and Mental Health Services Administration

SERT: serotonin transporter

TPH: tryptophan hydroxylase

TH: tyrosine hydroxylase

VTA: ventral tegmental area

vGLUT: vesicular glutamate transporters

VMAT: vesicular monoamine transporters

VOMA: Voluntary Oral Methamphetamine Administration
CHAPTER 1: INTRODUCTION
1.1 Methamphetamine addiction: The negative consequences on behavior and cognition

Methamphetamine (MA) abuse has an approximate $23.4 billion annual cost to society. Of this total, 71% can be attributed to intangible costs, including psychological stress and deficits. Studies on MA’s effects on cognitive and psychological functions have increased in the last 10 years, with conflicting clinical studies showing both cognitive enhancements and cognitive deficits after acute exposure to MA. Conversely, clinical studies on chronic MA exposure indicate long-lasting and progressive cognitive decline in these MA abusers. Furthermore, the addictive and neurotoxic nature of the drug produces long-term cognitive impairments in abstinent individuals. Thus, the time course of MA abuse and abstinence may play a major role in the progression of cognitive dysfunction. Chronic MA users develop cognitive deficits in sustained attention, episodic memory, information processing, and impulse control. Finally, hallucinations and cognitive impairment are clinical symptoms of MA psychosis in patients who show a sensitization to MA. Thus, research should seek to characterize the milieu of effects that MA can have on cognition, distinguishing between cognitive effects of acute vs. chronic MA exposure, as well as the specific cognitive capacities that are affected. Methamphetamine (MA) abuse is a costly and detrimental health risk in the U.S. and abroad. In the U.S., the number of individuals who reported abusing MA in 2012 was approximately 1.2 million people (4.7 percent of the population). Amphetamine-type stimulants (ATS) now rank second only to cannabis as the most common illicit drugs used worldwide, representing approximately 34 million users. North America continues to be a significant market for ATS, particularly amphetamine and methamphetamine. Based on these statistics, it becomes increasingly important that we understand the risk factors associated with MA addiction, and develop new animal models to better address treatment outcomes for addicts. It is well known that MA abusers show a long-
lasting reduction in dopamine terminals and transporters, both of which increase the risk for Parkinson’s Disease 33-34. Chronic MA abuse also results in neurodegeneration of frontal cortex, midbrain regions and hippocampus, which are all associated with memory deficits 35. Additionally, an increase in markers of neuroinflammation, including activated microglia was reported in MA abusers 36. Several animal studies have recapitulated many of these neurochemical and behavioral characteristics associated with MA abuse [reviewed in 2; briefly summarized in ]. Many animal studies show that neurotoxic or binge-dosing regiments of MA produce rapid increases in microglia 37 and glial fibrillary acidic protein (GFAP) levels within the striatum and hippocampus 38-40 shortly after administration.
Table 1: Molecular changes characterized in animal models of MA abuse. An overview of neurotoxic, dopaminergic and glutamatergic changes in the striatum and hippocampus of rodents highlighting the MA administration methods, the molecular markers used, and the timing of tissue collection.

<table>
<thead>
<tr>
<th>Neurotoxicity Effect</th>
<th>Markers/Method</th>
<th>MA Administration Protocol</th>
<th>Tissue Collection</th>
<th>Brain region</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased pyknotic nuclei</td>
<td>BRDU/cell counts</td>
<td>I.P. 30mg/kg</td>
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<td>Striatum</td>
<td>41</td>
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<tr>
<td>Increased apoptosis</td>
<td>TUNNEL staining/cell counts</td>
<td>I.P. 10mg/kg, 20mg/kg, 30mg/kg, 40 mg/kg</td>
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<td>Striatum</td>
<td>42</td>
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<td>Decreased cell density</td>
<td>Cresyl Violet stain/cell counts</td>
<td>I.P. 30mg/kg</td>
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<tr>
<td>Increased astrocytosis</td>
<td>GFAP IHC/Cell counts</td>
<td>10mg/kg x4</td>
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<td>Striatum</td>
<td>43</td>
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<tr>
<td>No increases in neurodegeneration</td>
<td>Fluorojade C IHC</td>
<td>6d Escalating doses (10-25mg/kg) + 30mg/kg on d7</td>
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<td>Striatum</td>
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<table>
<thead>
<tr>
<th>Dopaminergic dysregulation</th>
<th>Markers/Method</th>
<th>MA Administration Protocol</th>
<th>Tissue Collection</th>
<th>Brain region</th>
<th>Study</th>
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<tbody>
<tr>
<td>Decreased DARPP-32 neurons</td>
<td>DARPP-32 IHC/cell counts</td>
<td>I.P. 30mg/kg</td>
<td>24 hrs</td>
<td>Striatum</td>
<td>42</td>
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<tr>
<td>Decreased D1 receptor levels</td>
<td>D1/Western Blot</td>
<td>I.P. 30mg/kg x2</td>
<td>6 wks</td>
<td>Hippocampus</td>
<td>1</td>
</tr>
<tr>
<td>Increased DAT levels</td>
<td>DAT/Western Blot</td>
<td>I.P. 30mg/kg x2</td>
<td>6 wks</td>
<td>Hippocampus</td>
<td>1</td>
</tr>
<tr>
<td>Decreased DAT levels</td>
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<td>10mg/kg x4</td>
<td>1 wk</td>
<td>Striatum</td>
<td>45</td>
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<tr>
<td>Decreased TH levels</td>
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<td>1 wk</td>
<td>Striatum</td>
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<table>
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<th>Glutamatergic dysregulation</th>
<th>Markers/Method</th>
<th>MA Administration Protocol</th>
<th>Tissue Collection</th>
<th>Brain region</th>
<th>Study</th>
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<tr>
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<td>GluN2B/Western Blot</td>
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<td>6 wks</td>
<td>Hippocampus</td>
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<tr>
<td>Decreased AMPA and NMDA receptor levels</td>
<td>GluA2 + GluN2B/Western Blot</td>
<td>I.P. 30mg/kg x2</td>
<td>6 wks</td>
<td>Striatum</td>
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<tr>
<td>Increased AMPA receptor levels</td>
<td>GluA2/Western Blot</td>
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<td>24 hrs</td>
<td>Striatum</td>
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</tr>
<tr>
<td>Increased NMDA receptor levels</td>
<td>GluN2B/Western Blot</td>
<td>6d Escalating doses (10-25mg/kg) + 30mg/kg on d7</td>
<td>24 hrs</td>
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</tr>
<tr>
<td>Decreased AMPA receptor levels</td>
<td>GluA2/Western Blot</td>
<td>30mg/kg</td>
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<tr>
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<td>24 hrs</td>
<td>Hippocampus</td>
<td>38</td>
</tr>
</tbody>
</table>
1.2 The Neurocircuitry of Methamphetamine addiction: The vulnerability of the Hippocampus

MA is known to directly affect the monoamine neurotransmitter systems. Clinical MA abusers show deceased density of dopamine transporters (DAT) in the striatum and frontal cortex, two regions associated with reward processing and drug addiction. However, chronic MA abuse also results in neurodegeneration of frontal cortex, midbrain regions and hippocampus and is associated with memory deficits. Additionally, the ventral hippocampus-ventral striatum circuit is known to participate in stimulant-induced expression of drug addiction behaviors, suggesting that stimulant abuse could alter hippocampal-dependent behaviors through changes in hippocampal synaptic plasticity. In fact, a recent study has found that neurotoxic doses of MA can alter DA and glutamate marker levels in the hippocampus, affecting hippocampal-dependent behaviors. Additional evidence of hippocampal vulnerabilities to MA come from proteomic studies that found the drug to increase major apoptotic, inflammatory, and neurodegenerative pathway activity in this region. Activation of inflammatory and degenerative pathways in the brain are hypothesized to underlie the progression and development of neurodegenerative diseases like Alzheimer’s and Parkinson’s disease. Thus, serving a variety of roles within drug abuse and exhibiting many important molecular vulnerabilities, the hippocampus is emerging as a pivotal region in the study of MA abuse and addiction.

1.3 The acute and lasting effects of Methamphetamine abuse on the brain

MA can produce a variety of neurobiological effects once it has been consumed. The primary mechanism of action of MA in the brain is to act on dopamine (DA) and serotonin (5-HT) synapses. MA spills neurotransmitter into the cytosol via interactions with VMAT2 (synaptic
vesicle channel pump), followed by reversing DAT function and decreasing SERT activity (DA and 5-HT reuptake transporters (respectively)), leading to increased neurotransmitter release and post-synaptic receptor activation 45. These processes underlie the euphoria produced by MA. However, these processes become dangerous very quickly. Once DA has been displaced to the cytoplasm, it rapidly auto-oxidizes to form reactive oxygen species (ROS) that include superoxide radicals, DA quinones 45. Increased MA-dependent ROS formation has been linked to decreased tryptophan hydroxylase (TPH) (5-HT rate-limiting enzyme) activity in the hippocampus through oxidation of TPH as early as 1 hour after MA treatment 53 and linked to increased tyrosine hydroxylase (TH) (DA rate-limiting enzyme) activity and DA synthesis that contribute to MA-induced neurotoxicity and damage to DA terminals. 54. Further evidence of ROS-induced damage from MA comes from 55 who showed that administration of neurotoxic doses of MA to rats caused DA oxidation to DA-quinones that bind to cysteinyl residues on proteins, increasing protein cysteinyl-DA levels in the striatum. Protein oxidation in the brain can result in various signaling, homeostatic and even behavioral deficits, as revealed by studies of protein oxidation using amphetamine derivative, ecstasy (MDMA), and ethanol 56. These mechanisms can cause DA terminal degeneration leading to reductions in dopamine availability, which can increase the risk of Parkinson’s Disease and its associated symptoms 33-34. Studies have also elucidated the effects of MA on the glutamate system, reviewed in 57-58. MA synergistically increases DA terminal depletions through glutamate activity 59, but also can increase glutamate release via reactive-nitrogen-species activation 60, and can cause glutamate excitotoxicity and calpain-dependent proteolysis via AMPA receptor activation 61 in the striatum. More recent studies have shown that neurotoxic doses of MA can cause decreases in AMPA receptor and synaptic scaffolding and trafficking markers, PSD-95 and PKMζ, in the
hippocampus which could underlie the spatial learning and memory deficits revealed in the same studies. These MA-induced effects on the DA and glutamate systems can have far-reaching effects on cognitive functioning and require detailed investigations in behavioral models of MA abuse (Fig 1).

The effects of MA on the brain and behavior depend on how similar its chemical structure is to DA, which allows the drug to enter DA terminals through DAT reuptake or through lipid membranes due to the high lipophilic nature of the compound. Once inside the axon, MA can sequester VMAT2, also likely due to the similarity to DA, and through pH changes in the synaptic vesicle microenvironment spills DA from synaptic vesicles into the cytoplasm.

Through interactions with DAT, MA can induce reverse transport of cytosolic DA into the synaptic cleft. This increased availability of DA at the synapse is what allows for increased DA release, and once the MA-dependent increases in DA release occur in the nucleus accumbens and prefrontal cortices (brain regions that compute reward), the individual feels euphoria. This mechanism is understood to be the underlying end of MA abuse by addicts.

Once DA has been displaced to the cytoplasm by MA and VMAT2-dependent mechanisms, it rapidly auto-oxidizes to form reactive oxygen species (ROS) that include superoxide radicals, hydroxyl radicals, hydrogen peroxide and DA quinones. Increased MA-dependent ROS formation has been linked to decreased tryptophan hydroxylase (TPH) (5-HT rate-limiting enzyme) activity in the hippocampus through oxidation of thiol groups of TPH as early as 1 hour after MA treatment. Further evidence of ROS-induced damage from MA comes from who showed that administration of neurotoxic doses of MA to rats caused DA oxidation to DA quinones that bind to cysteinyl residues on proteins, which lead to an increase in protein
cysteiny1-DA levels in the striatum. These increases in oxidation of DA occurred only under conditions resulting in neurotoxicity, suggesting that the oxidation of DA may play a major role MA-induced damage to DA terminals. It is well known that MA abusers show a long-lasting reduction in dopamine terminals and transporters both of which increase the risk of Parkinson’s Disease 33-34.

There is evidence that links the 5-HT transporter (SERT) to MA-induced damage of 5-HT terminals. A number of SERT inhibitors, including fluoxetine, have been shown to block MA-induced reductions in TPH activity and prevent 5-HT depletion in the striatum, hippocampus and cortex 45 suggesting a role for SERT in MA-induced 5-HT terminal degeneration. Clinical Positron emission tomography (PET) studies have shown decreased levels of serotonin transporter (SERT) in the orbitofrontal and occipital cortices in individuals who were currently abstinent from MA 65-66. These individuals also showed increased aggression, highlighting how MA abuse and addiction can alter emotional and psychiatric health.

MA neurotoxicity is also driven by excitotoxic damage following glutamate release and activation of glutamate receptors 67. MA-induced glutamate release in the striatum is thought to be initiated by DA D1 receptor-dependent stimulation of striatonigral GABAergic pathway that causes increases in GABA release in the substantia nigra pars reticulata, inhibition of nigrothalamic GABAergic outflow via activation of GABAA receptors, and followed by disinhibition of thalamocortical afferents with resulting increases in striatal glutamate release 68.
Microglia are the resident immune cells in the brain that protect against injury and long-term damage 69. In the healthy brain, microglial cells exist in a quiescent state and they monitor the neuronal environment for any signs of injury and assist in housekeeping duties as it becomes necessary 69-70. However, in response to trauma, injury, or disease, microglial cells increase in size, migrate to the site of the injury, and undergo a series of signaling and phagocytic events that enable them to respond adequately to begin the process of repair 69-70. While microglial activation is essential for immune responses and restoration of normal neuronal function, the persistent or unceasing activation of microglial cells can result in neurotoxic consequences. Indeed, multiple lines of evidence have suggested that activated microglia release a variety of cytokines, reactive oxygen and nitrogen species and PGs that are known to cause neuronal damage during responses to injury 70-71 and, therefore, might be involved in neurodegeneration through pro-inflammatory processes. Importantly, prostaglandin-J2 (PGJ2), the most neurotoxic of all PGs, is a direct product of pro-inflammatory activity via COX-2 dependent mechanisms, and can feed-back onto COX-2 pathways to upregulate pro-inflammatory activity 72. A goal of this proposal is to determine the levels of PGJ2 in VOMA mice, in order to delineate the state of inflammatory processes in the brain due to MA abuse. In fact, an increase in markers of neuroinflammation, including activated microglia has been reported in MA abusers 36. Another report shows that the pro-inflammatory marker COX-2 increases in the striatum 73 occur as a result of MA use and is an obligatory factor in the neurotoxicity induced by the drug.
Figure 1. Model of Methamphetamine-induced effects on the brain and cognition. Neuroglial activation potentiates a neuroinflammatory response that can perpetually affect synaptic plasticity and in turn cognition.
1.4 The role of escalating exposure on Methamphetamine-induced effects

Because binge or bolus neurotoxic MA administration are not thought to model the typical pattern of human MA use, several groups of investigators focused their attention on using escalating MA dosing to investigate its effects on the brain. For example, one study administered MA on an escalating dose regimen, where injections were given 3x/day for 13 days starting at 0.1 mg/kg bw (s.c.) and increased by 0.1 mg/kg with every injection, so that on the 13th day, the animals received 3.9 mg/kg. This was done with the idea that some addicts would gradually escalate their doses and frequency of intake. Assessing the neurochemical deficits associated with MA in this dosing paradigm potential protective effects that occur as a result of repetitive dosing spread out across days. These reports reveal that differing doses across consecutive days may provide some neuroprotection and/or delay the long-term damage.

1.5 Susceptibility in adolescent and female populations to Methamphetamine abuse

A significant body of literature has shown that select populations, females and adolescents, exhibit susceptibilities to psychostimulant-addiction. It has been reported that MA use in female populations can be attributed to factors including weight loss, desire for increased energy and work output, and decreased exhaustion. Data on adolescent trends of abuse are sparse, but available studies suggest complex psycho-social dynamics at play in the progression of MA abuse in these populations. Furthermore, data on MA treatment admissions show that younger age groups are admitted at a higher rates and that in these younger groups, females are admitted at higher rates compared to males. Mechanistic studies on the effects of MA in these populations indicate neurobiological resilience to neurotoxic regimens of MA, although the underlying mechanisms of this resilience in adolescents remain unclear. One hypothesis for sex differences
in MA abuse highlights estrogen’s facilitation of neuronal and behavioral development of drug addiction processes. 16-18. Models to explain the susceptibility of adolescent psychostimulant abuse are focusing on developmental trajectories of cortico-limbic circuits and the impact of environmental stress on psychosocial-dependent behavioral dynamics 81-82. However, not much is known about the long-term trajectories of hippocampal behaviors and molecular profiles stemming from MA abuse in these populations.
CHAPTER 2: CHRONIC VOLUNTARY ORAL METHAMPHETAMINE INDUCES
DEFICITS IN SPATIAL LEARNING AND HIPPOCAMPAL PROTEIN KINASE
MZETA WITH ENHANCED ASTROGLIOSIS AND CYCLOOXYGENASE-2 LEVELS

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2.1 Abstract

Methamphetamine (MA) is an addictive drug with neurotoxic effects on the brain producing cognitive impairment and increasing the risk for neurodegenerative disease. Research has focused largely on examining the neurochemical and behavioral deficits induced by injecting relatively high doses of MA [30 mg/kg of body weight (bw)] identifying the upper limits of MA-induced neurotoxicity. Accordingly, we have developed an appetitive mouse model of voluntary oral MA administration (VOMA) based on the consumption of a palatable sweetened oatmeal mash containing a known amount of MA. This VOMA model is useful for determining the lower limits necessary to produce neurotoxicity in the short-term and long-term as it progresses over time. We show that mice consumed on average 1.743 mg/kg bw/hour during 3 hours, and an average of 5.23 mg/kg bw/day over 28 consecutive days on a VOMA schedule. Since this consumption rate is much lower than the neurotoxic doses typically injected, we assessed the effects of long-term chronic VOMA on both spatial memory performance and on the levels of neurotoxicity in the hippocampus. Following 28 days of VOMA, mice exhibited a significant deficit in short-term spatial working memory and spatial reference learning on the radial 8-arm maze (RAM) compared to controls. This was accompanied by a significant decrease in memory markers protein kinase Mzeta (PKMζ), calcium impermeable AMPA receptor subunit GluA2, and the post-synaptic density 95 (PSD-95) protein in the hippocampus. Compared to controls, the VOMA paradigm also induced decreases in hippocampal levels of dopamine transporter (DAT) and tyrosine hydroxylase (TH), as well as increases in dopamine 1 receptor (D1R), glial fibrillary acidic protein (GFAP) and cyclooxygenase-2 (COX-2), with a decrease in prostaglandins E2 (PGE2) and D2 (PGD2). These results demonstrate that chronic VOMA
reaching 146 mg/kg bw/28d induces significant hippocampal neurotoxicity. Future studies will evaluate the progression of this neurotoxic state.
2.2 Introduction

Methamphetamine (MA) abuse is a costly and detrimental health risk in the U.S. and abroad. In the U.S., the number of individuals who reported abusing MA in 2012 was approximately 1.2 million people (4.7 percent of the population) \(^{30}\). Amphetamine-type stimulants (ATS) now rank second only to cannabis as the most common illicit drugs used worldwide, representing approximately 34 million users \(^{31}\). North America continues to be a significant market for ATS, particularly amphetamine and methamphetamine \(^{32}\). Based on these statistics, it becomes increasingly important that we understand the risk factors associated with MA addiction and develop new animal models to better address treatment outcomes for addicts. It is well known that MA abusers show a long-lasting reduction in dopamine terminals and transporters, both of which increase the risk for Parkinson’s Disease \(^{33-34}\). Chronic MA abuse also results in neurodegeneration of frontal cortex, midbrain regions and hippocampus, which are all associated with memory deficits \(^{35}\). Additionally, an increase in markers of neuroinflammation, including activated microglia was reported in MA abusers \(^{36}\). Several animal studies have recapitulated many of these neurochemical and behavioral characteristics associated with MA abuse [reviewed in \(^2\)]. Many animal studies show that neurotoxic or binge-dosing regimens of MA produce rapid increases in microglia \(^{37}\) and glial fibrillary acidic protein (GFAP) levels within the striatum and hippocampus \(^{38-40}\) shortly after administration.

While there are many MA paradigms for rodents currently used, what is needed is a model that capitalizes on the voluntary MA consumption without the need for conditioning to administer the drug or surgery that can restrict the types of behavioral assessments used. Here we provide data
on a voluntary oral methamphetamine administration (VOMA) model useful for determining the progressive nature of voluntary abuse-associated neurotoxicity in a rodent model. Our goal was to assess the effectiveness of this VOMA model in producing hippocampal-dependent memory and learning deficits, as well as MA-associated neurochemical changes. To evaluate cognitive behavior we used the radial arm maze (RAM), which is a hippocampal-dependent task used to assess both long-term reference and short-term working memories.

Following behavioral assessments, we quantified changes in (1) dopamine-related markers involving tyrosine hydroxylase (TH), dopamine transporter (DAT), and the Dopamine 1 receptor (D1R); (2) glutamate receptor alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) GluA2 subunit; (3) atypical protein kinase C iota/lambda (PKCι/λ), protein kinase Mζta (PKMζ) and the post-synaptic density 95 (PSD-95) protein; and (4) markers of inflammation involving astrogliosis measured by GFAP, cyclooxygenase-2 (COX-2), and prostaglandins (PG) E2, D2 and J2. We focused on these molecular markers since MA selectively damages DA terminals and produces excitotoxicity effects involving AMPA receptors in addition to elevated levels of inflammation. Astrogliosis is associated with MA neurotoxicity and several other toxic insults in the brain. Finally, as a synaptic plasticity and memory marker, we focused on the atypical kinase PKMζ that is important for spatial learning and long-term memory, and which is increased concomitantly with improved memory across several memory paradigms [reviewed in].

Our results show that 28d on VOMA produces significant deficits in hippocampal-dependent short-term working and long-term reference learning on the RAM. These behavioral deficits
were associated with decreases in PKMζ, GluA2 and PSD-95. VOMA also produced decreases in DAT and TH, with a concomitant increase in D1R levels. Both GFAP and COX-2 increased, with decreases in PGE2 and PGD2. Thus, these results highlight the accumulating negative effects of chronic VOMA in addition to its usefulness in characterizing the progression of the MA-induced neurotoxicity.

2.3 Material and Methods

2.3.1 Mice

Male C57BL/6 mice from Taconic Farms (Germantown, NY) were purchased at 8 weeks of age. Mice were randomly assigned to 2 treatment conditions: MA (n = 5) and Control (n = 5). We have used similar sample sizes to evaluate behavioral performance and protein expression as previously reported 1, 90, 92. Mice were housed individually to control for social effects of group housing 93 that could affect the voluntary consumption of methamphetamine in our model. Mice were kept on a 12/12 h light/dark cycle at the Hunter College animal facility for one week prior to beginning any behavioral assessments with food and water ad libitum prior to behavioral shaping. All housing conditions conform to the Hunter College guidelines outlined by the Institutional Animal Care and Use Committee (IACUC).

2.3.2 Methamphetamine Treatment
We used a new voluntary oral methamphetamine administration (VOMA) model over 28 consecutive days (experimental days 5-32). A sweetened oatmeal flake (Maypo, Homestat Farm, Dublin, OH) was moistened with 8-10 μl of either MA (2.5 mg/ml) or water. This produces a palatable sweetened oatmeal flake that contains 1mg MA/kg of body weight (bw) per serving (adjusted to mice ranging in weight between 20-25 g). During MA administration, all mice were transferred to individual mouse cages that were lined with absorbent paper. Each cage was designated to a specific mouse throughout the experiment to ensure context specificity. Cages were wiped clean and lined with fresh paper after each use. All mice remained in their paper-lined cage for 30 min prior to treatment. During a 3 hour (hr) period, from 13:00 h to 16:00 h, mice were presented with individual MA or water moistened oatmeal flakes. A new presentation was delivered in a clean petri dish every 15 min. For each mouse, the number of consumed flakes during the 3 hr period was noted. Directly after VOMA, all mice were fed 4g of mouse chow daily at 18:00 h. This feeding schedule ensured that mice would be willing to consume oatmeal flakes during the VOMA treatment time-window, for 28 consecutive days. This feeding schedule also circumvented the significant circadian effects reported on VOMA administration without disrupting weight gain throughout the duration of the experiment.

2.3.3 Radial 8-arm Maze Shaping

The radial 8-arm maze (RAM) was used to assess both working memory and spatial learning performance. The maze consists of a center platform (15.24 cm diameter) with 8 equivalently sized arms radiating outward. Each arm is 38 cm in length, 6.35 cm wide and has a submerged food cup (2.0 cm diameter) at the end of each arm. Maypo was mixed in water to make a wet
mash used as a food reward (0.02 g portions), as previously described for mice and rats. Prior to working memory assessments, all animals were shaped on the RAM during days 1-2 of the study. Mice were food restricted to 85% of free feeding weight before being placed on the RAM for 10 min to acclimate to the maze and room cues. 1 hr later, all mice were given a second trial with sweetened oatmeal in the food cups. After 1 day of shaping (2 trials per day), mice were eating the food rewards and finding all 8 baits within a 15 min maximum latency.

2.3.4 Working Memory Assessment

The working memory assessment (WMA) occurred during experimental day 37. Mice were tested for 1 day (3 consecutive trials). Each trial started with all food cups baited. To begin each trial, mice were confined for 30 s to the center platform with a plastic cylinder. Between trials mice were confined to the center platform while the arms were re-baited and the maze cleaned. The sequence of arms entered to retrieve the food rewards was recorded. Mice were allowed to collect baits from up to 3 sequential arms in any direction around the radial arm maze. Under rare instances when a mouse adopted a chaining strategy by entering consecutive arms in one direction around the maze, the 4th sequential arm was blocked to disrupt this non-spatial strategy. Errors were recorded as re-entries into arms where the food reward had already been collected. Working memory was assessed by a % correct score for each trial, which was calculated by the number of total arm entries required to collect all 8 food rewards divided by 8 (the total number of rewards collected). All mice remained on the maze until all 8 baits were collected. Maximum latency for each trial was set at 15 min.
2.3.5 Spatial Learning/Cognitive Flexibility Assessment

During experimental days 40-49, all mice were trained on a reference and working memory version of the RAM as previously reported 1. This paradigm had 4 baited and 4 un-baited arms in a pattern that was specific to each animal and remained constant throughout the experiment. Mice were given 6 consecutive trials per day for 10 days (60 trials total). Between trials mice were confined to the center platform while the arms were re-baited and the maze cleaned. The sequence of arm entries was recorded. A reference memory error reflected an entry into an arm that was never baited, while working memory errors reflected re-entries into an arm where the bait had already been collected. Mice were only allowed to enter up to 3 sequential arms to prevent the non-hippocampal dependent chaining strategy. This version of the RAM required mice to learn room cues associated with the baited and un-baited arm sequence. The training room and room cues were identical to that used for the WMA. 24 hrs after the last RAM trial, all mice were given one reminder trial on the maze 1 hr prior to tissue retrieval. Hippocampi from each mouse were removed, snap frozen and stored at −80°C until processed further.

2.3.6 Preparation of Tissue Fractions

Hippocampi were micro dissected and fractionated into cytosolic and synaptic fractions as previously reported 1. Briefly, tissues were thawed and homogenized in a TEE (Tris 50mM; EDTA 1mM; EGTA 1mM) buffer containing a Sigma Fast, protease inhibitor cocktail (Sigma Aldrich) diluted to contain AEBSF (2 mM), Phosphoramidon (1 mM), Bestatin (130 mM), E-64 (14 mM), Leupeptin (1 mM), Aprotinin (0.2 mM), and PepstatinA (10 mM). Tissues were
homogenized in 200 μl of the TEE-homogenization buffer using 20 pumps with a motorized pestle. Homogenates were transferred to Eppendorf tubes and centrifuged at 3000 x g (5 min at 4°C), to remove un-homogenized tissue. The resulting supernatant was centrifuged at 100,000 g for 30 min. After ultracentrifugation, the supernatant was collected and stored as the cytosolic fraction. The remaining pellet was re-suspended in 100 μl of homogenizing TEE buffer containing 0.001% Triton X-100, incubated on ice for 1 hr and then centrifuged at 100,000 g for 1 hr at 4°C. The resulting supernatant was stored at the plasma-membrane fraction. The resulting pellet was re-suspended in 50 μl of TEE buffer and stored as the synaptic fraction. The Pierce bicinchoninic acid assay (BCA; Thermo Scientific, Rockford, IL, USA) was used to determine protein concentration for each sample. Samples were reduced with 4x Laemmli sample buffer equivalent to 25% of the total volume of the sample and then boiled and stored frozen at -80°C.

2.3.7 Immunoblots

Samples (20 μg) were loaded on to a Tris/Glycine 4–20% mini gel to resolve GAPDH (37 kDa), α-Tubulin (55 kDa), PKMζ (55 kDa), PKCι/λ (68 kDa), GluA2 (102 kDa), PSD-95 (95 kDa), COX-2 (72 kDa), GFAP (53 kDa), TH (60 kDa), D1 (49 kDa) and DAT (69 kDa). Every gel contained 4 lanes loaded with the same control sample designated as all brain sample (ABS). ABS was used to standardize protein signals between gels. Gels were transferred to nitrocellulose membranes in the IBlot® Dry Blotting System (Life Technologies; Carlsbad, CA, USA) for 7 min. Nitrocellulose membranes were then incubated in blocking solution containing 5% sucrose in Tris Buffered Saline with Tween-20 (TBST; 0.1% Tween-20 in TBS) for 30 min at room temperature. Samples were incubated overnight with the following primary antibodies:
GluA2 (1:2000; Chemicon, Temecula, CA, USA), PKMζ/ PKCζ (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), GAPDH (1:2000; Abcam Inc., Cambridge, MA, USA), PSD-95 (1:1000; Cell Signaling Technology, Danvers, MA, USA), COX-2 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), GFAP (1:5000; Abcam Inc., Cambridge, MA, USA), TH (1:2000; ab152, Millipore, Billerica, MA, USA), D1 (1:2000; Abcam Inc., Cambridge, MA, USA), DAT (1:1000; EMD Millipore, Temecula, CA, USA) and α-Tubulin (1:2000; Calbiochem, San Diego, CA, USA). Membranes were washed in TBST for 20 min and probed with Horseradish Peroxidase (HRP) conjugated secondary antibodies: Goat Anti-Mouse IgG (H + L)-HRP Conjugate #1706516 (BioRad); Goat Anti-Rabbit IgG (H + L)-HRP Conjugate #1706515 (BioRad); Rabbit Anti-Goat IgG H&L (HRP) (ab6741-Abcam). Membranes were incubated with Enhanced Chemiluminescence (ECL) substrate and exposed on CL-X Posure Film (Thermo Scientific; Rockford, IL, USA). Films were scanned, and then densitometry was performed with NIH ImageJ §6.

2.3.8 Preparation of Samples for Prostaglandin Quantification

For prostaglandin quantification, pre-weighed hippocampal tissues were homogenized in 0.9 ml of phosphate buffered saline using a BeadBug microtube homogenizer. Following homogenization, a 10-mg wet weight equivalent of homogenate was removed and diluted to a final volume of 10 mg per 0.9 ml before being further diluted 1:1 with 1% formic acid.

Deuterated internal standards were added (identified in italics in the table below) to the diluted homogenate and loaded on a 2 ml Biotage SLE+ cartridge. After a 5 min incubation, the cartridges were eluted twice with 6 ml of t-butylmethyl ether. The eluent was spiked with 20 μl of
a trap solution consisting of 10% glycerol in methanol with 0.01mg/ml butylated hydroxytoluene. The samples were dried for 45 minutes in a speed vacuum at 35°C, the walls of the tubes were washed with 1 ml of hexane and re-dried until a small aqueous residue remained. The residue was dissolved in 50 μl of 80:20 water:acetonitrile with 0.1 mg/ml butylated hydroxytoluene and spin filtered with a 0.22μm Millipore Ultrafree® filter. Samples were transferred to vials and 30 μl of sample were analyzed. Prostaglandin standard curves were spiked into PBS and prepared identically to the samples. Area ratios were plotted and unknowns determined using the slopes.

2.3.9 LC-MS/MS for Prostaglandin Quantification

Samples were analyzed using a 5500 Q-TRAP hybrid/triple quadrupole linear ion trap mass spectrometer (Applied Biosystems, Carlsbad, CA) with electrospray ionization (ESI) in negative mode. The mass spectrometer was interfaced to a Shimadzu (Columbia, MD) SIL-20AC XR auto-sampler followed by 2 LC-20AD XR LC pumps. The scheduled MRM transitions were monitored within a 1.5 min time-window (supplemental Table 1). Optimal instrument parameters were determined by direct infusion of each analyte. The gradient mobile phase was delivered at a flow rate of 0.5 ml/min, and consisted of two solvents, 0.05% acetic acid in water and acetonitrile. The analytes were resolved on a Betabasic-C18 (100x2 mm, 3 μm) column at 40°C using the Shimadzu column oven. Data was acquired using Analyst 1.5.1 and analyzed using Multiquant 3.0.1(AB Sciex, Ontario, Canada).

2.3.10 Statistics
For behavioral analyses, a repeated measure Two-Way ANOVA was used (Prism GraphPad 7.0a Statistical Package, La Jolla, California). Post-hoc analyses used Bonferroni-corrected t-tests. Western blot and PG mass-spec analyses between MA and control treatments used independent t-tests. To characterize the generalizability of our results and support the magnitude of effects in our studies independent of sample size, effect sizes are reported. Effect sizes were calculated as generalized eta squared ($\eta^2_G$) for behavioral data and Hedge’s unbiased g (g) for post-hoc analyses and molecular data, using IBM SPSS Statistics for Mac (Armonk, NY: IBM Corp.), and Microsoft Excel® as previously described 97. For our studies, benchmark coefficient thresholds for large effects 97 were taken into account and inflated in order to restrict significance. The thresholds for large effect sizes in our studies were as follows: when $\eta^2_G$ was 0.2 or higher, and when $g$ was 0.8 or higher. Effect sizes below these criteria were deemed moderate.

2.4 Results

2.4.1 Shaping and Methamphetamine administration

The study began with shaping animals for a total of 2 days. After 4 total days of acclimation, subjects were randomly assigned to either a water/control group or MA for 28 consecutive days (days 5-32). The WMA was performed on day 37 after 5 days of forced abstinence from MA. This was followed by 10 days of cognitive flexibility assessment on the reference/working memory version of the RAM (days 40-49). Tissue was collected on day 50 (Figure 2A). Over the 28d period mice consumed an average of 5.23 mg/kg of MA/day. Figure 2B reflects the average
weekly consumption of MA. Control mice ate every presentation of water-Maypo. Figure 2C shows the average weekly weights for both conditions. A two-way ANOVA shows no significant difference between the groups \( F(1,32) = 1.55, p > 0.05; \eta^2 = 0.023, \text{ non-significant effect size} \) indicating that the Maypo consumption, with or without MA, was not influenced by differences in body weight between conditions.
Figure 2. Timeline for the 50d study, Average Methamphetamine (MA) Consumption and Body Weights. (A) Days 1-2: behavioral shaping on RAM. Days 5-32: VOMA for 28d. Day 37: working memory assessment. Days 40-49: cognitive flexibility assessment (spatial learning). Day 50: RAM test and tissue harvest. (B) The total amount of MA consumed over the 28 day VOMA period, grouped into 4-week averages for Control and Methamphetamine (Meth) groups. (C) Mice were weighed during the consumption period of VOMA. No significant differences were found between the two groups when analyzed by two-way ANOVA [F(1,32) = 1.55, p > 0.05; $\eta^2_\text{G} = 0.023$, non-significant effect size]
2.4.2 Radial-Arm-Maze Working and Reference Memory Assessments

Figure 3A shows the % correct scores in two separate analyses to illustrate the differences in number of errors committed during a trial. Collecting the first 4 baits, when the working memory load is low, shows a much higher % correct score compared to when the working memory load is high, collecting baits 5-8. An overall 2-way ANOVA reflecting treatment condition (MA or saline) and baits (1-4 or 5-8) shows a significant effect of bait \( [F(1,8) = 50.67, p < 0.001; \eta^2_G = 0.76, \text{significant effect size}] \). The Bonferroni post-hoc analysis shows significant within-group differences in both control (Bonferroni corrected \( t \)-test=3.14, \( p < 0.05; g= 1.57 \)) and VOMA (Bonferroni corrected \( t \)-test=6.93, \( p<0.001; g= 4.85 \)) mice when comparing group performances between collecting baits 1-4 and baits 5-8. Between groups, there were no significant differences between treatments for baits 1-4 (Bonferroni corrected \( t \)-test = 0.39, \( p > 0.05; g= 0.23 \)) In contrast, VOMA produced a significant increase in working memory errors while collecting baits 5-8 compared to controls (Bonferroni corrected \( t \)-test= 3.41, \( p < 0.01; g= 1.92 \)). Figure 3B shows the latency to complete the working memory trials. There were no significant differences between treatment conditions for latency to complete working memory trials (Figure 3B).
Figure 3: VOMA impairs spatial working memory. (A) Two-way Repeated ANOVA showed an overall effect of bait group (** p<0.001). Post hoc analyses reveal that both control and MA mice performed significantly worse to retrieve baits 5-8 compared to baits 1-4 (# indicates different from control bait 1-4, p<0.05; ! indicates different from MA bait 1-4, p<0.001). Baits 5-8 reveal a significant difference between groups (^ indicates different from control in same condition p < 0.01). (B) There was no significant difference in latency to complete the trial between the groups.
Figure 4 shows the behavioral analyses for RAM acquisition across 10 training days (experiment days 40-49 shown on graphs). An overall 2-way ANOVA for % correct scores (Figure 4A) shows a significant interaction effect of treatment on training days \( F(9,72) =5.12, p < 0.01; \eta^2_G = 0.29 \) and time \( F(9,72) =33.38, p < 0.01; \eta^2_G = 0.73 \). Post hoc tests show significant differences between treatment conditions for training day 8 (Bonferroni corrected t-test = 3.45, \( p < 0.01; \ g = 1.97 \) ) and training day 9 (Bonferroni corrected t-test = 4.28, \( p < 0.01; \ g = 2.44 \) ). To determine whether there are significant differences in asymptotic performance level between groups, we assessed % correct scores during training days 6-10 (experiment days 45-49). This analysis shows an overall effect of drug treatment \( F(1,8) = 10.99, p < 0.05; \eta^2_G = 0.40 \), reflecting differences in the level of peak performance. As predicted with asymptotic performance, there was no significant effect of training days. Consistent with % correct score analyses, reference memory errors shown in Figure 4B reflect an overall significant interaction effect of treatment on training days \( F(9,72) = 3.75, p < 0.01; \eta^2_G = 0.25 \), and a significant effect of training days \( F(9,72) =25.00, p< 0.01; \eta^2_G = 0.69 \). During asymptotic performance (training days 6-10 experiment days 45-49), reference memory errors show an overall significant effect of treatment \( F(1,8) = 13.8, p < 0.01; \eta^2_G = 0.33 \). Analysis of working memory errors (Figure 4C) shows an overall significant effect on training \( F(9,72) = 5.25, p < 0.01; \eta^2_G = 0.35 \), but no significant effect between treatment conditions. During asymptotic performance (training days 6-10, experiment days 45-49), there was a significant effect of treatment \( F(1,8) = 5.513, p<0.05; \eta^2_G = 0.20 \) for working memory errors. Together the data in Figure 4A-C demonstrate that MA is significantly impairing the level of peak performance across multiple analyses on this spatial learning task. Analysis of latency to complete the training trials shows a significant interaction effect of treatment on training days \( F(9,72)=4.92, p <0.01; \eta^2_G = 0.23 \) and an overall significant decrease
over training days \( F(9,72) = 22.01, p < 0.01; \eta^2 = 0.57 \). Post-hoc tests show a significant difference between treatment conditions on training day 1 (Bonferroni corrected t-test = 3.31, p<0.05; g = 1.89).
Figure 4. VOMA impairs spatial learning. All statistical analyses involved two-way ANOVA across 10 consecutive training days (experiment days 40-49 shown on graphs) and treatment conditions. Differences between treatment conditions during asymptotic performance (training days 6-10, experiment days 45-49) were analyzed separately. (A) % Correct shows a significant effect of training days (^ p < 0.0001) and treatment conditions (# p < 0.0001). Bonferroni-corrected post hoc analyses revealed significant differences between treatment conditions on training days 8 and 9, (experiment days 47-48) (** p < 0.01; *** p<0.001). During asymptotic performance (training days 6-10) there is a significant treatment effect (! p<0.05). (B) Reference memory errors show a significant effect on training days (^ p < 0.01), a significant interaction (#
p < 0.01), and a significant treatment effect during asymptotic performance (training days 6-10, ! p<0.01). (C) Working memory errors show a significant effect of training days (^ p < 0.01), without a significant difference between treatment conditions except during asymptotic performance (training days 6-10, ! p<.05). (D) Latency to complete the trials show a significant effect of training days (^ p < 0.01), and a significant interaction between treatment conditions (# p < 0.01). Bonferroni post-hoc tests show a significant difference between treatment conditions on training day 1 (p<0.05).
2.4.3 Hippocampal Dopaminergic Marker Expression

Western blot analyses for dopaminergic markers showed a significant decrease in cytosolic TH ($t= 2.67, \ p<0.05, \ g= 1.59$; Figure 5A). In the synaptic fraction, there was a significant increase in D1R expression ($t= 3.16, \ p<0.01, \ g= 1.81$, Figure 5B) and a decrease in synaptic DAT expression ($t= 2.16, \ p<0.05, \ g= 1.29$, Figure 5C).
Figure 5. Chronic VOMA affects dopaminergic marker expression. Compared to controls, VOMA decreases cytosolic TH (*p<0.05; A). In the synaptic fraction, D1R significantly increased (**p<0.01; B) and DAT significantly decreased (*p<0.05; C) compared to controls. Representative blots show control (left band) and MA (right band) treatments. See supplementary figures for original images of blots.
2.4.4 Hippocampal Inflammatory Marker Expression

Inflammatory markers showed a significant increase of synaptic COX-2 ($t_8 = 1.93, p<0.05, g=1.09$, Figure 6A) and cytosolic COX-2 ($t_7 = 2.03, p<0.05, g=1.211$, Figure 6B). Cytosolic GFAP was also significantly elevated compared to controls ($t_7 = 3.86, p<0.01, g=-2.30$, Figure 6C). GFAP and COX-2 increases suggest a raise in proinflammatory activity in the hippocampus. MA-induced excitotoxicity can mediate increases of GFAP to allow for astrocyte activation in response to MA-induced damage. COX-2 is an inducible inflammatory activator that can signal other pathways, including prostaglandin synthesis, which can be protective or toxic to neurons. Figure 6D-F shows analyses of PGE2, D2 and J2 in picograms per milligram of wet weight of tissue, indicating that PGD2 levels in control mice are at least 10-fold higher than PGE2 and PGJ2. Both PGE2 and PGD2 significantly decrease (33% and 40% respectively) with VOMA compared to controls ($t_8 = 3.95, p < 0.05; g=2.26$, Figure 6D. $t_8=3.73, p< 0.01; g=2.13$, Figure 6E.). PGJ2, which is a PGD2 metabolite, did not change significantly between treatment conditions (Figure 6F).
Figure 6. Chronic VOMA increases neuroinflammation marker expression. In the synaptic (A) and cytosolic (B) fractions COX-2 levels significantly increased compared to controls (*p<0.05). (C) Cytosolic GFAP increased compared to controls (**p<0.01). See supplementary figures for original images of blots. (D,E). PGE2 and PGD2 significantly decreased with VOMA compared to controls (*p<0.05; picograms/milligram). (F). PGJ2 expression did not change significantly between treatment conditions. Representative blots show control (left band) and MA (right band) treatments.
2.4.5 Hippocampal Synaptic Plasticity Marker Expression

Compared to controls, VOMA produced a significant decline in glutamatergic and synaptic plasticity markers including GluA2 ($t_6=8.53$, $p<0.01$, $g=5.24$, Figure 7A), synaptic PSD-95 ($t_8=3.38$, $p<0.01$, $g=1.93$, Figure 7B) and cytosolic PKMζ ($t_8=2.55$, *$p<0.05$, $g=1.46$, Figure 7C). Cytosolic PKCι/λ expression was not altered by VOMA treatment (Figure 7D).
Figure 7. Chronic VOMA decreases memory associated marker expression. (A) In the synaptic fraction, VOMA decreased GluA2 (***p<0.01) and PSD-95 compared to controls (***p<0.01; B). (C) In the cytosolic fraction, VOMA decreased PKMζ compared to controls (*p<0.05). (D) VOMA did not significantly affect cytosolic PKCι/λ expression. Representative blots show control (left band) and MA (right band) treatments. See supplementary figures for original images of blots.
2.5 Discussion

2.5.1 VOMA as a model for studying MA addiction

VOMA is a new model for assessing both the chronic and acute effects of MA. MA mixed in a palatable sweetened oatmeal mash provides a paradigm that does not require surgery or conditioning. This model permits the animal unrestricted movement that allows the experimenter to test various behavioral learning paradigms. Our results demonstrate that chronic voluntary consumption of MA results in significant spatial learning and memory deficits together with significant changes in markers reflective of MA neurotoxicity. One of the main challenges in MA research is designing an experiment that models aspects of human MA addiction. We find that this VOMA model is useful for characterizing aspects of addiction, as mice are able to titer the amount of drug administered, as reflected by a consistent level of MA consumed over several weeks. Our results indicate that the C57BL/6 mice will voluntary consume about 1.743 mg/kg bw/hr during a 3 hr MA administration period. These data suggest that if 5mg/kg bw MA was delivered as a bolus dose to a mouse of this strain, even such a seemingly low dose is considerably higher than would be consumed voluntarily. Whether there is a difference between an injection of 5 mg/kg bw compared to voluntarily consuming this amount over a 3 hr period remains to be determined. Other MA models have used appetitive paradigms or drinking paradigms that also demonstrate the short-term behavioral and neurochemical changes associated with small doses of voluntary MA consumption. In our current study, we examine the behavioral and neurochemical effects of chronic voluntary MA as a way to more accurately model human MA addiction.
Throughout 28 days of VOMA, consumption rates increase over the first 2 weeks and stabilize during the last 2 weeks. Previous self-administration models may only reveal a trend to increase consumption due to the brief (2 weeks or less) administration window. It is unclear how other self-administration models would compare to VOMA across longer periods administration (4 weeks or more). Moreover, it is important to consider how oral administration via a sweetened medium might affect VOMA. Control mice in our study consumed every presentation of water-Maypo (12 baits/day over 28 days), revealing a preference for sweetened food by this mouse line. However, mice that consumed water-Maypo laced with MA consumed less than half of the baits presented to them each day on average (see Results). This reveals that although a sugar preference could motivate consumption of the MA-baits, mice rapidly adapt a strategy to consume only as much MA-Maypo as they prefer. Mice on VOMA could still develop a preference to MA as a result of the sweetened medium, but more work is needed to determine this. Future studies should examine how the drug’s medium affects consumption rates across long periods of MA access.

The use of an oral administration design is validated by previous work indicating that the mean half-elimination life of oral MA is not significantly different from that of the intravenous route, and that peak plasma concentration is delayed via the oral route by 3 hrs in humans, compared to other routes 101-102. This suggests that VOMA can model the delayed effects of MA on the brain, and very adequately reveal the prolonged neurotoxic effects of the drug. Additionally, previous work has revealed that although the injection route may be preferred by a subset of highly-addicted individuals, non-injection routes make up a high percentage of MA use in the United
States Nonetheless, our study models oral MA administration, a route not likely preferred by MA-addicted humans. Non-injection MA administration might be preferred by individuals who have not yet developed an addiction. In order to fully understand the distinct populations that abuse MA, more research is needed to clarify the onset and progression of this addiction in humans as it relates to administration routes. Future VOMA studies should examine the progression and relapse of MA consumption in VOMA in order to accurately characterize the addictive phenotype produced by our model.

2.5.2 MA-induces deficits in both spatial working and reference memory performance

Our results show that total MA consumption of 146.4 mg/kg bw/28 consecutive days result in a significant spatial working memory performance deficit. Our previous study reports working memory deficits following neurotoxic doses of MA (30 mg/kg bw delivered once per week for 2 weeks; 60 mg/kg bw total). These working memory deficits developed over a 4 week abstinence period. The working memory deficit observed in our current study may have developed much earlier based on the levels of MA consumed. However, reports by others suggest that lower doses of MA delivered chronically may have less harmful or delayed cognitive effects, compared to bolus or neurotoxic doses. The latter studies are consistent with the protective effects observed when escalating doses of MA are delivered over days that show reduced cognitive and neurochemical deficits, compared to neurotoxic effects and the resulting cognitive deficits that result from a bolus dose. Rapid deficits in working memory performance identified 24 hrs following a neurotoxic dose of MA were previously reported. However, without repeated assessments over time, it is difficult to determine whether the working memory deficits are long
lasting or are reflective of the short-interval between MA treatment and working memory testing. It is possible that shortly after MA administration, mice are low on seeking novelty, an effect that is rectified weeks after MA treatment as demonstrated in rat performance on the spontaneous alternation task 109.

Following the working memory assessment, mice were tested for cognitive flexibility on the RAM. For this assessment, both working and reference memory errors were scored. The MA treated mice exhibited a lower asymptotic performance level for % correct scores and an increase in the number of reference and working memory errors compared to mice given control treatment. This is consistent with several other studies reporting spatial memory deficits following a short abstinence period 110-112. Others have shown that chronic use of MA can actually stave off MA-induced deficits, which can then be initiated following long-periods of abstinence. In this case, cognitive deficits were observed only following long-term abstinence (1-4 weeks) 1, 104, 113-114. The degree to which VOMA produces neuroprotection and/or can delay the neurotoxic effects of MA remains to be determined.

2.5.3 Chronic VOMA decreases TH and DAT while increasing D1R levels

Our previous studies revealed that acute neurotoxic doses of MA (2x 30 mg/kg) followed by 6 weeks of forced abstinence produce long-lasting decreases in dopamine marker expression in the hippocampus 1. In our current study, we found that VOMA for 28d also produced significant decreases in these markers in the hippocampus. Previous work has shown that within days of injecting neurotoxic doses of MA, the striatum exhibits a comparable decrease in DA levels 115-
MA-induced reductions of TH and DAT in the striatum may indicate degeneration of DA axonal terminals. This explanation is supported by increased reactive astrogliosis associated with MA treatment. We hypothesize that decreased DAT and TH together with an increase in astrogliosis reflects a degeneration of DA terminals within the hippocampus. Whether our VOMA paradigm produces similar effects in the striatum remains to be determined.

VOMA also induced a significant increase in D1R expression. This receptor is known to mediate some of the MA-driven neurotoxic effects in the striatum and PFC. Selective D1R antagonists delivered prior to injections of binge doses of MA produce neuroprotective effects against the MA-induced decreases in DA, TH and DAT. Moreover, increases in MA-induced extracellular DA causes acute increases in striatal glutamate as a result of D1R mediated disinhibition of corticostriatal glutamate release. Together these findings suggest that increased D1R expression is detrimental and/or exacerbates the neurotoxic damage from MA. In contrast, it has been suggested that D1R activation is a neuroprotective response. Dopamine suppresses glutamatergic hippocampal and entorhinal neurotransmission by activation of the D1R. Additionally, D1R activation can reduce NMDA receptor mediated Ca^{2+} currents in hippocampal neurons, thus decreasing excitotoxicity. The D1R also plays a role in reducing calcium currents in striatal neurons, and in producing inhibition on N-type voltage-gated calcium channels. Therefore, it is possible that alterations in other receptor levels dictate whether increased D1R-levels are beneficial or detrimental. Future studies will evaluate the changes in NMDA and mGluA receptor subunits, which could add to understanding the consequences of these neurochemical changes. For example, antagonism of NMDA, AMPA or
metabotropic (mGluR5) receptors can block MA-induced toxicity in the striatum 133-135 or hippocampus 136.

2.5.4 Neuroinflammatory marker levels increase in the hippocampus following chronic VOMA

We show that compared to controls, GFAP and COX-2 protein levels are elevated in the hippocampus after 28d of VOMA. We propose that these changes in inflammatory markers reflect a neurodegenerative response to MA. COX catalyzes the conversion of arachidonic acid to prostaglandins (PG) and thromboxanes. COX-1 is constitutively expressed, whereas COX-2 is induced upon stimulation by various proinflammatory agents (endotoxins, cytotoxins) 99, 137. Elevated levels of COX-2 in the hippocampus increase the extracellular concentration of glutamate, exacerbating glutamate-associated excitotoxicity 138-141. Our results are consistent with what is observed following neurotoxic doses of MA 142. Conversely, COX-2 KO mice are resistant to MA-dependent dopamine depletion in the striatum that results from high doses 143, suggesting that COX-2 up-regulation exacerbates the neurotoxic effects of MA 144-145. Other reports show temporary GFAP increases in the hippocampus 24h after neurotoxic doses of MA, which return to basal levels by 7d post injection 39, 142. These data suggest that the rapid increases in GFAP induced by a neurotoxic dose of MA may reflect activation of a neuroregenerative response, as this marker is expressed during neurogenesis in the subgranular zone of the dentate gyrus 146. Based on these findings, it is possible that the accumulating (low doses) of MA during VOMA prolong the short-term increase in GFAP that has been observed following neurotoxic doses. Future studies should characterize the time course for the onset and progression of GFAP expression in the hippocampus after VOMA.
Following a regimen of neurotoxic MA administration (4× 10 mg/kg, 2 h apart, i.p.), mice exhibit cytotoxic brain edema driven by increases in hippocampal and striatal aquaporin-4 channels on astrocytes. Increased GFAP expression in the hippocampus following VOMA suggests that after chronic MA abuse, the hippocampus is vulnerable to breakdown of the blood-brain barrier resulting in cytotoxic edema. Astrocytes are one part of the complex neurovascular niche and aquaporins play a pivotal role in regulating water and cerebro-spinal fluid (CSF) flow into and out of the neuronal space. It is unclear how and when this dysregulation in aquaporin-mediated shifts in water/CSF emerges and is resolved after chronic MA abuse. Future studies should examine the role that aquaporin-mediated cytotoxic edema plays in VOMA-induced neural and cognitive deficits.

PGE2 and PGD2 levels decrease following VOMA. PGD2 is the most abundant of the PGs in the brain followed by PGE2, and both PGs induce secretion of nerve growth factor and brain-derived neurotropic factor. In addition, both PGD2 and PGE2 were found to have neuroprotective effects in the CNS. These studies suggest that decreased levels of PGD2 and PGE2, together with increased expression of COX-2 could mediate a neurotoxic response to VOMA. It is possible that the decrease in PGE2 reflects an increase in the 15-hydroxyprostaglandin dehydrogenase (15-PGDH), a key enzyme in the metabolism of PGE2. An ALS mouse model shows that 15-PGDH increases throughout the progression of the disease specifically in GFAP positive astrocytes, while PGE2 only increased at the end stage of the disease. No other studies have measured PGs in the hippocampus following MA. One study examined binge doses of MA on PGE2 in the striatum showing no changes in PGE2 levels up to
48 hrs following MA. Further work is needed to delineate how these different MA models affect PGs across various brain regions.

PGJ2 is a product of spontaneous dehydration of PGD2, which is the most abundant prostaglandin in the brain and the one that changes the most under pathological conditions. PGJ2 is a highly neurotoxic prostaglandin, as it impairs both the ubiquitin-proteasome pathway and mitochondrial function. This PG also up-regulates COX-2, most likely leading to a positive feedback loop between itself and COX-2. In supplementary studies, we have observed that PGJ2 levels increase following 6 weeks of abstinence after acute neurotoxic doses of MA (2x 30 mg/kg; supplementary Figure 1) and also increases following 4 weeks of abstinence after a 2 week VOMA period (25 mg/kg total MA consumed over 14d; supplementary Figure 2). However, mice given 28d of VOMA did not exhibit increases in PGJ2 (Figure 6F). These data suggest that abstinence may be playing a role in the increased expression of PGJ2.

This finding is consistent with previous studies indicating that abstinence from MA can exacerbate memory deficits.

Our results suggest that the duration of abstinence and of MA-administration could play opposing roles in promoting or suppressing PGJ2 levels in the hippocampus. We hypothesize that a long period of abstinence from MA promotes PGJ2 expression, but that a longer period of MA administration mitigates it through increased dopamine release. This hypothesis is consistent with the understanding that dopamine is anti-inflammatory by inhibiting the activation of NOD-like receptor containing Pyrin domain-3 (NLRP3) inflammasome by D1R activation.

Additional studies have shown that low doses of MA, which increase dopamine release, are
neuroprotective following oxygen-glucose deprivation. However, it remains to be determined how abstinence from MA contributes to increases in PGJ2.

2.5.5 MA decreases synaptic memory markers

Our results show that the levels of PKMζ and AMPA receptor subunit GluA2 decrease following VOMA. PKMζ is important for both late-phase LTP and long-term memory maintenance across various learning paradigms. It is also important to note that increased GluA2 synaptic expression is important for spatial memory, as is the trafficking of AMPA receptors by PKMζ. These studies are consistent with the decline in PKMζ and GluA2 levels following VOMA. We also assessed the protein levels of PKCι/λ, another atypical kinase known to support spatial memory in the conditional-PKMζ KO mouse, suggesting that PKCι/λ is active when PKMζ is compromised. PKCι/λ expression was not significantly altered following VOMA, suggesting that deficits in PKMζ may not reflect neurotoxic damage, but rather reflect poor learning.

GluA2 is one of the four AMPA receptor subunits impermeable to Ca2+ and is the dominant heteromeric conformation. Reduced GluA2 levels were shown to increase neurotoxicity in amyotrophic lateral sclerosis motor neurons. We hypothesize that decreased GluA2 and PKMζ mediate spatial learning impairment as well as the elevated neurotoxic effects of MA. This is consistent with chronic MA treatment. In contrast, studies by others examining acute doses of neurotoxic MA report increased levels of GluA2 shortly after treatment, suggesting that elevated hippocampal GluA2 levels may be contributing to a neuroprotective response by
MA. However, after several weeks of abstinence, hippocampal GluA2 levels return to baseline. Together these results suggest that early after MA, hippocampal GluA2 increases as a neuroprotective mechanism, while following chronic MA a decline in hippocampal GluA2 could exacerbate MA neurotoxicity. These results are also consistent with our behavioral data, indicating that GluA2 decline in the hippocampus could underlie the spatial memory deficits found in VOMA mice.

Our results also show a significant decrease in PSD-95 protein levels. PSD-95 is primarily located in the neuronal spine head and its density reflects synaptic efficacy. Moreover, increases in PSD-95 spine density are associated with improved memory performance. These findings are consistent with the poor memory performance and reduced hippocampal PSD-95 level in the MA treated mice. PSD-95 levels also increase in the forebrain following MA-induced place preference, suggesting PSD-95 involvement in memory rather than a secondary effect of MA-induced toxicity.

2.6 Conclusions

Our data demonstrate that chronic VOMA leads to significant deficits in spatial learning and short-term working memory, which is consistent with reports by others. Additionally, our results show that low doses of MA consumed over a 3 hr period can produce significant neurotoxic changes in the hippocampus, reproducing the neurochemical effects observed in studies using bolus or neurotoxic doses of MA. Chronic VOMA also leads to significant decreases in TH and DAT and an increase in D1R expression as seen with neurotoxic and binge doses of MA. These
neurochemical changes are accompanied by increases in both COX-2 and GFAP that were previously reported. In concert with the decline in PGE2 and PGD2 levels, these data may reflect extensive neurotoxic damage attributed to MA. Finally, decreases in synaptic markers relevant to spatial long-term memory involving both GluA2 and PKMζ are consistent with neurotoxicity-induced deficits in working memory and spatial learning. The time course for the onset and progression of these various neurochemical changes in the VOMA model remains to be determined. These data are consistent with what is observed in MA addicts showing low levels of dopamine, DAT and TH associated with cognitive impairment. Together these studies support the significance of this VOMA mouse model in identifying risk factors and exploring potential remediation therapies against MA addiction.
2.7. Supplementary Figures

Figure 8. Supplemental Figure 1. Prostaglandin levels in hippocampus after 14d VOMA followed by 4wks of abstinence. (A) PGE2 and (B) PGD2 levels did not differ between treatment conditions. (C) PGJ2 levels significantly increased in MA condition compared to controls (t9=2.23; *p=0.026, g=1.34)

Figure 9. Supplemental Figure 2. Prostaglandin J2 levels increase in mice hippocampi 6 weeks after two bolus doses of MA (2x 30 mg/kg). (A) PGE2 and (B) PGD2 levels did not differ between treatment conditions. (C) PGJ2 levels significantly increased in MA 6-week condition compared to MA 3-day condition (t5=3.24; *p=0.023, g=2.09)
Table 2: MRM transitions for prostanoids and deuterated internal standards for PG Mass Spectrometry. The scheduled MRM transition values monitored within a 1.5 min window.

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CHAPTER 3: TWO-WEEKS OF VOLUNTARY ORAL METHAMPHETAMINE ADMINISTRATION PRODUCES ACUTE SPATIAL-MEMORY DEFICIT AND INCREASES NEUROINFLAMMATION DURING ABSTINENCE

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To be submitted to Behavioural Brain Research as: Jorge A. Avila, Nicoletta Memos, Tytus Andrejewski, & Peter A. Serrano (submission in 2020). Two-weeks of voluntary oral Methamphetamine administration produces acute spatial-memory deficit and increases neuroinflammation during abstinence
3.1 Abstract

Methamphetamine (MA) is a neurotoxic drug of abuse. It can produce neurodegenerative processes in the brain that impair behavioral and cognitive functions. The mechanisms that underlie MA-induced neurodegeneration are well characterized, but less is known about how MA induces behavioral and cognitive deficits. Furthermore, little is known about the progression of behavioral deficits immediately after MA abuse and during abstinence. To address these gaps in knowledge, our lab has developed a voluntary oral administration model of MA (VOMA) using mice. Male C57Bl6 mice were put through VOMA for 14 days followed by a prolonged period of abstinence. During VOMA, mice were allowed to voluntarily consume up to 16mg/kg of MA each day. During and after VOMA, mice were put through a battery of cognitive and behavioral assessments to test drug sensitization, spatial memory capacities, and long-term retention. Our results show that VOMA produces subsets of MA consumers in mice, accurately modeling voluntary MA abuse in humans. Our behavioral data reveals that VOMA produced an acute sensitization to MA, and an acute working spatial memory deficit directly after VOMA. However, VOMA did not produce a long-term spatial learning or retention deficit during abstinence. Hippocampal tissue analyses of mice from different timepoints reveal that abstinence significantly increases neuroinflammation, and significantly contributes to modulations in synaptic dopamine transporter and 5-5-hydroxytryptamine receptor 2A (5HT2a) expression. Our study characterizes the progression of behavioral changes that follow MA administration, revealing that mice do not exhibit cognitive deficits during abstinence. Neurochemical analyses reveal that abstinence contributes to major changes in hippocampal neuroinflammation and monoamine protein expression. These findings provide new insight into the complex behavioral resilience and neurochemical susceptibility exhibited by MA subjects during abstinence.
3.2 Introduction

Methamphetamine (MA) is a highly neurotoxic and addictive psychostimulant. Studies show that MA is increasing in popularity and abuse in the United States\(^1\). MA exposure during critical developmental periods can impair cognition in rodents\(^2\), as well as humans\(^3\), and can significantly affect neurochemistry\(^4\). Chronic MA users develop hallucinations\(^5\) as well as cognitive deficits in attention, memory, information processing, and impulse control\(^6\)-\(^9\). MA abuse can also trigger negative emotional states such as depression during drug withdrawal and abstinence\(^10\). These deficits may be attributed to neurodegeneration of the frontal cortex, midbrain and hippocampus\(^11\). Additionally, increased MA exposure correlates with greater locomotion in mice\(^12\)-\(^13\) and can also affect various unconscious physiological behaviors such as food intake\(^14\). In order to determine the progression of behavioral changes following MA intake, the current study used a new model of methamphetamine administration labeled Voluntary Oral Methamphetamine Administration (VOMA)\(^15\). Previous MA administration animal models have used binge and neurotoxic dosing via injections\(^16\). These models do not accurately represent the human model since people are not likely to take binge or neurotoxic doses in a short periods of time. Instead, humans may opt to spread out similar MA doses over a longer span of time. The VOMA model allows mice to titer the amount of MA they consume in a day and over many weeks. For this particular study, we exposed mice to VOMA for 2 weeks and a long abstinence period of up to 7 weeks prior to tissue collection or behavioral assessments.

The trajectories in behavioral and neurochemical changes during abstinence from MA remain unclear. MA abuse can produce long-term cognitive impairments in abstinent individuals\(^17\)-\(^19\). It has also been shown that long periods of abstinence from MA can improve the recovery of
decision making-skills and emotional symptoms which may help individuals to avert relapse. Yet, other reports indicate that relapse can still occur after prolonged abstinence, as a result of exposure to specific environmental cues. The underlying neurochemical pathways that result in relapse during abstinence remain unclear. Previous work has implicated glutamatergic signaling in the nucleus accumbens as pivotal in addiction models, and recent evidence has also linked hippocampal neurogenesis to context-dependent MA memory during abstinence. A thorough understanding of the neurochemical mechanisms underlying MA relapse during abstinence would provide pharmacological targets to assure successful recovery from addiction. Since the abstinence and relapse are also vital to studying addiction, our experiments focused on tissue analyses from two timepoints following VOMA, to characterize the progression of neurochemical changes in this animal model of drug abuse.

As a result of MA-induced neurotoxicity, neuroinflammatory pathways are activated in the brain involving astrocytes and pro-inflammatory enzymes. The contribution of abstinence to neuroinflammatory pathway activation and/or silencing is unclear. Parallel neurochemical changes following MA abuse include those that also occur in psychiatric disorders, including changes to dopamine and serotonin signaling. Previous literature has revealed a link between serotonin signaling, via the 5HT2a receptor, with cognition, hallucinogenic drugs and psychiatric disorders. Additionally, MA abusers also show decreased density of dopamine transporters (DAT) in the striatum and frontal cortex, as markers for degeneration of DA innervations in the brain. It is unclear when DAT function is compromised following MA exposure and under what circumstances this function is restored.
during abstinence. Our study characterizes changes in these markers following VOMA and after abstinence.

3.3 Methods

3.3.1 Animals

All experimental conditions, housing, and drug administration procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Hunter College. Two (2) cohorts of male C57Bl6 mice (N=71 total; cohort 1 n=22, cohort 2 n=49) purchased from Taconic Biosciences were used for this study. Upon purchase, animals were received at Hunter College’s Animal facility aged 6 weeks. Mice were individually housed, kept on a 12h/12h light/dark cycle from 7:30h to 19:30h, and fed standard rodent chow ad libitum for 1 week prior to handling and starting baseline assessments. Mice were housed individually to control for social effects of group housing 93 that could affect the voluntary consumption of methamphetamine in our model.

3.3.2 Food restriction

In order motivate mice to consume MA baits, strict food-restriction schedules were instituted for both MA and control mice. Standard rodent chow was delivered at 18:00h, at a ratio of 1g of chow per 5g of body weight, every day for the duration of the study. This feeding schedule ensured that mice would be willing to consume MA-oat baits during the VOMA administration window each day without disrupting weight gain or loss during the experiment, as previous work has reported significant circadian effects in a similar VOMA administration design 94.
3.3.3 VOMA

From two (2) cohorts of mice, n=17 mice were placed into control treatment and n=54 mice were placed into VOMA. Daily body weights were recorded, and 1 or more dosing solutions were formulated for each weight class, restricted to within 5 grams of body-weight variability. Working MA dosing solutions of 0.8 to 0.9 mg/mL (in water) was made and prepared for administration. Mice were housed in standard polycarbonate cages for drug administration procedures. Each cage was used for the same mouse throughout the experiment to minimize housing stressors. Methamphetamine (0.007 mL at 1mg/kg and ~0.8mg/mL) was dissolved into oatmeal bran chips (Maypo Inc.) and served 16 times per day to each mouse, at 15-minute intervals. Control mice received water absorbed by oat chips. Experimenters administered individual MA-oatmeal chips to each mouse and tracked consumption before delivering new MA-oat baits. Administration room was kept isolated from surrounding noise and activity. VOMA and control administration was carried out between 12:00h and 18:00h every day for the duration of the experiment.

3.3.4 Sensitization-induced mobility in test cage

A subset of mice (n=12 control, n=13 low consumers and n=9 high consumers) were used for tests of sensitization measures. Mice were analyzed for mobility in their VOMA cage to measure sensitization prior to receiving MA. Mobility was defined as time mice spent running, jumping, walking, interacting with nests, and climbing on the bars of the cage. Three-minute videos were recorded of each subject. Mobility of each subject determines degree of sensitization and anticipation of MA delivery on day 8 (after one week of VOMA) and day 15 (one day after the
VOMA period). Mice did not receive MA on day 15, but rather were only exposed to their MA cage to capture videos and then promptly returned to their home cage.

3.3.5 Radial Arm Maze Shaping and Baseline Working Memory Assessments
The radial 8-arm maze (RAM) was used to assess both working memory and spatial learning. The maze consists of a center platform (15.24 cm diameter) with 8 equivalently sized arms radiating outward. Each arm is 38 cm in length, 6.35 cm wide with a submerged food cup (2.0 cm diameter) at the end of each arm. Ensure® was used as a food reward (0.007mL per bait). Prior to the start of VOMA, all animals were shaped on the RAM. Mice were food restricted to ~85% of free feeding weight. On day 1 of RAM shaping, mice were placed on the RAM for 5min to acclimate to the maze and room cues, with food baits available around maze and inside food cups. On day 2 of shaping, mice were given 15min each to explore the maze and the food baits on ends of maze-arms and inside cups. On day 3 baseline working memory performance was recorded. Mice were given 15min each to collect small baits at the ends of arms. Performance on days 3 was used to balance groups across treatments.

3.3.6 Radial Arm Maze Working Memory Assessment
Working memory assessment (WMA) was assessed 2 days after end of VOMA and 4 weeks into abstinence, for a subset of mice not slated for tissue collection (n=11 control, n=13 low consumers and n=8 high consumers). One (1) trial was used to assess performance at each time point. Before starting each trial, all food cups were baited with Ensure®. To begin a trial, mice were confined for 30s to the center platform with a plastic cylinder. The sequence of arms entered to retrieve the food rewards was recorded. To prevent a non-spatial strategy, mice were
allowed to collect baits from up to 3 sequential arms (in any one direction around the radial maze) before the experimenter interrupted the chaining strategy. Errors were recorded as re-entries into arms where the food reward has already been collected. Maximum latency for each trial was set at 15min.

3.3.7 Radial Arm Maze Spatial Learning / Cognitive Flexibility Assessment

28d after VOMA, a subset of mice (n=6 control, n=5 low consumers and n=6 high consumers) were trained on a reference memory version of the RAM as previously reported\(^1\). This paradigm has 4 baited and 4 un-baited arms in a pattern specific to each animal and remained constant throughout the experiments. Mice were given 6 consecutive trials per day for 10 days (60 trials total). Between trials mice were confined to the center platform while the arms were re-baited, and the maze cleaned. The sequence of arm entries was recorded. A reference memory error reflects an entry into an arm that was never baited, while working memory errors reflect re-entries into an arm where the bait had already been collected. Mice were only be allowed to enter up to 3 sequential arms (in any one direction around the radial maze) to prevent the non-hippocampus dependent chaining strategy. This version of the RAM requires mice to learn room cues associated with the baited and un-baited arm sequence. The training room and room cues were the same to those used for the WMA.

3.3.8 Radial Arm Maze Retention Memory test

The Retention Memory test used the same parameters and arm sequences used during the cognitive flexibility/reference memory assessment. Three (3) trials using the same baited
sequences from the learning assessment were used to test the memory performance of each mouse that underwent the reference learning paradigm.

### 3.3.9 Tissue Collection

Following the end of VOMA and 4 weeks days of abstinence, designated mice underwent controlled cervical dislocation of the skull from the spine and brain tissue was harvested. Hippocampi from each mouse were microdissected, snap frozen and stored at −80°C until processed further.

### 3.3.10 Fractionation

Following tissue collection, a subset of mice tissues from each consumption group were used for western blotting analyses (n=5 control, n=2 low-abstinence consumers, n=4 low+abstinence consumers, n=3 high-abstinence and n=5 high+abstinence consumers). Collected tissue was fractionated into cytosolic and synaptic fractions as previously reported189. Briefly, tissues were thawed and homogenized in a TEE (Tris 50mM; EDTA 1mM; EGTA 1mM) buffer containing Sigma Fast, a protease inhibitor cocktail (Sigma Aldrich) diluted to contain AEBSF (2 mM), Phosphoramidon (1 mM), Bestatin (130 mM), E-64 (14 mM), Leupeptin (1 mM), Aprotinin (0.2 mM), and PepstatinA (10 mM). Tissues were homogenized in 200µl of TEE-homogenization buffer using 20 pumps with a motorized pestle. Homogenates were transferred to Eppendorf tubes and centrifuged at 3000 x g (5 min at 4° C), to remove un-homogenized tissue. The resulting supernatant was centrifuged at 100,000 g for 30 min. After ultracentrifugation, the supernatant was collected and stored as the cytosolic fraction. The remaining pellet was re-suspended in 100µl of homogenizing TEE buffer containing 0.001% Triton X-100, incubated on
ice for 1hr and then centrifuged at 100,000 g for 1hr at 4°C. The resulting supernatant was stored at the plasma-membrane fraction. The resulting pellet was re-suspended in 50 μl of TEE buffer and stored as the synaptic fraction. The Pierce bicinchoninic acid assay (BCA; Thermo Scientific, Rockford, IL, USA) was used to determine protein concentration for each sample. Samples were reduced with 4x Laemmli sample buffer equivalent to 25% of the total volume of the sample and then boiled and stored frozen at -80°C.

3.3.11 Western Blotting Analysis

Hippocampi were collected from mice directly following VOMA and after 4 weeks of abstinence (mice from each consumer groups were allocated to –Abstinence and +Abstinence sub-groups, balanced for MA consumption). The tissue was separated into synaptic fractions as previously reported. Samples (20 μg) were loaded on to a Tris/Glycine 4–20% mini gel to resolve: GFAP (55 kDa), GAPDH (37 kDa), DAT (69 kDa), COX-2 (72 kDa), 5-HT2a (53 kDa). Every gel contained 3-4 lanes loaded with the same control sample designated as all brain sample (ABS). ABS was used to standardize protein signals between gels. Gels were transferred to nitrocellulose membranes in the IBlot® Dry Blotting System (Life Technologies; Carlsbad, CA, USA) for 7 min. Nitrocellulose membranes were then incubated in blocking solution containing 5% sucrose in Tris Buffered Saline with Tween-20 (TBST; 0.1% Tween-20 in TBS) for 30 min at room temperature. Samples were incubated overnight with the following primary antibodies: GFAP (1:2000; Abcam Inc., Cambridge, MA, USA), GAPDH (1:2000; Abcam Inc., Cambridge, MA, USA), DAT (1:2000; EMD Millipore, Temecula, CA, USA), COX-2 (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and 5-HT2a (1:2000; EMD Millipore, Temecula, CA, USA). Membranes were washed in TBST for 20 min and probed with Horseradish Peroxidase.
(HRP) conjugated secondary antibodies: Goat Anti-Mouse IgG (H + L)-HRP Conjugate #1706516 (BioRad); Goat Anti-Rabbit IgG (H + L)-HRP Conjugate #1706515 (BioRad); Rabbit Anti-Goat IgG H&L (HRP) (ab6741-Abcam). Membranes were incubated with Enhanced Chemiluminescence (ECL) substrate and exposed on CL-X Posure Film (Thermo Scientific; Rockford, IL, USA). Films were scanned, and then densitometry was performed with NIH ImageJ.

3.3.12 Statistics

For behavioral analyses, a repeated measure t-test, a repeated measures ANOVA and two-way ANOVA was used. Post-hoc analyses used a Bonferroni-corrected t-test. For data-sets that included missing values due to tissue collection attrition designs, a Type III Mixed Effects model analysis design was used. Molecular analyses used a One-way ANOVA and Bonferroni corrected post-hoc comparisons. Prism GraphPad 7.0 (La Jolla, California) was used for all statistical analyses.

3.4 Results

3.4.1 VOMA models naturalistic MA consumption

The experiment was conducted over 10 weeks, with VOMA running for 14d during study weeks 1 and 2. Behavior Assessments were carried out throughout the experiment. Tissue collection for western blot analyses occurred after 14-day VOMA and after 4 weeks of abstinence (Fig11A). Consumption of each subject was recorded throughout the 14-day VOMA period (Fig11B). Tracking of consumption revealed distinct consumer classes in mice. Subjects were split into two distinct groups for respective behavioral and neurochemical analyses: high and low consumers.
High consumers averaged 2.36 mg/kg per day (n=34; 33.15 mg/kg total over 14d). Low consumers averaged 1.39 mg/kg per day (n=20; 19.4 mg/kg total over 14d). Both groups’ consumption rates dropped from week 1 to week 2, suggesting a failure in producing sustained MA preference in this experiment. A Two-way repeated measures ANOVA of MA consumption rates between the groups revealed an effect of day (F13, 676=21.69, p<0.001) and effect of group (F1,52=33.94, p<0.001). Post hoc comparisons revealed significant differences on days 1, 2, 3, 4, 6, 7, 8, 9, 10, 11 and 12 (p>0.05). Weights of each subject was measured throughout the whole experiment (Fig1C). A Type III mixed effects model was run to analyze body weights of all groups throughout the experiment. A significant effect of day was observed (F9,420=121.1, p<0.001). No significant effects between groups were observed.
Figure 10. Experimental design, Methamphetamine consumption and Body weights. (A)

Over 10 weeks, mice were assessed for various behavioral measures, put through 14 days of VOMA, and tissue collected (red asterisks*) at designated timepoints. (B) Consumption of each subject was recorded throughout the 14-day VOMA period. Due to the wide range of consumption rates, mice were split into two distinct groups: high (n=34) and low (n=20) consumers. High consumers averaged 2.36 mg/kg per day (33.15 mg/kg total over 14d). Low consumers averaged 1.39 mg/kg per day (19.4 mg/kg total over 14d). Both groups’ consumption rates dropped from week 1 to week 2, suggesting a failure in producing sustained MA preference in this experiment. (C) Weights of each subject was measured throughout the whole experiment. No significant difference was found between each of the groups.
3.4.2 VOMA consumers show transient sensitization

The mobility of mice in their respective drug-administration cages prior to starting any VOMA procedures may serve as a measure of sensitization and potentially, anticipation of MA administration. Three (3) minute videos were captured for n=12 control, n=13 low consumers and n=9 high consumers inside their drug administration cages before the start of any VOMA procedures. Blinded observers quantified each video for mobility of each mouse. A One-way ANOVA of mobility at the end of week 1 revealed a significant overall effect of group \( F_{2,31}=6.699; p<0.005 \). Post hoc analyses revealed significant differences between High consumers (**) vs. low consumers (p<0.05) and, separately, High consumers vs controls (p<0.005) (Fig 12A). A One-way ANOVA of mobility at the end of week 2 revealed no significant group effects (Fig 12B).
Figure 11. VOMA-induced sensitization. VOMA-Cage mobility prior to start of VOMA delivery indicates sensitization to MA and potentially anticipatory awareness of VOMA delivery. (A) Week 1 mobility analysis revealed a significant overall effect of treatment. Post hoc analyses revealed significant differences between High consumers (n=9; **) vs. low consumers (n=13; p<0.05) and controls (n=12; p<0.005). (B) Week 2 mobility analyses revealed no significant overall effect of treatment.
3.4.3 VOMA produces acute spatial working-memory deficit not present during abstinence

Working memory assessments were run prior to and after VOMA. Baseline working memory assessments were used to balance groups into either VOMA or control. A subset of mice for used to test working memory performance over the course of the study (n=11 control, n=13 low consumers and n=8 high consumers). Baseline performance for these mice shows no appreciable difference between groups (Fig 13A; 2days, 1 trial per day). A Two-way ANOVA of WM Baseline performance between control mice and Low Consumers revealed a significant effect of bait (F1,22=158.6; p<0.001) and no significant effect of treatment. A Two-way ANOVA of WM Baseline performance between control mice and High Consumers revealed a significant effect of bait (F1,17=107.8; p<0.001) and no significant effect of treatment. A Two-way ANOVA of WM Baseline performance between High Consumers and Low Consumers revealed a significant effect of bait (F1,19=133.3; p<0.001) and no significant effect of treatment. Directly following VOMA, 1 WM trial was run for all mice (Fig 13B). A Two-way ANOVA of WM performance after VOMA between control mice and Low Consumers revealed a significant effect of bait (F1,22 =82.49; p<0.001) and no significant effect of treatment. A Two-way ANOVA of WM performance after VOMA between control mice and High Consumers revealed a significant effect of bait (F1,17 =72.03; p<0.001) and a significant effect of treatment (F1,17 =5.096; p<0.05). Post-hoc comparisons of performance between groups in each bait-group revealed a marginal difference between groups on baits 5-8 (t34=2.194, p=0.07). A Two-way ANOVA of WM performance after VOMA between High Consumers and Low Consumers revealed a significant effect of bait (F1,19 =154.4; p<0.001) and a significant effect of treatment (F1,19 =6.176; p<0.05). Post-hoc comparisons of performance between groups in each bait-group revealed a significant difference between groups on baits 5-8 (t38=2.46, p<0.05). Following a 4-
week abstinence period, 1 more WM trial was run for all mice (Fig 13C). A Two-way ANOVA of WM performance between control mice and Low Consumers revealed a significant effect of bait (F1,22 =61.27; p<0.001) and no significant effect of treatment. A Two-way ANOVA of WM performance between control mice and High Consumers revealed a significant effect of bait (F1,17 =50.48; p<0.001) and no significant effect of treatment. A Two-way ANOVA of WM performance between High Consumers and Low Consumers revealed a significant effect of bait (F1,19 =34.69; p<0.001) and no significant effect of treatment.
Figure 12. Working Memory Assessment. Working memory performance was tracked for a subset of mice across the study. (A) Baseline performance is not different between treatment groups. (B) After VOMA, 1 WM test revealed that High consumers performed significantly worse than control and low consumers (*). (C) After 4 weeks of abstinence, WM performance was no different between treatment groups.
3.4.4 Abstinence does not affect RAM spatial learning

A subset of mice (n=6 control, n=5 low consumers and n=6 high consumers) were chosen to undergo further cognitive testing on the RAM. Mice underwent spatial-reference memory training over a 10d period (Fig 14A). A Two-way ANOVA of learning performance between control mice and Low Consumers revealed a significant effect of day (F9,81=12.96, p<0.001) but no significant effect of treatment. A Two-way ANOVA of learning performance between control mice and High Consumers revealed a significant effect of day (F9,72=12.24, p<0.001) but no significant effect of treatment. A Two-way ANOVA of learning performance between High Consumers and Low Consumers revealed a significant effect of day (F9,63=11.06, p<0.001) but no significant effect of treatment. A Two-way ANOVA of reference-memory errors between control mice and Low Consumers revealed a significant effect of day (F9,81=21.12, p<0.001) but no significant effect of treatment (Fig 14B). A Two-way ANOVA of reference-memory errors between control mice and High Consumers revealed a significant effect of day (F9,72=14.43, p<0.001) but no significant effect of treatment. A Two-way ANOVA of reference-memory errors between High Consumers and Low Consumers revealed a significant effect of day (F9,63=11.08, p<0.001) but no significant effect of treatment. A Two-way ANOVA of working memory errors control mice and Low Consumers revealed a significant effect of day (F9,81)=3.739, p < .001), no significant effect of treatment, and a significant interaction of day and treatment effect (F9,81=2.408, p<0.05). Post hoc comparisons found no significant differences between groups. A Two-way ANOVA of working memory errors control mice and High Consumers revealed a significant effect of day (F9,72)=5.888, p < .001) but no significant effect of treatment. A Two-way ANOVA of working memory errors High Consumers and Low
Consumers revealed a significant effect of day \((F_{9,63} = 4.236, \, p < .001)\) but no significant effect of treatment (Fig 14C).
Figure 13. Reference Memory Assessment. (A) Reference memory assessment shows an overall effect of day within first five days of learning but not within the last five days of learning, after running a Two-way ANOVA. This indicates a more drastic increase in learning in the first five days compared to the last four. (B) All mice showed a gradual decrease in reference errors over time that was not significant between groups. (C) Working memory errors made throughout 19d of training were not different between groups. Also gradually decreased, indicating a general improvement of memory and performance throughout the experiment.
3.4.5 Abstinence does not affect RAM retention memory

Eight (8) days after the end of the RAM reference-training phase, mice were put back on the maze to complete a retention test of the memory learned during the training period within 3 trials. A One-way ANOVA of the performance on the RAM revealed no significant differences between groups (Fig 15A). A One-way ANOVA of the reference-memory errors revealed no significant differences between group (Fig 15B). A One-way ANOVA of the working-memory errors revealed no significant differences between groups (Fig 15C). These results highlight the behavioral resilience and plasticity that subjects experience during abstinence.
Figure 14. Retention Memory Test. Eight (8) days after the end of RAM training, a retention test was run for all mice. (A) Performance on the tests was not significantly different between the treatment groups. (B) Reference memory errors during the retention test were not significantly different between the treatment groups. (C) Working memory errors during the retention test were not significantly different between the treatment groups.
3.4.6 Abstinence upregulates neuroinflammation in the hippocampus

Western blot analyses of hippocampal tissue followed. A One-way ANOVA of GFAP revealed an overall effect of group (F4,16 =5.734; p<0.01) and controlled post hoc analyses revealed that the high consumption group significantly decreased GFAP compared to controls (*; p<0.05) and that abstinence in the same consumption group brought GFAP levels closer to control (Fig 16A). A One-way ANOVA of COX-2 across groups revealed an overall effect of treatment (F4,16 =8.897; p<0.001) and controlled post hoc comparisons revealed significant differences between control mice and the high consumption mice that underwent abstinence (*; p<0.01) (Fig 16B). These results highlight the progression of neuroinflammation in the brain that occurs during abstinence.
Figure 15. Neuroinflammation increases after prolonged abstinence following VOMA. (A) VOMA decreases GFAP levels in the hippocampus of high consumers and abstinence modulates its levels closer to control. (B) Abstinence increases COX-2 levels significantly in high consumers compared to other treatment groups.
3.4.7 Abstinence decreases DAT and increases 5-HT2a in the hippocampus

A One-way ANOVA of hippocampal DAT revealed an overall effect of treatment (F4,14 =5.902; p<0.01) and post hoc analyses revealed a significant difference between control mice and High consuming mice that underwent abstinence (**; p<0.01) (Fig 17A). A One-way ANOVA of hippocampal 5HT2A revealed an overall effect of treatment (F4,13 =17.4; p<0.001) and post hoc analyses revealed a significant difference between control mice and High consuming mice that underwent abstinence (**; p<0.01) (Fig 17B). These results highlight the progressive dysregulation of monoamine protein expression that occurs as a result of abstinence.
Figure 16. Synaptic Monoamine proteins are modulated after prolonged abstinence. (A) Hippocampal DAT decreases appreciably directly in high consumers after VOMA but significantly decreases after a prolonged abstinence period. (B) Hippocampal 5HT2a does not change following VOMA, but abstinence increases its expression significantly in high consumers.
3.5 Discussion

3.5.1 Acute VOMA models naturalistic MA abuse

To investigate the progression of behavioral, and neurochemical changes produced by MA and abstinence, we used the VOMA model to put mice through 14 days of MA administration prior to starting abstinence. High consumers averaged 2.36 mg/kg per day (33.15 mg/kg total over 14d). Low consumers averaged 1.39 mg/kg per day (19.4 mg/kg total over 14d). Additionally, both groups’ consumption rates dropped from week 1 to week 2, suggesting that this model produced declining MA preference. This claim is supported by our data showing different sensitization-induced mobility across the drug administration weeks (Fig 12). The distribution of Low and High MA consumers in this study confirms our hypothesis that VOMA can model naturalistic MA abuse. Recent studies have begun to delineate the genetic risks associated with MA preference\textsuperscript{201-202}. However, we cannot discount environmental factors within our study design affecting MA preference. Pavlovian conditioning studies in humans have shown that extinction procedures can quickly decrease preference for MA\textsuperscript{203}, while the number of drug presentation only modestly increase drug-induced responding\textsuperscript{204}. Thus, more work is needed to understand the variables that produce various rates of drug consumption within VOMA.

3.5.2 Behavioral changes following MA abuse

Our behavioral analyses following VOMA revealed that High consuming mice exhibited an acute spatial working-memory deficit directly after the drug administration period but not during abstinence. This suggests that the cognitive deficits induced by 14 days of VOMA are only transient and further, that the adolescent mouse brain has sufficient plasticity to overcome this deficit. This claim is strengthened with the data from the spatial learning paradigm where MA
mice showed no reference memory or retention deficits during a prolonged abstinence period (Fig 14). Future studies should compare the long-term cognitive effects of this VOMA design on adult vs adolescent mice. Previous studies have shown that MA administration can produce cognitive deficits after 1 or 3 weeks of abstinence following 16mg/kg MA administered over an 8hr period\textsuperscript{104}, \textsuperscript{113} and throughout 3 weeks of abstinence following 14 days of 24mg/kg MA administration per day (but not directly after MA administration)\textsuperscript{114}. Additionally, a comparative study examining the effects of 13 days of escalating doses of MA on single-day binge MA (up to 16mg/kg MA) vs a single-day binge alone showed that the addition of escalating MA doses over multiple days prevented cognitive deficits produced by binge MA after 1 week of abstinence\textsuperscript{74}. A previous experiment from our lab observed cognitive deficits following MA administration after 7 weeks of abstinence following 2x 30mg/kg MA doses (but not before)\textsuperscript{1}. Thus, it seems that MA can negatively affect cognition depending on the MA administration design and the timing of assessments following MA administration. 14 days of VOMA may prove insufficient to produce any robust cognitive deficits within the timeline of the present study, as the administration design spreads out doses over many hours per day, over 2 weeks, similar to previous studies using escalating MA doses\textsuperscript{74, 205}. Nevertheless, having observed neurochemical changes after 4 weeks of abstinence in the present study, the cognitive deficits could have developed shortly after the tissue was collected. Future studies should characterize the cognitive performance of mice under this model at later timepoints, comparable to those of previous studies\textsuperscript{1}.

3.5.3 DAT as a clinical marker of interest for MA abuse and abstinence

Our current study shows that VOMA decreases DAT in High consumers that received abstinence. High consumers that received no abstinence before tissue collection showed decreased DAT levels.
but not to the extent of abstinent mice. These results indicate that abstinence plays a major role in the reduction of DAT levels following VOMA. Our previous studies showed that acute neurotoxic doses of MA (2x 30 mg/kg) followed by 6 weeks of forced abstinence produce long-lasting decreases in dopamine marker expression in the hippocampus. Previous studies of DAT availability in the brains of human MA users show recovery of DAT levels as well as sustained decreases in levels during abstinence. Reduction in dopamine terminals and transporters are known to be an increased risk-factor for Parkinson’s Disease, suggesting that VOMA can model neurodegenerative changes in the brain. Decreases in DAT levels can indicate decreases in DA terminal function and density in the brain. We hypothesize that decreased DA availability following MA abuse can lead to activation of compensatory mechanisms, such as increased 5HT2a-dependent DA release. Previous studies indicate that 5HT2a crosstalk with DA pathways can affect synaptic plasticity, learning and memory. However, the role of 5HT2a upregulation during abstinence following VOMA is unclear and should be explored in future research. Additionally, MA can increase astrocytosis via DAT-dependent neurotoxicity, as mice lacking the transporter do not show increased GFAP mRNA or immunostaining. In the present study, GFAP levels are decreased after VOMA and return to control levels after abstinence (Fig 16), suggesting that astrocyte-dependent neurotoxicity was attenuated in these mice. Previous research shows that GFAP levels increase following MA exposure, within a week after a bolus 30mg/kg MA dose and following a 5-day, 3x daily binge of 5mg/kg MA, with concomitant decreases in DAT levels. VOMA may produce a delayed and longer-lasting shift in astrocyte activity that could polarize glial signaling to the neurotrophic/A2 end of the spectrum, resulting in partial neuroprotection of DA terminals in the long-term. Future research should
characterize the effects of A1 and A2 astrocyte signaling on DAT levels and DA terminal health following MA exposure.

3.5.4 5HT2a signaling in MA abuse

Our study also reveals that abstinence increases 5HT2a levels in the hippocampus. In the context of drug addiction, 5HT2a is known to play a major role in the development of cocaine sensitization, and chronic MA upregulates 5HT2a in the medial prefrontal cortex, perirhinal cortex, and dorsal hippocampus, brain regions pivotal to executive function and memory. Current studies on the potential of 5HT2a as a pharmacological target within studies of MA abuse are inconclusive, but previous work has shown that this receptor underlies the reinforcing properties of MDMA. In the 5HT2a receptor is also known to play roles in learning and memory as well as aberrant cognitive processes as seen in psychiatric disorders. Whereas presynaptic 5HT2a may regulate neurotransmitter release, post-synaptic localization may underlie pivotal molecular signaling needed to promote learning and memory, such as diacylglycerol-IP3 and ERK pathways. The 5HT2a-dependent signaling that follows chronic MA abuse has not been explored. However, the increased levels of 5HT2a observed during a long-term abstinence in the current study suggest that the changes on this receptor may underlie the behavioral performance observed on the RAM tasks. The result also suggests that these mice may have developed a latent re-sensitization during abstinence as a result of the increased 5HT2a. Future studies should characterize the changes in this receptor in addiction-related brain regions, such as the NAc, PFC and others. Additionally, several lines of evidence have linked auto-receptor, 5HT1b, function to MA addiction. We hypothesize that in the context of chronic voluntary MA abuse, 5HT2a-dependent drug sensitization is
modulated in direct response to 5HT1b auto-receptor activity. This idea is consistent with preliminary, unpublished data from 5HT1b KO VOMA studies showing that female KO mice consume less MA over 28 days compared to male KO mice (Appendix I). This result suggests that 5HT1b KO mice have a loss of auto-receptor function in the brain that may require less MA to produce euphoric effects and/or that these mice must adopt increased self-regulation due to aversive effects of MA abuse. Future studies should examine the effects of VOMA on 5HT2a expression in these mice across sexes.

3.5.5 Chronic neuroinflammatory pathways in the context of MA

Analyses of COX-2, the inducible pro-inflammatory enzyme responsible for downstream inflammatory activity, revealed that High consumers that received 4-weeks of abstinence showed significantly increased levels compared to controls. Non-abstinent mice showed comparably lower COX-2 levels compared to controls. Unpublished data from studies using bolus (60mg/kg total) MA have revealed that 7 weeks of abstinence upregulates COX-2 and ubiquitin expression in the hippocampus (Appendix II). These data suggest that inflammation might be exacerbated during long periods of abstinence, despite not showing differences shortly after MA exposure. MA-induced neurotoxicity can activate microglia early on after MA abuse, that can induce inflammatory molecular signaling detrimental to neuronal function (reviewed in45). Acute neuroinflammation is essential to neuronal survival, but repetitive activation of neuroinflammatory responses may contribute to neurodegenerative processes, as seen in many neurodegenerative disease models. Pro-inflammatory pathways include cyclooxygenases, specifically COX-2 which produces neurotoxicity after MA abuse. COX-2 upregulation can form a chronic inflammatory feedback loop with prostaglandin J2 (PGJ2). PGJ2 is a highly neurotoxic
prostaglandin, as it impairs both the ubiquitin-proteasome pathway and mitochondrial function, leading to aggregation of ubiquitinated proteins and failure of protein turnover. This mechanism provides one pathway that neuroinflammation can transition from acute to chronic states. Previous work using our VOMA model investigated the changes in prostaglandin levels in mice hippocampi. Mass spectrometry analyses revealed that a cohort of male C57 mice that consumed an average total of 25.8 mg/kg MA over 14 days of VOMA showed increased PGJ2 levels in the hippocampus after 4-weeks of abstinence. In the same study, mice that were injected with 60 mg/kg MA over 2 weeks also showed increased PGJ2 levels in the hippocampus after a prolonged abstinence period. Previous studies have shown that chronic unpredictable stress and MA can interact to significantly increase COX-2 in the cerebral vasculature within 1 week of abstinence. Recent evidence indicates that microglial and COX-2 play major roles in dopaminergic neurotoxicity 1 week after an ethanol and MA challenge. Our current study reveals that even low doses of VOMA over 2 weeks can produce a delayed increase in COX-2, observed 4 weeks into abstinence. This delayed neuroinflammatory response may reflect a latent susceptibility to MA-induced changes. Future studies should investigate the effects that increased COX-2 may have on parallel immune pathways such as astrocyte activity, which is known to participate in crosstalk with the primary proinflammatory mediators, microglia.

3.5.6 Neuroglial activity following MA abuse

Finally, analyses of GFAP expression, a marker of astrocyte activation, revealed that VOMA significantly decreases its levels and that abstinence brings its expression comparably closer to baseline levels (Fig 16). MA-induced reductions of DAT may indicate degeneration of DA axonal terminals. This explanation is supported by increased activation of astrocytes associated with
MA treatment. Increases in GFAP have been observed at 24h post neurotoxic MA administration and decrease 7 days after injection. Increases in GFAP may indicate a regenerative response in the brain, and suggests that this neuroinflammatory response works to regenerate MA-induced neurochemical deficits. Previous work on MA-induced effects on neuroglia have found that acute MA can induce a polarized neurochemical activation in microglia to M1, or proinflammatory, while decreasing their M2, or neurotrophic, activity. Similarly, two types of astrocyte populations have been reported during neuroinflammation: A1 astrocytes are pro-inflammatory and neurotoxic, and A2 astrocytes secrete neurotrophic factors to promote neuroprotection. Previous work in brain injury models indicate that various pathways could promote neuroprotective activity by astrocytes. Future research should elucidate the expression of A1 and A2 astrocyte populations following VOMA and abstinence.

3.6 Conclusion and Clinical Implications

Our study characterizes a time-course of behavioral and molecular deficits that occur following voluntary oral administration of methamphetamine. Our VOMA study provides evidence that low doses of voluntary MA do not produce long-lasting behavioral deficits. Neurochemical analyses showed that VOMA produced only a significant decrease in GFAP levels. Tissue collected after 1 month of abstinence revealed more changes in monoamine protein and neuroinflammatory marker expression. These results suggest that despite receiving low doses of MA, VOMA can model long-lasting neurochemical changes that may reflect susceptibilities to neurodegenerative processes present during abstinence. Our data provide new avenues of research to investigate the neurochemical changes that ensue during abstinence periods, and its effects on behavior and cognition in-vivo.
CHAPTER 4: ADOLESCENT FEMALE MICE SHOW MEMORY DEFICITS AND GREATER VULNERABILITY TO CHRONIC METHAMPHETAMINE NEUROTOXICITY THAN MALE ADOLESCENTS

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To be submitted to Neuroscience as: Jorge A. Avila, Nicoletta Memos, Abdurrahman Aslan, Tytus Andrejewski, Maria E. Figueiredo-Pereira, Victoria N. Luine, & Peter A. Serrano (submission in 2019). Adolescent female mice show memory deficits and greater vulnerability to chronic methamphetamine neurotoxicity than male adolescents
4.1 Abstract

Studies show that females exhibit increased susceptibilities to psycho-stimulant addiction compared to males. Additionally, adolescence may contribute to female-specific vulnerabilities to addiction. However, little is known about the underlying mechanisms in the brain that produce this vulnerability in adolescent female populations. To model methamphetamine addiction, we used a mouse model for voluntary oral methamphetamine administration, (VOMA). Previous work suggests VOMA can model voluntary consumption that mirrors human binge patterns over 28 consecutive days, unlike other models that utilize bolus doses over shorter periods of time. Additionally, our current study used an escalating-dose regimen over the first 10d to maximize the administration rate over the remaining 18d of VOMA. Over the course of 28d, we found that male mice consumed an average 4.67 mg/kg/day over 28d and female mice consumed an average 5.84 mg/kg/day over 28d. In order to evaluate differential neurotoxic effects of MA between sexes, we focus on medium consumers of MA whose consumption rates across both sexes were not significantly different: male mice consuming an average 7.68 mg/kg/day over 28d and female mice consuming an average 8.2 mg/kg/day over 28d. Following VOMA, behavioral assessments revealed that VOMA did not alter depression in the tail suspension task but decreased anxiety in both sexes on the elevated plus maze (EPM). VOMA females, but not males, showed increased exploratory behavior on the EPM. In a hippocampal dependent working memory task (radial arm maze), adolescent female, but not male, VOMA mice show working memory deficits. The sex-specific behavioral changes occurred while both sexes voluntarily consumed equivalent amounts of MA. The behavioral results occur in tandem with sex-specific neurochemical changes in the hippocampus, specifically involving glycogen synthase kinase-3 beta (GSK3β)-associated molecular activity. Male, but not female, adolescent
mice exhibit neuroprotective molecular changes in response to VOMA including increases in dopamine receptor 1 (D1), estrogen receptor alpha (ERα), phosphorylated Akt (pAkt), and pGSK3β/GSK3β ratio. Further, females show an increase in D-prostanoid receptor 1 (DP1) levels and a concomitant increase in kappa opioid receptor (κOR) in the nucleus accumbens (NAc) suggesting a response to increased neuroinflammation that males do not exhibit. These results suggest that an escalation MA administration protocol may potentiate the sex-differences in neurochemical resilience observed in male, but not female adolescent mice. The results further indicate that female mice show molecular deficits in the brain compared to males. Our study reveals that female mice show specific susceptibilities to methamphetamine challenges and offer potential molecular mechanisms for this phenomenon.
4.2 Introduction

Methamphetamine is a highly addictive psychostimulant, showing higher rates in adolescent females compared to adult female lifetime users. Clinical reports indicate that females begin using MA recreationally at a younger age, and transition more quickly to MA addiction that leads to intravenous use compared to males. Female MA users also reported greater severity of drug use and psychological burden than males, including increased rates of depression, psychosis, and suicide. In males, but not females, drug craving was correlated with depression and anxiety. Recent reports show females have sustained vulnerability to the neurotoxic effects of MA following prolonged abstinence, showing greater reductions in hippocampal volumes and wide-spread reduction in grey matter compared to control females, whereas no difference was observed between METH-abusing and control male subjects.

Rodent studies have also identified sex-differences in MA self-administration. Female rats show accelerated MA self-administration compared to males and more rapidly reinstated drug-seeking compared to males. While few studies have identified signaling mechanism associated with sex-dependent drug seeking behaviors, even fewer studies have identified the neurochemical effects of long-term or chronic MA on adolescent male and female mice. Therefore, the current study investigated the chronic behavioral and neurochemical consequences of voluntary oral methamphetamine administration (VOMA) in adolescent male and female mice.

Our study focuses on select signaling pathways and synaptic markers that are known to be selectively affected by MA. Previous studies have identified acute and chronic deficits in
dopamine related markers following binge and VOMA models 118, 189, 258. Thus, we examined the
effects of the dopamine transporter (DAT), the dopamine precursor tyrosine hydroxylase (TH)
and the dopamine receptor 1 and 2 (D1, D2) in hippocampus following chronic MA. The effect
of neurotoxic or binge dosing of MA shows increased susceptibility in males than females as
observed by more extensive striatal DA reduction and larger decreases in DAT 78, 259-261. These
studies suggest a role for estrogen as an underlying neuroprotective mechanism and are
consistent with the report on BALB/c mice showing greater MA-induced vulnerability in females
during diestrus (low estrogen) compared to proestrus (high estrogen) 260. Based on these
findings, we evaluated the long-term consequences of chronic VOMA on ERα and its
downstream activation of Akt and GSK3β signaling pathways. Since MA produces different
effects on dopaminergic markers in female and male mice, these findings provide important new
information related to the signaling pathway differences in toxicity induced by chronic MA
exposure. To examine potential dysphoric effects between males and females during 2 weeks of
abstinence after chronic 28d MA, we evaluated the expression of the κ-opioid receptor (κOR)
and D1 in the NAc.

Additionally, our study focuses on the sex-differences in hippocampal molecular signaling.
Previous studies have revealed that rodents activate distinct molecular cascades across the sexes
following hippocampal-dependent spatial training 262-263. Available evidence indicates that
female spatial learning is influenced greatly by fluctuations in gonadal hormone availability
throughout the estrous cycle, with optimal performance observed during the proestrus phase 264,
and is thought to be driven by proliferative molecular signaling in the hippocampus 264. More
recently, it has been shown that female mice exhibit heightened corticosterone responses
following MA challenges compared to males, driven by hippocampal and hypothalamic HPA-axis dependent glucocorticoid signaling. These findings suggest that females may be specifically vulnerable to MA-induced cognitive deficits and neurochemical shifts.

We focus our analysis on groups of males and females that voluntarily consumed equivalent amounts of MA. Our results show that following 28d VOMA, females show a deficit in spatial working memory performance, but males do not. VOMA males show increased hippocampal D1 with a concomitant increase in ERα and enhanced downstream signaling including pAkt and the pGSK3β/GSK3β ratio, but females do not. VOMA females show elevated D-prostanoid receptor 1 (DP1) and a decrease in DP2 suggesting elevated neuro inflammation. In the NAc, females show lower levels of D1 and increased κOR in VOMA females compared to female controls. These findings highlight the differential effects and elevated vulnerability of the adolescent female to the neurotoxicity of chronic MA and possibly enhanced dysphoric effects during abstinence which contribute to our understanding of the neural mechanisms that may underlie differential vulnerabilities to MA between sexes.

4.3 Methods

4.3.1 Animals

Male (n=20) and female (n=20) C57/bl6 mice were purchased from Taconic Biosciences and were received at Hunter College’s Animal facility at 6 weeks of age. Mice were individually
housed, kept on a 12h/12h light/dark cycle from 7:30h to 19:30h, and fed ad libitum for 1 week prior to handling.

4.3.2 Food restriction

At the start of their 2nd week in the facility, mice were transitioned from *ad libitum* food to being fed daily rodent chow that weighed 20% of the average body weight (approx. 4g chow for males and 3.5g chow for females per day). For RAM assessments, weights were recorded for 5d and diets were restricted until mice reached 85% of their baseline BW. Working memory assessment on the 8-arm Radial Maze, lasted 4d total with shaping and baseline assessments included. Following RAM memory assessments, mice were fed daily rodent chow that weighted 20% of the average BW. Feeding mice daily chow weighing 20% of their BW allowed for a continuous increase in BW reflecting growth (Fig 1).

4.3.3 Radial 8-arm Maze Shaping/Baseline

The radial 8-arm maze (RAM) was used to assess spatial working memory. The maze consists of a center platform (15.24 cm diameter) with 8 equivalently sized arms radiating outward. Each arm is 38 cm in length, 6.35 cm wide with a submerged food cup (2.0 cm diameter) at the end of each arm. Maypo (Homestat Farm, Dublin, OH) was mixed in water to make a wet mash that that was used as a food reward (0.02 g portions), as previously described for mice 189 and rats 88. On day 1 of RAM shaping, mice were placed on the RAM for 5 min to acclimate to the maze and room cues, with food baits available around maze and inside food cups. On day 2 of shaping,
mice were given 15 min each to explore the maze and the food baits on ends of maze-arms and inside cups. On days 3 and 4 of shaping, mice were given 15 min each to collect small baits at the ends of arms. Performance on days 3 and 4 was recorded and used to balance groups across conditions based on baseline RAM performance.

4.3.4 Working Memory Assessment

After 4d of abstinence mice underwent working memory assessments (WMA). Mice were tested over 2 days (1 trial/day). Each trial started with all food cups baited. To begin each trial, mice were confined for 30s to the center platform with a plastic cylinder. The sequence of arms entered to retrieve the food rewards was recorded. To prevent a non-spatial strategy, chaining, mice were allowed to collect baits from up to 3 sequential arms before the experimenter interrupted the chaining strategy. Working memory errors were recorded as re-entries into arms where the food reward has already been collected. Maximum latency for each trial was set at 15 min.

4.3.5 Tail Suspension

After 5 days of abstinence, mice underwent behavioral assessments. To assess for depressive phenotypes, we used the tail-suspension test. Mice were hung by their tails and suspended three feet in the air. Mice were kept suspended for six minutes and videos were recorded. Video scorers were blinded to the animals’ experimental condition. Scorers tracked and recorded the
time spent immobile for each mouse. Immobility was considered any lack of voluntary taxis or body movements, including the torso, limb, or head.

4.3.6 Elevated Plus Maze

After 7d of abstinence, mice were placed on the elevated plus maze (EPM) assessment. This behavioral assay assesses anxiety and related behavioral phenotypes. Lighting conditions on and around the EPM inside the testing room were kept consistent for all mice. Lux was maintained high (average 200 lux on and around EPM) to measure for anxiolytic effects of chronic MA exposure. Following a 15-minute acclimation to the testing room, each mouse was placed onto the center of the EPM, facing an open arm opposite from the experimenter. The mouse was allowed to explore the EPM for 8 minutes. Videos were recorded for each mouse. Video scorers quantified the number of open and number of closed arm entrances for each mouse. Arm entries were scored when all four mouse paws crossed into the 75% remaining length of an arm (from the center to the end).

4.3.7 MA formulation

A stock solution 40mg/mL of methamphetamine hydrochloride (Sigma Aldrich) was formulated using de-ionized water. Daily body weights were used to calculate working volumes of Ensure-MA solutions for each weight class. From 40mg/mL stock, Ensure-MA solutions were delivered to mice in 7μL containing a maximum dose of 1mg/kg bw MA.
4.3.8 Voluntary oral methamphetamine administration

A cohort of 40 mice (n=20 per sex) were assigned into drug and control groups, balanced across treatments with bw and baseline working memory performance. VOMA groups contained n=14 mice per sex and Control groups (no MA, Ensure only) contained n=6 mice per sex. On the 15th day following their arrival, mice began the Escalation phase of VOMA: 3 days of 1 dose/day at 0.25 mg/kg MA per bait; 3 days of 4 doses/day at 0.25 mg/kg MA per bait; 2 days of 16 doses/day at 0.25 mg/kg MA per bait; and 2 days of 16 doses/day at 0.5 mg/kg MA per bait. From day 11 to day 28 of VOMA, mice received 16 doses/day at 1 mg/kg MA per bait. Doses of MA were delivered at 15 min intervals for a total period of administration lasting 4 h. Following each bait presentation, the number of consumed bait was recorded for each animal. Control mice had untreated Ensure delivered at the same bait/hour/day rate as MA treated mice.

4.3.9 Tissue sample collection and fractionation

Following 28d of VOMA and 15 days of abstinence where behavioral testing was done, hippocampal and nucleus accumbens (NAc) tissue from all animals were collected on day 15 of abstinence. Tissues were flash frozen on dry ice. To fractionate tissue into cytosolic and synaptic fractions as previously reported. Briefly, tissues were thawed from frozen and homogenized in a TEE (Tris 50 mM; EDTA 1 mM; EGTA 1 mM) buffer containing a SigmaFast, protease inhibitor cocktail (Sigma Aldrich) diluted to contain AEBSF (2 μM), Phosphoramidon (1 μM), Bestatin (130 μM), E-64 (14 μM), Leupeptin (1 μM), Aprotinin (0.2 μM), and Pepstatin A (10
μM). Tissues were homogenized in 200 μl of the TEE-homogenization buffer using 20 pumps with a motorized pestle. Homogenates were transferred to Eppendorf tubes and centrifuged at 3,000 g (5 min at 4°C), to remove unhomogenized tissue. The resulting supernatant was centrifuged at 100,000 g for 30 min. After ultracentrifugation, the supernatant was collected and stored as the cytosolic fraction. The remaining pellet was resuspended in 100 μl of homogenizing TEE buffer containing 0.001% Triton X-100, incubated on ice for 1 h and then centrifuged at 100,000 g for 1 h at 4°C. The resulting pellet was resuspended in 50 μl of TEE buffer and stored as the synaptic fraction. The Pierce bicinchoninic acid assay (BCA) (Thermo Scientific, Rockford, IL) was used to determine protein concentration for each sample. Samples were reduced with 4x Laemmli sample buffer equivalent to 25% of the total volume of the sample and then boiled and stored frozen at -80°C.

4.3.10 Protein quantification and western blot assessments

Samples (20 μg) were loaded onto a Tris/Glycine 4-20% midi gel to resolve gapDH (36 kDa), D1 (49 kDa), D2 (50 kDa), TH (60 kDa), DAT (69 kDa), ERα (66 kDa), Akt (56 kDa), phosphorylated s473-AKT (60 kDa), GSK3β (46 kDa), phosphorylated s9-GSK3β (46 kDa), PKCι/λ (68 kDa), PKMζ (55 kDa), COX-2 (72 kDa), DP1 (46 kDa), DP2 (~64 kDa), and κOR (~58 kDa). Every gel contained 3-4 lanes loaded with the same control sample, (all brain samples, ABS). ABS were used to standardize protein signals between gels. Gels were transferred to nitrocellulose membranes in IBlot® Dry Blotting System (Life Technologies; Carlsbad, CA) for 9 minutes. Nitrocellulose membranes were then incubated in blocking solution containing 5% sucrose in Tris Buffered Saline with Tween-20 (TBST; 0.1% Tween-20 in TBS).
for 30 minutes at room temperature. Samples were incubated with the following primary antibodies for 18-36hrs: gapDH (1:2000; Abcam Inc., Cambridge, MA, USA), D1 (1:2000; Abcam Inc., Cambridge, MA, USA), D2 (1:2000; Abcam Inc., Cambridge, MA, USA), TH (1:2000; EMD Millipore Corp., Billerica, MA, USA), DAT (1:2000; EMD Millipore Corp., Billerica, MA, USA), ERα (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA), Akt (1:2000; Cell Signaling Technology, Danvers, MA, USA), phosphorylated s473-AKT (1:2000; Cell Signaling Technology, Danvers, MA, USA), GSK3β (1:2000; Cell Signaling Technology, Danvers, MA, USA), phosphorylated s9-GSK3β (1:2000; Cell Signaling Technology, Danvers, MA, USA), PKMζ/PKCι/λ (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA), COX-2 (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA), DP1 (1:500; Cayman Chemical, Ann Arbor, Michigan, USA), DP2 (1:2000; ThermoFisher Scientific, Waltham, MA, USA), and κOR (1:2000; Abcam Inc., Cambridge, MA, USA). Membranes were washed in TBST for 20 min and probed with Horseradish Peroxidase (HRP) conjugated secondary antibody. Membranes were incubated with Enhanced Chemiluminescence (ECL) substrate and exposed on CL-XPosure Film (Thermo Scientific; Rockford, IL). GapDH was used as a control to standardize for protein concentration loaded on gels. Films were scanned for quantification with NIH Image J.

4.3.11 Statistical analyses

For behavioral and molecular analyses, a 1-way ANOVA across treatment groups 2-way ANOVA (sex x drug treatment) were used (Prism GraphPad 6.0 Statistical Package, La Jolla, California). Post-hoc analyses used controlled comparisons Bonferroni-corrected t tests.
4.4 Results

4.4.1 Behavioral Shaping

Analyses of RAM baseline and post-VOMA performance revealed one female mouse as an outlier from the rest of the group (z-scores 1.6 SDs and 1.5 SDs, respectively) and thus is not included for subsequent behavioral or molecular assays; total female control n=5. A timeline is provided to depict the duration of and the description for each phase of in-vivo assessments, to include RAM assessments and VOMA [Fig18A].

4.4.2 Voluntary Ensure Consumption

Across 28d of VOMA, n=6 male and n=5 female mice underwent control exposure to the Ensure, vehicle. For the first 3 days, mice were exposed to a total of 1 Ensure bait per day. For the next 3 days, mice were exposed to a total of 4 baits per day at 1 bait every 15min. From Day 7 onward, control mice received 16 Ensure baits per day at 1 bait every 15min. Over the course of the 28d VOMA, control mice ate all Ensure baits. There were no differences in Ensure consumption between control male and control female mice [Fig 18B].

4.4.3 Voluntary Oral Methamphetamine Consumption

Having placed n=14 mice per sex on VOMA (total n=28) [Fig18C], we observed that approximately 2/3 [n=9 mice per sex] of mice fell into a consistent and equivalent administration
rate, and that the remaining 1/3 of mice consumed significantly different (higher and lower vs the average consumer). A two-way repeated measures ANOVA of MA consumption between Low male VOMA consumers [n=5; 2.611 mg/kg/day over 28d +/-0.39 SEM] and medium male VOMA consumers [5.811 mg/kg/day over 28d +/-0.59 SEM] over 28d of VOMA showed a significant overall effect of consumption rate [F_{1,12}=15.46, p<0.01]. A two-way repeated measures ANOVA of MA consumption between Low female VOMA consumers [n=2; 1.283 mg/kg/day over 28d +/-0.15 SEM] and medium female VOMA consumers [6.01 mg/kg/day over 28d +/-0.62 SEM] over 28d of VOMA showed a significant overall effect of consumption rate [F_{1,9}=17.41, p<0.01]. A two-way repeated measures ANOVA between High female [n=3; 8.4 mg/kg/day over 28d +/-0.91 SEM] and average female consumers [see above for statistics] over 28d of VOMA showed a significant overall effect of consumption rate [F_{1,10}=5.719, p<0.05]. To compare equivalent rates of voluntary MA consumption between sexes, our study focuses on the behavioral and molecular results from the 2/3 of mice that displayed this average (medium) consumption rate across males and females.
Figure 17. Escalation VOMA model produces equivalent, sustained MA consumption in both male and female mice. The study lasted 8 weeks, with acclimation and shaping during weeks 1-2; VOMA during weeks 3-6; behavioral analyses during weeks 7-8 (A). Control mice expose to vehicle (Ensure) only, consumed all baits delivered over 28 days of the VOMA study design (B). Escalation VOMA produces equivalent consumption of MA across medium male (average 5.8mg/kg MA/day over 28d) and medium female mice (average 6.0 mg/kg MA/day over 28d) (C). A Two-way ANOVA within-subjects analysis revealed an overall interaction effect of sex and day but no significant post-hoc effects on any given day. From a cohort of 28 mice (n=14 from each sex) enrolled into VOMA, n=9 mice of each sex fell into the medium consumption group, while n=5 of each sex fell into high and/or low consumption groups. Body weight were tracked across all study weeks and a mixed model ANOVA revealed only overall sex and week differences between group body weights (D).
Following the escalation phase of VOMA, our analyses show that medium male mice consume an average of 7.68mg/kg MA/day (+/− 0.31 SEM) and medium female mice consume an average 8.2mg/kg MA/day (+/−0.12 SEM) over the last 18d of VOMA; 5.8mg/kg/day MA (+/−0.59 SEM) and 6.0mg/kg MA/day (+/− 0.62 SEM) over 28d for medium male and medium female mice, respectively. When analyzing daily MA consumption between average male and average female mice over the 28d VOMA regimen (Figure 18C), a two-way repeated measures ANOVA shows no significant overall effect between sexes (F_{1,16}=0.08, p=0.782), but both show a significant overall effect of day (F_{27,432}=56.6, p<0.001) and a significant overall interaction effect of sex and day (F_{27,432}=1.82, p<0.01). Upon closer analysis of the last 18d of VOMA, there was no significant overall effect between sexes (F_{1,16}=0.25, p=0.62) but a significant effect of day (F_{17,272}=4.569, p<0.001) and a significant interaction effect of sex and day (F_{17,272}=2.12, p<0.01). There were no post-hoc effects between groups.

### 4.4.4 Body weights

Body weights were tracked daily for all groups across all 8 weeks of the study [Fig 18D]. A repeated measures multivariate ANOVA of body weights across weeks revealed a significant overall effect of time (F7,27=133.1, p<0.001), a significant overall effect of sex (F1,33=150.1, p<0.001), no overall effect of MA (F1,33=0.697, p>0.5), no overall effect of MA dose by sex (F1,33=0.106, p>0.5), and a significant overall interaction effect of time by MA dose (F14,56=1.886, p=0.048) and a significant overall interaction of time by sex (F7,27=4.089,
p<0.01). These results indicate that within the study, body weights were affected more so in relation to time and were different between sexes. There is no strong indication that MA exposure affected the body weights of mice at any given point in the study. The overall interaction of time by MA dose is likely due to the lower body weights of High Female VOMA mice.

4.4.5 Tail suspension and Elevated Plus Maze Assessments

Possible effects of VOMA on depression were assessed by the tail suspension test. A two-way ANOVA of time spent immobile during the tail suspension test [Fig 19A], across sex and drug treatment, revealed no significant overall effect of sex (F1,25=2.67, p>0.1), no significant overall effect of drug (F1,25=0.7, p>0.4), and no significant overall interaction effect of sex by drug (F1,25=0.6, p>0.4). This result indicates that Escalation VOMA had no effect on eliciting depressive phenotypes in these mice. Effects of VOMA on locomotion and anxiety were examined on the EPM. A two-way ANOVA of closed-arm entries [Fig 19B] on the EPM revealed a significant overall effect of sex (F1,25=5.67, p<0.05), no significant overall effect of drug (F1,25=0.039, p>0.8), and no significant overall interaction effect of sex by drug (F1,25=0.5, p>0.4). Post-hoc analyses revealed a significant difference between male and female VOMA mice (t25=2.53, p<0.05). A two-way ANOVA of open-arm entries on the EPM [Fig 19C] revealed no significant overall effect of sex (F1,25=1.35, p>0.2), a significant overall effect of drug (F1,25=5.96, p<0.05) and no significant overall interaction effect of sex by drug (F1,25=0.006, p>0.9). These results show that VOMA increased exploratory and ambulatory
behavior in females (increased closed arm entries) while both sexes exhibited less anxiety following VOMA (increased open arm entries).
Figure 18. Escalation VOMA increases ambulatory behavior on the elevated plus maze.

Following VOMA, mice underwent the tail-suspension test and results revealed no effect of drug or sex on immobility in this assessment, indicating that VOMA does not produce a depressive phenotype in either sex (A). Analysis of elevated plus maze activity shows female VOMA mice were more ambulatory as seen in their number of entries into closed arms (B) and in analyzing the entries into open arms, VOMA mice had significantly more than control mice (C). (*p<.05)
4.4.6 Working Memory Assessment

Working-memory performance on the radial arm maze was analyzed across bait pairs, i.e. arms visited to collect the first set of baits (baits 1-2), second set baits (baits 3-4), third set of baits (baits 5-6) and fourth set of baits (baits 7-8). Within each bait-pair trial, the number of correct arms (always n=2) was divided by the total number of arms visited to collect the bait pair. A repeated measures three-way ANOVA of RAM performance between treatments (medium VOMA and control), sexes and across all bait pairs revealed an overall effect of bait pair (F3,75=106.4, p<0.001), no overall effect of treatment (F1,25=0.2018, p>0.6), no overall effect of sex (F1,25=0.32, p>0.5), no overall interaction effect of bait pair and sex (F3,75=2.12, p>0.1) a marginal overall interaction effect of bait pair and treatment (F3,75=2.65, p=0.055), no overall interaction effect of treatment and sex (F1,25=1.53, p>0.2) and no overall interaction effect of sex, treatment and bait pair (F3,75=0.6, p>0.6). To more closely examine group comparisons, two-way ANOVAS were run to compare RAM performance across sex and treatments. A repeated measures two-way ANOVA of VOMA mice performance [Fig 20A] for each bait pair revealed no significant overall effect of sex (F1,16=1.75, p>0.2), a significant overall effect of bait pair (F3,48=90.55, p<0.001) and a significant overall interaction effect of sex by bait (F3,48=3.48, p<0.05). Post-hoc analyses revealed a significant effect of sex between VOMA mice on bait-pair 5-6 (t64=2.94, p<0.05). A repeated measures two-way ANOVA of the performance of Control mice [Fig 20B] within each bait pair revealed no significant overall effect of sex (F1,9=0.31, p>0.5), a significant overall effect of bait-pair (F3,27=32.3, p<0.001) and no significant overall interaction effect of sex by bait (F3,27=0.35, p>0.79). Post hoc analyses revealed no significant differences between groups on any given bait trial. However, in
VOMA mice there was a significant difference between sexes midway during the assessment when the working memory load is heavy (bait pair 5-6) with females showing poorer performance than males. In addition, female mice exhibited a significantly greater number of working-memory errors committed during the RAM assessment, as a percent of controls, compared to males (t16=2.49, p<0.05) [Fig 20C]. These results highlight that Escalation VOMA affects working memory in females but not males.
Figure 19. Radial Arm Maze working memory assessments. The 8-arm radial arm maze working memory assessment was used to test the short-term memory capacities of mice following VOMA. VOMA produced sex differences in performance of baits 5-6 (of total 8), with male mice performing better than female mice (A). Control treatment showed no significant sex differences in performance throughout the assessment (B). Female VOMA mice exhibit increased percent working-memory errors compared to male VOMA mice (C). (*p<.05)
Whole hippocampi were processed for western blot analyses. Cytosolic and synaptic fractions were processed for dopamine marker expression, as MA is known to preferentially affect monoaminergic pathways like the dopamine system. All values were corrected for gapDH. A two-way ANOVA of synaptic DAT [Fig 21A] revealed no significant effect of sex (F1,14=3.6, p>0.05), or drug (F1,14=1.17, p>0.2), but a significant interaction effect of sex by drug (F1,14=6.19, p<0.05). Post-hoc analyses revealed significant differences between male control mice and male VOMA mice (t14=2.65 p<0.05) and between male and female control mice (t14=2.97, p<0.05). A two-way ANOVA of cytosolic TH [Fig 21B] revealed a significant effect of sex (F1,21=18.68, p<0.001), no significant effect of drug (F1,21=0.14, p>0.7), or interaction effect of sex and drug (F1,21=0.056, p>0.8). Post-hoc analyses revealed significant differences between male and female mice in control (t21=2.96 p<0.05) and VOMA (t21=3.2 p<0.01) treatments. A two-way ANOVA of synaptic D1 [Fig 21C] revealed a significant effect of sex (F1,23=11.66, p<0.01), a significant effect of drug (F1,23=11.77, p<0.01), and a significant interaction effect of sex and drug (F1,23=9.14, p<0.01). Post-hoc analyses revealed significant differences between male VOMA mice and male control mice (t23=4.1 p<0.001) and male VOMA mice and female VOMA mice (t23=5.8 p<0.001). A two-way ANOVA of synaptic D2 [Fig21D] revealed no significant effect of sex (F1,14=1.399, p>0.2), or drug (F1,14=0.1045, p>0.7), and a significant interaction effect of sex and drug (F1,14=8.051, p<0.05). Post-hoc analyses revealed a significant difference between male VOMA and male control mice (t14=2.9 p<0.05).
Figure 20. Escalation VOMA produces sex differences in hippocampal D1, D2 and DAT expression levels. Male and female mice exhibit different basal levels of hippocampal DAT and escalation VOMA decreases levels in males but not females (A). Male mice show higher levels of TH across control and VOMA conditions compared to females (B). Escalation VOMA increases hippocampal D1 levels in male mice but not in female mice (C). Escalation VOMA decreases hippocampal D2 levels in male mice but not in female mice (D). (*p<.05; **p<.01)
4.4.8 ERα and Akt/GSK3β signaling pathways

Hippocampal cytosolic and synaptic fractions were analyzed for neuroprotective signaling pathways and estrogen receptor alpha (ERα) to examine the role of hormonal fluctuations on VOMA-induced neurochemical changes. All values were corrected for gapDH. A two-way ANOVA of synaptic ERα [Fig 22A] revealed no significant effect of sex (F1,19=1.528, p>0.2), a significant effect of drug (F1,19=4.97, p<0.05), and a significant interaction effect of sex and drug (F1,19=8.317, p<0.05). Post-hoc analyses revealed significant differences between male VOMA mice and male control mice (t19=2.57 p<0.01) and male VOMA mice and female VOMA mice (t19=3.48 p<0.01). A two-way ANOVA of cytosolic AKT [Fig 22B] between revealed no significant effect of sex (F1,19=0.086, p>0.7), a significant effect of drug (F1,19=4.692, p<0.05), and a significant interaction effect of sex and drug (F1,19=7.91, p<0.05). Post-hoc analyses revealed significant differences between male VOMA mice and female VOMA mice (t19=2.47 p<0.05) and female control mice and female VOMA mice (t19=3.5 p<0.01). A two-way ANOVA of cytosolic phospho-AKT [Fig22C] revealed a significant effect of sex (F1,19=16.85, p<0.001), a significant effect of drug (F1,19=10.07, p<0.01), and no significant interaction effect of sex and drug (F1,19=0.2687, p>0.05). Post-hoc analyses revealed significant differences between male VOMA mice and female VOMA mice (t19=3.68 p<0.01) and female control mice and female VOMA mice (t19=2.59 p<0.05). A two-way ANOVA of synaptic GSK3β [Fig 22D] revealed a significant effect of sex (F1,19=21.95, p<0.001), no significant effect of drug (F1,19=0.065, p>0.8), or interaction effect of sex and drug (F1,19=1.81, p>0.1). Post-hoc analyses revealed significant differences between male VOMA mice and female VOMA mice (t19=4.81 p<0.001). A two-way ANOVA of synaptic phospho-
GSK3β [Fig 22E] revealed a significant effect of sex (F1,19=6.841, p<0.05), no significant effect of drug (F1,19=1.904, p>0.1), or interaction effect of sex and drug (F1,19=0.175, p>0.6). Post-hoc analyses revealed no significant differences between groups. A two-way ANOVA of synaptic phospho-GSK3β/GSK3β corrected ratios [Fig 22E] revealed no significant effect of sex (F1,20=1.159, p>0.2), a significant effect of drug (F1,20=4.956, p<0.05), and no significant interaction effect of sex and drug (F1,20=4.026, p>0.05). Post-hoc analyses revealed significant differences between male VOMA mice and female VOMA mice (t20=2.5 p<0.05) and between male VOMA mice and male control mice (t20=2.9 p<0.05).
Figure 21. Escalation VOMA produces sex differences in hippocampal ERα-GSK3β-pathway activity. Male and female mice exhibit different levels of hippocampal ERα pathway activity following Escalation VOMA, with male mice showing increased expression of neuroprotective markers ERα (A), AKT (B), and pAKT (C) over female VOMA mice. AKT activation is known to directly mediate activation of GSK3β, whose phosphorylation is neuroprotective and dephosphorylated state is pro-apoptotic. Escalation-VOMA increases total GSK3β in females (D) but not males, dysregulates pGSK3β, and increases pGSK3β to total GSK3β ratios in males but not females, indicating that escalation produces sex-specific neuroprotection only in males. (*p<.05; **p<.01)
4.4.9  D-prostanoid receptor 1, 2 (DP1/DP2 receptor)

Hippocampal cytosolic and synaptic fractions were analyzed for prostaglandin receptor expression, to examine the effects of VOMA on prostaglandin-mediated signaling. All values were corrected for gapDH. A two-way ANOVA of cytosolic COX-2 [Fig 23A] revealed a significant effect of sex (F1,21=4.569, p<0.05), a significant effect of drug (F1,21=5.422, p<0.05), and no significant interaction effect of sex and drug (F1,21=0.787, p>0.3). Post-hoc analyses revealed no significant differences between groups. A two-way ANOVA of synaptic DP1 [Fig23B] revealed a significant effect of sex (F1,17=11.01, p<0.01), no significant effect of drug (F1,17=0.288, p>0.5), or interaction effect of sex and drug (F1,17=3.09, p>0.05). Post-hoc analyses revealed a significant difference between male VOMA mice and female VOMA mice (t17=4.1 p<0.01). A two-way ANOVA of synaptic DP2 [Fig 23C] revealed no significant effect of sex (F1,22=1.159, p>0.2), drug (F1,22=2.92, p>0.5), or interaction effect of sex and drug (F1,22=2.386, p>0.05). Post-hoc analyses revealed a revealed no significant differences between groups.
Figure 22. Escalation VOMA produces sex differences in hippocampal DP1 but not COX-2 or DP2 levels. In response to Escalation VOMA, Hippocampal COX-2 levels do not change significantly compared to controls (A). However, DP1 levels increase in female mice but not in male mice (B). Hippocampal DP2 levels do not show significant changes in male or female mice, with or without VOMA (C). (**)p<.01
4.4.10 Hippocampal PKCι/λ and PKMζ

Hippocampal cytosolic fractions were analyzed for atypical protein kinase c expression, PKMζ, known to play a major role in learning and memory \(^{170}\), in order to examine the underlying mechanisms for VOMA induced memory deficits. All values were corrected for gapDH. A two-way ANOVA of cytosolic PKCι/λ [Fig 24A] revealed no significant effect of sex (F1,22=0.209, p>0.6), drug (F1,22=0.014, p>0.9), or interaction effect of sex and drug (F1,22=0.532, p>0.4). A two-way ANOVA of cytosolic PKMζ [Fig 24B] revealed a significant effect of sex (F1,23=6.775, p<0.05), no significant effect of drug (F1,23=0.436, p>0.5), or interaction effect of sex and drug (F1,23=1.447, p>0.2). Post-hoc analyses revealed a significant difference between male VOMA mice and female VOMA mice (t23=2.98 p<0.05).
Figure 23. Escalation VOMA produces sex differences in hippocampal PKMζ. Escalation VOMA produces no differences in PKCι/λ across male or female mice (A). Escalation VOMA produces sex differences in hippocampal PKMζ levels, with females showing a significant reduction compared to males following VOMA (B). (*p<.05)
Hippocampal and NAc synaptic fractions were analyzed for κOR expression in order to characterize the molecular signaling underlying addiction as perpetuated by VOMA. All values were corrected for gapDH. A two-way ANOVA of hippocampal synaptic κOR [Fig 25A] revealed no significant effect of sex (F1,13=1.358, p>0.2), no significant effect of drug (F1,13=1.752, p>0.2), and a significant interaction effect of sex and drug (F1,13=12.32, p<0.01). Post-hoc analyses revealed significant differences between male control mice and female control mice (t13=3.06 p<0.05) and between male control mice and male VOMA mice (t13=3.74 p<0.01). A two-way ANOVA of NAc synaptic D1 [Fig 25B] revealed a significant effect of sex (F1,17=40.69, p<0.001), no significant effect of drug (F1,17=4.23, p>0.05), or interaction effect of sex and drug (F1,17=0.9, p>0.3). Post-hoc analyses revealed significant differences between male control mice and female control mice (t17=3.32 p<0.01) and between male VOMA mice and female VOMA mice (t17=6.34 p<0.001). A two-way ANOVA of NAc synaptic κOR [Fig 25C] revealed no significant effect of sex (F1,17=0.3478, p>0.5), no significant effect of drug (F1,17=0.026, p>0.8), and a significant interaction effect of sex and drug (F1,17=8.153, p<0.05). Post-hoc analyses revealed a significant difference between male VOMA mice and female VOMA mice (t17=2.98 p<0.05).
Figure 24. Escalation VOMA produces increased NAc κOR pathway activity. In response to Escalation VOMA, Hippocampal κOR levels are significantly decreased in male mice but not in female mice (A). Accumbal D1 levels are significantly decreases in female mice, with and without VOMA compared to males (B). Accumbal κOR levels are significantly increased in female mice after VOMA (C) indicating that differences in D1 signaling can be explained partly by κOR-dependent signaling that affects the dopamine system in this region. (*p<.05; **p<.01)
4.5 Discussion

and females (6-8 weeks of age). Based on medium VOMA consumers [average 5.8-6mg/kg MA/day over 28d; 7.6-8.2 mg/kg/day over the last 18d] across males and females, we created cohorts that consumed equivalent amounts of MA during the 28d MA-treatment period. Our results show sex-specific effects on behavior and neurochemical expression of various markers in the hippocampus. Behavioral results show that VOMA did not affect depression, was associated with increased anxiety in both sexes, and VOMA-females developed working-memory deficits, but males did not. Analysis of dopamine markers show increased D1 in VOMA males compared to control males and VOMA females. There were no changes in DAT, TH or D2. Additionally, ERα increased in VOMA males including the downstream activation of pAkt and the ratio pGSK3β/GSK3β but not in females. These results indicate that VOMA males, but not females, have enhanced neuroprotection. In contrast, VOMA females show enhanced DP1 and reduced DP2, a PGD2 receptor specific expression pattern reflecting heightened response to neuroinflammatory insults that is not observed in males. VOMA females also demonstrate enhanced δ-opioid receptor expression in the NAc indicative of negative symptomology associated with drug withdrawal but males did not. These results highlight the sex-specific vulnerability of chronic VOMA and the differential signaling pathways that are activated between sexes during adolescence and suggest a role of sex-specific treatments for addiction.

4.5.1 Chronic VOMA impairs female working memory but not males
impulse control 25-27, 267. Many of these results are also shown in rodent studies 22, 268. MA-induced working memory deficits in male mice show sustained effects for up to 7 weeks after a neurotoxic dose of MA and following 28d VOMA without escalation 189, 258. Our working memory assessment of adolescent mice shows working memory deficits in VOMA females as compared to control females, but such VOMA-induced deficits were not present in males. This effect is consistent with other reports identifying female enhanced sensitivity to MA 253-254, 269. However, following chronic or long-access to MA, both males and females show memory deficits but females exhibited higher MA intake and greater relapse to MA-seeking 253. Taken together, increased drug consumption combined with greater escalation demonstrates that females are more sensitive to the primary rewarding aspects of psychostimulant drugs which can disrupt reward driven memory tests 269. The superior performance of VOMA males compared to VOMA females may be the result of the MA-induced increase in D1 expression. For example, increased striatal D1 has been shown to engender a readiness to respond to reward in animals pretreated with MA 270. Increased cortico-striatal D1 activity is predictive of reward-based learning 271, and increased sensitivity in this D1 pathway can modulate working memory performance 272. Hippocampal-dependent working memory is also modulated by discrete changes in D1 activation 273.

4.5.2 VOMA differentially affects Dopamine-related markers across sexes
Our results show that VOMA did not alter TH, D2 or DAT expression in females, while males show decreases in DAT and D2 with increases in D1 compared to controls. This patterns suggests differential neurotoxic effects between sexes as D2 and DAT are modulated by MA, and D2 antagonist have been shown to reduce neurotoxic effects of binge doses 118 as do DAT
inhibitors 274. Thus, lower levels of D2 and DAT could reflect neuroprotection in males. Previous studies have shown that escalating doses of MA offer some neuroprotection and/or slow the expression of neurotoxicity 19-20, 74. This interpretation is consistent with our previous VOMA study in males where we identified decreases in both TH and DAT following 28 VOMA without escalation 189. Our results with TH and DAT also indicate a sex-difference between control groups. This effect is in contrast to DAT levels reported in human studies showing higher DAT expression in females than males 275-277. Rodent studies have been less consistent, with some studies demonstrating greater DAT expression in females 278-280, and other showing no basal differences 281-283. Additionally, some studies have shown that male DAT expression in striatum is more responsive to early life stress 284 and to high doses of MA 78. In striatum, DA concentration are not different between sexes however; males show great numbers of TH immunoreactive cells in the substantia nigra pars compacta compared to females 285-286. While no evaluation of TH or DAT across sexes has been done in the hippocampus, these current data indicate sex-specific expression differences in hippocampus for dopamine markers.

VOMA also induced a significant increase in D1R expression in males but not females. Several reports show that MA-induced D1 activation is neuroprotective in the hippocampus by suppressing glutamatergic neurotransmission 130 and can reduce NMDA receptor mediated Ca2+ currents in hippocampal neurons, 131, in striatal neurons 127, and in producing inhibition on N-type voltage-gated calcium channels 132. In contrast, reports show that a D1R antagonist can reduce the neurotoxic effects of binge doses of MA 118. MA-induced D1R activation can also exacerbate glutamate toxicity 129. Therefore, it is possible that alterations in other receptors dictate whether increased D1R-levels are beneficial or detrimental. It is interesting that males
significantly upregulate D1R but females do not. To date, we believe this is the first report identifying differential expression of MA-induced D1 across adolescent animals, suggesting that D1 could be a useful sex-specific therapeutic target for MA neurotoxicity.

4.5.3 The role of dopamine induced expression on estrogen receptor α (ERα)

Our results show males increase ERα following VOMA but females do not. Several reports have shown that estradiol can elevate ERα expression and have neuroprotective effects on MA and on mitigating MA-induced cognitive deficits. However, fewer studies have investigated the role of dopamine on estrogen receptors as we have here. While we do not know the mechanism explaining why this sex difference occurs, several reports show that ERs can also be activated in the absence of ligand. In vitro studies show that in the absence of estradiol, ERs are activated by several different neurochemical factors involving: EGF, IGF-I, caveolin-1, and activators of the protein kinase A and protein kinase C pathways resulting in ER-dependent gene transcription. Uterine EGF and IGF-I also result in ER-dependent gene transcription that can be blocked by an ER antagonist in vivo. Furthermore, ERs can be activated in vitro in the absence of estradiol by dopamine, and in vivo. Based on these studies, we suggest that the elevated expression of D1 in VOMA-treated males is presumably enhancing the ERα expression in a sex-specific manner.

4.5.4 VOMA-induced sex differences in GSK3β and Akt signaling pathways associated with ERα
Estradiol and the IGF-1 receptor (IGF-1R) interact through ERα, allowing for the formation of a multimolecular complex composed by ERα, IGF-1R and components of this downstream signaling pathway such as, PI3K, Akt, and GSK3β. This ERα/IGF-1R interaction regulates PI3K-Akt-GSK3β-β-catenin signaling by estradiol. It has been shown that inhibition of PI3K signaling abolishes the hippocampal neuroprotective actions of estradiol. In addition, the Akt activation by estradiol controls expression of the anti-apoptotic molecule Bcl-2 via the cAMP-response element-binding protein (CREB). Downstream, Akt phosphorylates the glycogen synthase kinase 3β (GSK3β) and BAD proteins, thereby inhibiting their pro-apoptotic functions inducing the activation of neuronal survival pathways. These studies are consistent with our results showing VOMA males increasing ERα together with sustaining higher levels of phosphorylated Akt and the pGSK3β/GSK3β ratio and support a role for the neuroprotective effects of the GSK3β pathway as sexually dimorphic with chronic MA. GSK3β is negatively regulated by the phosphorylation on its serine 9 (Cohen and Frame, 2001). In contrast, 40mg/kg IP MA following 7d abstinences produces greater neurotoxicity in males with decreased GSK3β and pAkt compared to females. This report suggests that at high doses of MA, females may have greater capacity for neuroprotection than males, while the opposite may be true for low or voluntary consumption rates of MA between sexes. Nevertheless, our results show a sex-specific effect of GSK3β. While no other reports have identified differences in expression levels of this marker, one report has identified differential activation of this signaling pathway mediated in leukemic stem cell therapy.

4.5.5 Enhanced D-prostanoid receptor 1 (DP1 receptor) in females following VOMA
We evaluated the expression of both the DP1 and DP2 receptors following VOMA as an indicator of response to neuroinflammation. Prostaglandins initiate signaling through multiple receptors that have both proinflammatory and anti-inflammatory effects. Prostaglandin D2 (PGD2), the most abundant prostaglandin in the brain, is considered anti-inflammatory when it signals through the D-prostanoid receptor 1 (DP1 receptor) present on myeloid cells but proinflammatory when it binds to the DP2/CRTH2 receptor on Th2 CD4 T cells. PGD2/DP1 signaling triggers G protein activation and cAMP production and has been associated with neuroprotection following diverse insults. In addition, a PGD2 derivative, 15d-PGJ2, also signals through the DP1 receptor, inhibiting the inflammatory response. Inhibiting the DP1 receptor retards resolution of inflammation in macrophages. Conversely, the activation of DP2 receptor is associated with activation of proapoptotic signaling. Based on these reports, our data suggest that the differential expression observed in VOMA-females is a response to enhanced neuroinflammation, this data further suggests that males are not activating this signaling pathway for neuroprotection. CRTH2 signaling leads to GSK3β phosphorylation in TH2 lymphocytes, suggesting a role for this receptor in neuroprotection. However, the cell-specific localization of this receptor could determine its protective or pro-inflammatory role as the activation of DP2/CRTH2 is associated with activation of proapoptotic signaling in cardiomyocytes. Based on these reports our data suggests that the differential expression of DP1 observed in VOMA-females is in response to enhanced neuroinflammation. The data further show that males are not activating this signaling pathway for neuroprotection. This hypothesis is consistent with our observations of increased neuroprotection via enhanced AKT-pGSK3β found in males but not females.
4.5.6 VOMA induces sex-specific downregulation of PKMζ in the hippocampus

Our analysis of sex-specific effects of VOMA include PKMζ and PKCι/λ, synaptic plasticity markers associated with learning and memory. PKMζ is important for both late-phase LTP and long-term memory maintenance across various learning paradigms. PKMζ plays major roles in the trafficking of AMPA receptors that sustain LTP processes. Our results show that PKMζ was significantly reduced in female VOMA mice compared to male VOMA mice and showed a trending decrease compared to controls. These results indicate that synaptic plasticity marker expression was differentially affected between sexes to produce the working memory deficit in female VOMA mice. Previously, it has been shown that PKCι/λ acts as a compensatory AMPAR trafficking enzyme in animal models where PKMζ was knocked-out, but in wildtype mice, PKMζ remains a pivotal enzyme regulating AMPAR trafficking, LTP, and memory. Still unknown is how PKCι/λ may undermine AMPAR trafficking and LTP deficits in models of stress and drug addiction, where PKMζ is consistently affected. The current study shows that PKCι/λ, unlike PKMζ, remains unchanged following VOMA in both male and female mice. This result suggests that following VOMA, PKCι/λ may act to partially rescue AMPAR and LTP deficits brought on by PKMζ. This hypothesis should be tested in future studies.

4.5.7 Methamphetamine-induced modulation of κ-opioid receptor (κOR) and D1 in NAc

We investigated κ-opioid receptor (κOR) expression in the NAc following VOMA as part of the ventral hippocampus-NAc circuit that participates in stimulant-induced expression of drug addiction behaviors. The reciprocal connections between the NAc and the hippocampus suggest that individual drug preferences could also influence the rewarding properties associated with spatial learning. Our results show that VOMA females increase κOR in the NAc compared...
to VOMA males. The expression of κOR in the NAc is associated with the negative affect of drug withdrawal and for the repeated, compulsive-like drug behavior. D1 activation regulates prodynorphin, the precursor of dynorphin, the endogenous ligand for κOR. κORs are located on striatal dopaminergic and glutamatergic terminals. Activation of dynorphin systems feedback to decrease dopamine release and contribute to the dysphoric syndrome associated with cocaine dependence, and long access to MA. Consistent with our data, chronic MA leading to escalating consumption depends on the activation of the κOR system in the NAc shell. Selective inhibition of κOR blocks the development and/or expression of escalation of intravenous heroin intake, excessive alcohol self-administration and MA self-administration. Conversely, κOR agonists have been shown to induce dysphoric-like states in rodents and humans. This state in the ventral striatum may be driving escalation of intake via a negative reinforcement mechanism. Our results suggest that females may have a vulnerability to dysphoric-mediated effects of κOR based on lower basal levels of D1 in the NAc compared to males. This hypothesis is consistent with demonstrating that escalation of cocaine intake is driven by decreases in phasic dopamine release in the ventral striatum and that normalization of this state by the dopamine precursor L-DOPA can eliminate the escalation of cocaine intake. Thus, κOR acts as a presynaptic regulator of dopamine release, and, when the κOR system becomes sensitized by repeated psychostimulant use, it creates a state of deficient presynaptic dopamine release altering not only the rewarding properties of drugs, but also the food reward earned during spatial learning.

4.6 Conclusion and Clinical Implications
To determine how chronic access to MA differentially affects the hippocampus and NAc across adolescent male and female mice, we used a recently developed VOMA model. The findings show that with 10 days of escalating dosing, the males show reduced neurotoxic effects of MA compared to previously published results without escalation. Additionally, the VOMA females show exacerbated working memory deficits compared to VOMA males and show a distinctly different pattern of neurochemistry compared to males. Males increase D1, ERα, pAkt, and pGSK3β/GSK3β ratio, suggesting neuroprotection, but females do not. Conversely, females show an increase in DP1 levels and a concomitant increase in κOR in NAc suggesting a response to increased neuroinflammation and elevated dysphoria that males do not. Presently little evidence exists to understand the differential drug seeking and vulnerabilities between sexes. Few studies have examined the long-term access drug seeking profiles in addition to their underlying mechanisms. The current work highlights differential activation of signaling pathways between males and females that have consumed voluntarily equivalent amounts of MA for 28d. The increased vulnerability of the
Figure 25. Escalation VOMA produces distinct behavioral and molecular responses between male and female mice. In response to Escalation VOMA, female mice exhibit compromised molecular signatures, including deficits in synaptic marker expression, increased neuroinflammatory activity, and decreased neuroprotective pathway activity that may underlie the cognitive deficits evidenced on the radial arm maze. Conversely, male mice that underwent Escalation VOMA exhibit increased neuroprotective activity, and no significant deficits in synaptic marker expression or neuroinflammatory activity, that result in no change in cognitive performance. This model has revealed a specific role for escalating methamphetamine doses to produce neuroprotection but only in male subjects. Female subjects are revealed to be susceptible to MA’s negative effects on cognition and the neurochemistry underlying behavioral performance.
5.1 VOMA as a naturalistic model of Methamphetamine abuse

The VOMA model has offered a new method to assess the voluntary consumption rates in a chronic MA exposure design. VOMA allows mice to titer the amount of drug administered in a given administration window, as reflected by the sustained level of MA consumed over several weeks. It is worth noting that within VOMA, mice do not consume all the MA baits delivered on any given day. As seen in our 28d and escalation models, on average, mice consume about half the total mg/kg MA made available. This indicates that although mice may want to consume MA via VOMA, this model does not produce mice that binge on MA, on any given day or throughout the administration period. Future studies should focus on the emotive, behavioral, and neural mechanisms that allow mice to self-regulate their MA consumption rates in VOMA.

Furthermore, the VOMA models have proven useful in producing specific hippocampal-dependent cognitive impairments, as assessed by our RAM tasks. This behavioral deficits indicate that chronic MA abuse has the potential to affect this brain region, and further, that VOMA is useful in characterizing these effects where other models, such as self-administration, may have failed. In contrast to previous work on the cognitive effects of neurotoxic MA, VOMA more closely models natural MA consumption as it may occur in some populations. Specifically, I hypothesize that although VOMA may not be able to accurately depict the most addicted, highest consuming MA abusers, it can model the regular, chronic and moderate MA abusers. These populations may include frequent partiers, women seeking to dramatically lose weight and increase productivity, and even professionals looking to boost their day-to-day work output.
Our experiments have revealed that with VOMA, we can model preference for MA as seen in human populations. The use of forced MA administration in animal models has provided mechanistic understandings of the molecular and physiological effects that the drug has on the brain. The current body of work has provided the next step in understanding the voluntary development of MA addiction. As my data shows, VOMA can produce a range in consumers (See Chapter 3). Intuitively, this result translates to our understanding of MA addiction in humans, as not all human MA consumers are considered highly dependent addicts. Thus, the true range in human MA consumption likely follows a normal distribution, with very low and very high consumers at the ends of the population, with the majority of consumers falling in between these extremes. From my experiments, it seems very plausible that we have been able to mimic this hypothetical human distribution in mice. Through various experimental set-ups, we have produced low consumers, average consumers, and (not reported) the occasional super consumers. As mentioned above, more work is needed to understand the neural, emotive and/or behavioral mechanisms that produce this distribution in consumption rates. Preliminary work on this question has been shown in Chapter 3, where intracellular AKT-GSK3β and KOR activity has been measured and found to be dysregulated in female VOMA mice. Future experiments should be designed to target these pathways pharmacologically to assess output measure in behavior, DA toxicity, synaptic plasticity and neuroinflammation.

Despite its reliance on oral-dosing, a route not considered to be wholly reflective of consumption by addicted individuals, VOMA is nonetheless a useful model that provides us with non-invasive and effective MA administration. Consistent deficits in RAM working memory, despite the design of VOMA experiments, indicate a loss of normal function in striatal-hippocampal signaling. This
is directly in line with decreases in dopamine marker expression in the hippocampus. These data show that VOMA is effective in producing the behavioral and molecular deficits produced by previous administration models. The critique against the oral route for this psychostimulant can be attributed to the notion that addicted individuals do not administer MA via this route, opting for intravenous or intranasal administration, as discussed above. However, data on human psychostimulant administration shows that oral administration makes a high percentage of abuse, and that the half-life of MA via oral administration is not different from that of other routes. Follow-up work on VOMA should aim to characterize the pharmacokinetics of MA following various dosing periods, such as after 1d VOMA, 7d VOMA, 14d VOMA and 28d VOMA at 1hr, 4hr, 8hr, and 24hr post final dose. Blood plasma and brain samples should be taken to assess for MA content and following VOMA for these periods of time. These follow-up experiments should help clarify the ability of VOMA to make MA available throughout the body and brain.

5.2 Abstinence exacerbates the neuronal damage associated with Methamphetamine abuse

MA is known to facilitate dopamine neuro-transmission which is the underlying molecular mechanism that produces euphoria and increases the risk for dependence and addiction. As MA is used to increase DA release more and more during abuse, toxicity of DA terminals and/or compensatory signaling can lead to decreased baseline DA activity in the brain. In-vivo, this decreased DA pathway activity can lead to physiological dependence to MA as well as psychological and behavioral shifts that mirror neurological diseases like depression and
Parkinson’s disease, among others. Thus, it seems that the time away from MA is as important to the progression of addiction and physiological changes that occur as a result of MA-abuse.

Abstinence, whether permanent (as in our animal models) or transient (as can be seen in human addicts that cannot abstain), is an important factor to consider in understanding the behavior and physiological changes that result from MA abuse. To reiterate, MA acts on the dopamine system, and is predominantly acting to increase DA neurotransmission. Although many of MA’s deleterious effects occur after this main molecular activity (including DA-quinone formation, DAT loss of function, etc.), special attention should be placed on the period of time following a period of abuse. In humans, this period could be the time between last period of abuse and the one to come. In our VOMA model, this is the length of time following last dose and prior to tissue collection.

Our findings are illuminating. Four (4) weeks of abstinence following VOMA or bolus-MA injections produces increased neurotoxic prostaglandin expression. This result is counter to what was expected, as MA alone should have increased chronic neuroinflammation. However, it was abstinence from the drug that produced this effect. The mechanisms surrounding how exactly abstinence can increase the brain’s susceptibility to damage is unclear. Besides a need for more in-depth molecular analyses of the development of this diseased state of the brain following abstinence, molecular targets should be narrowed down to prevent neurodegenerative processes such as this from being initiated. One future experiment should include the use of DAT agonists (to increase the normal function of the molecule) and anti-inflammatories during or prior to VOMA, as my data has shown these two pathways to become dysregulated in the brain after abstinence. My hypothesis is that DAT loss of function (i.e. inverse agonism and depletion) could
perpetuate the effects of abstinence after MA abuse, but that either with DAT-agonists or anti-inflammatories, we could prevent total DAT loss of function or prevent the downstream effects of increased DA availability in the synaptic cleft and then overall preventing a diseased state from developing in the brain. This is in line with the understanding that DAT regulates dopamine availability at synapses, and that dysregulation of this molecule disrupts dopamine-mediated signaling. In the context of addiction, this MA-induced DAT loss of function could underlie the molecular mechanisms of MA physiological dependence.

5.3 Escalating doses prevent the cognitive and neurochemical deficits associated with Methamphetamine abuse in male mice

To our surprise, the escalation VOMA model (Chapter 4) was not able to produce the behavioral nor the neurochemical deficits in male adolescent mice produced in the static dose 28d VOMA model (Chapter 2), despite having similar total MA consumption over 28d (see Fig 27). As was discussed in Chapter 2, 28d VOMA using a static dose design produced spatial working memory and learning deficits in male mice. However, escalation VOMA did not produce greater cognitive deficits, or any at all. Upon analysis of hippocampal tissue samples, our neurochemistry results from escalation VOMA male mice aligned with the in-vivo work, revealing non-significant changes in tyrosine hydroxylase, no changes in neuroinflammation, and no changes in synaptic plasticity marker expression. This was in contrast to findings from the 28d static VOMA model, where DA toxicity, neuroinflammation, and synaptic plasticity marker expression was all affected.
Figure 26. Total MA consumed in 28d VOMA (Chapter 2) vs Escalation VOMA (Chapter 3) in Male mice. There was no significant difference between the two groups.
The behavioral data suggest that although mice are consuming at high rates during VOMA, they have not yet reached stages of addiction that produce craving and depression. Thus, we can conclude that although VOMA may produce sensitization and high rates of voluntary consumption, the timeline or dose-design of this model is not sufficient to produce the latter stages of addiction associated with drug seeking, craving and dysfunction. Rather these data suggest that Escalation VOMA produces the early stages of MA abuse and addiction. Additionally, our model uses young adult mice (aged 8 weeks at start of VOMA), and our behavioral data could reflect a resilience in young mice to develop the behavioral deficits associated with MA exposure in older mice. More work needs to be done to compare how old and young mice respond to VOMA.

This suggests that high rates of MA abuse may be maximally achieved through gradual introduction to the drug. Escalation better models how humans might initiate MA abuse, by gradual introduction to small doses of the drug. Our Escalation VOMA study has offered a more robust method to produce sustained MA consumption, as evidenced by the decreased variability in daily mg/kg MA consumed over the last 18d of the study. Additionally, this design is also able to produce Low consuming mice. Of 14 male mice that went into VOMA, 9 mice fell into our medium consumer group and 5 fell into the low consumer group. This distribution highlights the strength of this design to produce high numbers of mice that sustain higher levels of MA consumption. The data indicate a wider and clear split in consumption between groups and provides a means to analyze genetic and behavioral variability (and/or drift) that produces higher and lower consumers.
Taken together, these results suggest that escalating doses have some intrinsic neuroprotective qualities that protect the male brain and male behavior from a chronic MA’s detrimental effects. The nature and source of this neuroprotection is unclear, but previous work has shown that this phenomenon occurs in binge injection models, 19-20, associated with decreases in MA binding to DAT and DA release. However, the underlying mechanisms that produce this change in DA sensitivity remain unknown. This highlights the importance of MA’s action on DAT as a key factor in the neurotoxicity following MA. Finally, it is unknown how escalating oral MA provides protection or tolerance to MA challenges via DAT. This remains to be uncovered.

Interestingly, escalation VOMA did re-capitulate decreases in DAT expression in the hippocampus, as seen in the 28d static VOMA model. DAT are modulated by MA and D2 antagonist have been shown to reduce neurotoxic effects of binge doses 118 as do DAT inhibitors 274. Thus, lower levels of DAT could reflect neuroprotection in males. Previous studies have shown that escalating doses of MA offer some neuroprotection and/or slow the expression of neurotoxicity 19-20, 74. This interpretation is consistent with the static 28d VOMA model where we identified decrease in both TH and DAT following 28 VOMA without escalation 189. Additionally, some studies have shown that male DAT expression in striatum is more responsive to early life stress 284 and to high doses of MA 78.

5.4 Female mice are susceptible to the cognitive and neurochemical deficits associated with Methamphetamine abuse
Our Escalation VOMA model has revealed substantial sex differences in response to chronic MA exposure. Male mice exhibit neuroprotective profiles, both behavioral and molecular, following Escalation VOMA, but female mice show susceptibilities at these levels. Key neuroprotective pathways are compromised in female mice.

Several reports show that MA-induced D1 activation is neuroprotective in the hippocampus by suppressing glutamatergic neurotransmission, by suppressing NLRP3 inflammasomes and by reducing NMDA receptor mediated Ca2+ currents in hippocampal neurons, and in striatal neurons. Alternatively, others have shown that a D1R antagonist can reduce the neurotoxic effects of binge doses of MA and that D1R activation following MA exposure can intensify glutamate toxicity. Thus, D1 alone cannot mediate neuroprotection or neuro-susceptibilities. Several reports have shown that estradiol can elevate ERα expression and have neuroprotective effects on MA and on mitigating MA-induced cognitive deficits. The mechanisms underlying VOMA-induced upregulation of ERα in males is unknown and warrants further investigation, but previous work suggests that protein kinase A and protein kinase C pathways, as well as dopamine in vitro and in vivo can all increase ER levels. Thus, we hypothesize that females exhibit vulnerabilities to MA as result of a failure to increase D1-dependent signaling to parallel neuroprotective pathways. The underlying sex-differences in DA-mediated signaling in response to MA, including the receptors activated, should be delineated in future studies.

The neuroprotective nature of ERα is derived from the signaling pathways that are initiated in response to its activity. Estradiol and the IGF-1 receptor (IGF-1R) interact through ERα, leading to a formation of a complex composed of ERα, IGF-1R and downstream signaling pathway.
components such as, PI3K, Akt, and GSK3β. Akt activation by estradiol regulates expression of the anti-apoptotic molecule Bcl-2 via the cAMP-response element-binding protein (CREB). Downstream, Akt phosphorylates GSK3β and BAD proteins, which inhibits their pro-apoptotic functions, leading to the activation of neuronal survival signaling. Our data provide evidence that these neuroprotective pathways are only active in males exposed to escalation VOMA, to produce a behavioral and molecular phenotype unlike that seen in female mice (Fig 28). GSK3β is negatively regulated by phosphorylation on its serine 9 site. In contrast, 40mg/kg IP MA following 7d abstinences produces greater neurotoxicity in males with decreased GSK3β and pAkt compared to females. This report suggests that high doses of MA may produce greater neuroprotection for females than males, contrary to our escalating dose VOMA model. This highlights the utility of our model to elucidate the effects of naturalistic MA administration to investigate the underlying molecular mechanisms surrounding neurotoxicity and neurodegeneration. Overall, this indicates that more work is needed to characterize the role of GSK3β pathways in MA-induced toxicity, cognitive changes and addiction in males and females.

To explore the sex differences in neuroinflammation following VOMA, we analyzed expression levels of DP1 and DP2 receptors, endogenous protein receptors to prostaglandin ligand activity. Prostaglandin D2 (PGD2), the most abundant prostaglandin in the brain, and a derivative 15d-PGJ2 are considered anti-inflammatory when it signals through the D-prostanoid receptor 1 (DP1 receptor) present on myeloid cells but proinflammatory when it binds to the DP2/CRTH2 receptor on Th2 CD4 T cells. PGD2/DP1 signaling triggers G protein activation and cAMP production and has been associated with neuroprotection following diverse insults. Inhibiting the DP1 receptor retards resolution of inflammation in macrophages. Conversely, the
activation of DP2 receptor is associated with activation of proapoptotic signaling. Our data suggests that the increased expression observed in VOMA-females was observed due to ongoing neuroinflammation, which is likely not as prevalent in male mice, if at all. CRTH2 signaling leads to GSK3β phosphorylation in TH2 lymphocytes, suggesting a role for this receptor in neuroprotection. However, the cell-specific localization of this receptor could determine its protective or pro-inflammatory role as the activation of DP2/Crth2 is associated with activation of proapoptotic signaling in cardiomyocytes.

We also assessed levels of PKMζ and PKCι/λ, synaptic plasticity markers associated with learning and memory, of which PKMζ has been shown previously to be utilized differentially across sexes for spatial memory functions. PKMζ was significantly reduced in female VOMA mice compared to male VOMA mice and showed a trending decrease compared to controls. PKCι/λ acts as a compensatory AMPAr trafficking enzyme in animal models where PKMζ was knocked-out, but in wildtype mice, PKMζ remains a pivotal enzyme regulating AMPAr trafficking, LTP, and memory. The current study shows that PKCι/λ, unlike PKMζ, remains unchanged following VOMA in both male and female mice. This result suggests that following VOMA, PKCι/λ may act to partially rescue AMPAr and LTP deficits brought on by PKMζ. These data reveal some of the molecular mechanisms that underlie the behavioral deficits exhibited by females in this VOMA model. The precise upstream mechanism that produce this result are unknown but could involve GSK3β-dependent activity, as it is known to regulate and be regulated by synaptic plasticity and is known to regulate AMPA-receptor subunit trafficking. Future studies should clarify the role of GSK3β in VOMA-induced cognitive and excitotoxic deficits.
Finally, our results show that VOMA females increase κOR in the NAc compared to VOMA males. κOR expression in the NAc is associated with the negative effects of drug withdrawal and with repeated, compulsive-like drug behavior 319-320. Interestingly, κOR and D1 are linked, as activation of D1Rs regulates prodynorphin, the precursor of dynorphin, the endogenous ligand for κOR 321. κORs are located presynaptically on striatal dopaminergic and glutamatergic terminals 322-323, acting to regulate/decrease neurotransmitter release. This action of κORs can contribute to the dysphoric syndrome associated with cocaine dependence 324-327, and long access to MA 319.

This state in the ventral striatum may be driving escalation of intake via a negative reinforcement mechanism 319, 330, with increased κOR activity leading to increased dysphoria which leads to increased drug administration to counter this syndrome with stimulant-induced euphoria. This mechanism could explain sustained and/or escalating drug administration rates that underlie physiological dependence under a voluntary administration context. Our results suggest that females may have a vulnerability to dysphoric-mediated effects of κOR based on lower basal levels of D1 in the NAc compared to males, and increased κOR in the same brain region. Future studies should elucidate the role of κOR in the NAc to negatively couple effects of MA on cognition, as is seen in our study. Additionally, κOR antagonists should be used to clarify its role on female-specific molecular vulnerabilities to dopaminergic signaling via D1.
Figure 27. Molecular Mechanisms of D1 and ERα- dependent neuroprotective signaling. In the context of methamphetamine, specific synaptic pathways initiate signaling to promote neuroprotection or neurotoxicity. Chapter 4 explores the contribution of these pathways in the context of neurochemical and behavioral effects in male and female adolescent mice.
CHAPTER 6: FUTURE DIRECTIONS
6.1 Delineating VOMA-induced deficits from abstinence-induced deficits

The studies included into this dissertation have characterized the effects of VOMA following a 14-day or 28-day drug administration period and abstinence periods. Data from Chapter 2 and 3 indicate that our VOMA model can produce a neuroinflammatory response in the hippocampus, following 2 weeks or 4 weeks of abstinence. It is unclear if the neuroinflammation observed is due to MA neurotoxicity or abstinence. Additionally, it is unknown how mice will respond to longer administration periods lasting longer than 28 days. To delineate the onset and progression of VOMA-induced deficits apart from deficits induced by abstinence, I propose the following:

1) A series of VOMA administration experiments to collect brain tissues at the end of 7, 28 and 44 days of VOMA without any abstinence.

   a) This will characterize the onset and progression of neurochemical changes in the brain that occur as a result of progressively longer periods of VOMA. I hypothesize that neurotoxicity in tissues will positively correlate with length of VOMA, with increasing neuroinflammatory responses observed as the time spent in VOMA increases.

   b) Alternatively, mice on VOMA could show an unchanged neurochemical profile as a result of MA-induced D1 activity\textsuperscript{127, 130-132} and fail to exhibit robust molecular deficits throughout the course of the study. This finding could provide pivotal information about the utility of the VOMA model and overall, the long-term effects of voluntary oral MA on neurochemistry. A measure to assess the feasibility of this proposed experiment will be by tracking daily consumption. From the studies provided, we know that neuroinflammation is present in mice when consumption rates are sustained at or above ~5mg/kg MA/day. In mouse populations consuming below this level, alternative approaches to answer this question should be used.
i) An alternative approach to delineate the neuroinflammatory response of VOMA apart from that of abstinence would be to expose mice to bolus injections of approx. 5mg/kg MA/day and per the given study time-course above.

c) Follow-up experiments should assess the cognitive performance of mice given 7 and 44 days of VOMA. These experiments will provide a time-course for behavioral changes produced by progressively longer exposure to MA via VOMA.

6.2 Mechanisms underlying abstinence-induced changes in behavior and neurochemistry following VOMA

The studies included into this dissertation have utilized a 2-week (approximately 14 days) or 4-week (approximately 28 days) abstinence period to carry out behavioral assessments and prior to specific tissue collections. Data from Chapter 3 suggests that abstinence can remediate cognitive deficits produced by MA, despite contradictory neurochemical changes that show activation of susceptible molecular signaling. It remains unclear what molecular signaling during abstinence can promote neurodegenerative or neuroprotective pathways in the brain. Thus, to clarify what molecular pathways should be targeted during abstinence in clinical settings, I propose the following:

1) A series of VOMA administration experiments to assess efficacy of pharmacological agents administered during abstinence in preventing the neurochemical changes produced during a 14-day, 28-day, and 44-day abstinence period

 a) A series of experiments using NSAIDs (targeting cyclooxygenases) and ERα agonist estradiol (starting administration right after VOMA) to test the efficacy of these pharmacological interventions in preventing the negative neurochemical changes associated with abstinence after MA exposure, such as decreases in DAT, increases in
PGJ2 levels, COX-2, and others. Control groups will receive no additional treatment for 44-days of abstinence.

b) Follow-up experiments should assess the behavioral changes associated with administering these drugs during abstinence, with behavioral assessments occurring during and after the abstinence period.

c) Alternatively, we could observe that all neurodegenerative signaling could resolve by the end of 44 days of abstinence in control groups receiving no NSAID or estradiol treatment. This could suggest that no pharmacological intervention is necessary for resolution of these neurochemical deficits and that additional treatment only speeds up potential recovery of neurochemical deficits. In this scenario, follow-up experiments should the efficacy of NSAID or estradiol treatment in preventing deficits induced by a series of low-dose and high-dose challenge exposures of MA, following abstinence. This follow-up experiment will test the efficacy of pharmacological intervention in reducing the neurochemical deficits associated with a challenge exposure of MA, as previous studies have shown that neuroprotection by low-dose MA exposure is eliminated by 31 days after low-dose MA exposure\textsuperscript{341}.

i) Alternative approaches should also be considered as per section 6.1.b above, if mice consume below 5mg/kg MA/day. Tissue analyses will be feasible from mice that receive similar doses in order to delineate the effects of compounding pharmacological interventions, as outlined above.
6.3 Mechanisms underlying neuroprotection or susceptibility following Escalation VOMA

The last study in this dissertation showed that male mice exhibit protection from MA’s detrimental behavioral and neurochemical effects as a result of Escalating doses of MA. This effect was not seen in female mice. Further investigation at the molecular level showed that male mice showed significant increases in hippocampal estrogen receptor alpha, an effect that female mice did not show. These results warrant further investigation in order to determine more clearly the necessity of ERα-to-GSK3β activity to promote neuroprotection in males. To do so, I propose the following:

1) A series of VOMA administration experiments with an added ERα antagonist manipulation in male mice to determine the necessity of ERα to promote behavioral and neurochemical resilience. An initial administration design could see the ERα antagonist delivered at the start, middle and end of the 28-day VOMA period.

a) These experiments will determine if male mice need to upregulate ERα in order to show resilience to MA challenges. I hypothesize that male mice co-administered with an ERα antagonist will show behavioral and neurochemical deficits after VOMA

i) Alternative approaches should also be considered as per section 6.1.b above, if mice consume below 5mg/kg MA/day. Tissue analyses will be feasible from mice that receive similar doses in order to delineate the effects of compounding pharmacological interventions, as outlined above.

2) A series of VOMA administration experiments with an added ERα agonist manipulation in female mice to determine the necessity of ERα to promote behavioral and neurochemical resilience in these mice. An initial administration design could see the ERα agonist delivered at the start, middle and end of the 28-day VOMA period.
a) These experiments will determine if female mice that are given ERα agonist exhibit neuroprotection, like male mice have already shown

i) Alternative approaches should also be considered as per section 6.1.b above, if mice consume below 5mg/kg MA/day. Tissue analyses will be feasible from mice that receive similar doses in order to delineate the effects of compounding pharmacological interventions, as outlined above.

The potential pitfalls from these experimental designs include the unknown variable of the 10-day escalation period of VOMA. This period, prior to the full-dose and access to MA, could prove to be a pivotal priming point for mice that enables activation or suppression of neuroprotective pathways. Thus, to answer this question, I propose the following:

3) A series of Escalation-VOMA administration experiments to collect tissues at 2 days, 4 days, 6 days, 8 days, and 10 days into the Escalation period. This design will enable us to capture the progression of neurochemical adaptation that mice are going through prior to engaging in full-access VOMA.

a) These experiments will determine how male and female mice are responding to escalating doses of MA in the different tissues collected. It will be important to collect a few brain tissue and potentially a few relevant peripheral tissues (like liver, intestines, and others) to determine the full scope of molecular changes that mice exposed to escalation VOMA undergo.

i) Alternative approaches should also be considered as per section 6.1.b above, if mice do not escalate as well as presented in Chapter 4. In this alternative experiment, injections of average daily escalating doses should be administered throughout the 10-day escalation period to accomplish the tissue collection design above.
APPENDICES
Figure 28. 5-HT1b Knockout mice show different rates of consumption across sexes. Mice aged 8-16 weeks at the start of VOMA were put through Escalation VOMA. Tracking of MA consumption revealed the female KO mice consumed significantly less over the 28 days of VOMA [Two-way repeated measures ANOVA: across gender: $F_{1.9}=3.685$, $p=0.08$; across days: $F_{27.243}=9.67$, $p<0.001$; interaction of gender and days: $F_{27.243}=2.23$, $p<0.001$]
Appendix II. Bolus MA increases chronic neuroinflammation after 6 weeks of abstinence

Figure 29. Chronic neuroinflammation after 6 weeks of abstinence following bolus MA.

Ubiquitin and COX-2 significantly increased 6 weeks after bolus (60mg/kg) MA treatment (*p<.05). Ubiquitin and COX-2 quantified by WB.
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