Davis, Delaney L., Neurobehavioral and biochemical consequences of chronic, low-dose methamphetamine exposure in male and female mice. Doctor of Philosophy (Biomedical Sciences), April, 2022, 121 pp., 9 tables, 19 figures, bibliography, 336 titles

Although prescription psychostimulants are effective in reducing attention deficit hyperactivity disorder (ADHD) symptomology, misuse of these drugs can pose serious risks such as potential abuse, dependence, and/or neurotoxicity. Of particular concern is that young adults have the highest prevalence of prescription stimulant misuse, with almost 10% of college students admitting to using amphetamine (e.g. Adderall) or methylphenidate (e.g. Ritalin) products. Despite these drugs being widely used for therapeutic and recreational use, the long-term effects of prescription stimulants have not been systematically evaluated in controlled clinical trials. Therefore, it is critical to conduct this research because young adults may be a vulnerable, at-risk population to the potential adverse consequences of long-term amphetamine use.

This dissertation research evaluates the biochemical and behavioral consequences of chronic exposure of the prototypical psychostimulant, methamphetamine (METH), in a rodent model. It is hypothesized that repeated doses of METH, within the therapeutic dosing range used in a clinical setting, will induce neurotoxicity through the interplay of biological mechanisms of oxidative stress, glutamate excitotoxicity, neuroinflammation and epigenetic alterations and increase susceptibility to addiction that will be exacerbated by aging processes. Overall, the body of results showed short-term alterations in brain biochemistry and behavioral function, that do not necessarily persist past 5 months after METH treatment.

In conclusion, this dissertation highlights the importance of long-term studies in addressing prescription stimulant misuse in an adult population to better understand the safety of these widely used and prescribed psychostimulants.

NEUROBEHAVIORAL AND BIOCHEMICAL CONSEQUENCES OF CHRONIC, LOW-DOSE METHAMPHETAMINE EXPOSURE IN MALE AND FEMALE MICE

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Presented to the Graduate Council of the School of Biomedical Sciences

University of North Texas Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

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Fort Worth, Texas

April 2022

ACKNOWLEDGEMENTS

I would like to thank my committee members, Dr. Ren-Qi Huang, Dr. Michael Gatch, Dr. Nicole Phillips, Dr. Derek Schreihofer and Dr. Rong Ma for their support and overseeing my research project. I would like to thank my mentor, Dr. Nathalie Sumien, for helping me through this long journey from research assistant to Ph.D. candidate. She has made me not only a better scientist, but a better person, merci pour tout. I would like to thank my friends (especially Carmen, Jonah, and Judy!) my D&D group, my partner, and my family for their continued support as well as my labmates through the years; Tom, Jess, Philip, Paapa, Daniel and Kay. Thank you to Cheryl, Elizabeth, Allie and Kiran for putting up with my general tomfoolery!

Many thanks to everybody who has ever listened to me talk on and on about lemurs and reciprocated in sharing pet pictures with me. Much love to my pets Diego, Catsby and Lily!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Psychostimulant use and misuse

Psychostimulant use is a major public health concern, as it presents considerable challenges to health and in the past decade, levels have continued to steadily increase. In the United States, almost 10.3 million people have misused psychostimulants and approximately 1/3 of these individuals further develop stimulant use disorder (SUD) (SAMHSA 2021). Chronic abuse of these drugs can cause severe long-term effects such as psychosis, cardiovascular events, and memory loss (Favrod-Coune and Broers 2010; Miquel et al. 2016). Psychostimulants are a broad class of sympathomimetic drugs that stimulate the both the peripheral and central nervous system and are a type of psychoactive drug. Acute administration of psychostimulants produce a wide range of behavioral changes, including increased wakefulness, attention, movement and arousal, anorexia, and euphoria which can in turn lead to the abuse of the drug(Martin et al. 1971; Cole 1967). Amphetamine type stimulants (ATS) are psychostimulants that can be categorized into two groups: illicit (e.g. methamphetamine or 3,4-methylenedioxy methamphetamine; MDMA) and licit (prescription stimulants) substances. Furthermore, prescription stimulant drugs are divided into different subtypes based on chemical structure: amphetamine products (phenylethylamines), methylphenidate products (piperidines) and anorectics (diphenylmethanes) (SAMHSA 2021). Commonly misused types of prescription stimulants include amphetamine (e.g. Adderall, dextroamphetamine) and methylphenidate (e.g. Ritalin, Concerta) products. Chronic stimulant medication for maintenance therapy has long

since been the most effective treatment for reducing symptomology of attention deficit and hyperactivity disorder (ADHD) (Weyandt et al. 2016). When used as prescribed, these drugs have proven to be safe and efficacious in children and adults with ADHD (Goodman et al. 2005; Spencer et al. 2007; Buitelaar et al. 2009). However, these drugs are also classified by the United State Drug Enforcement Agency (DEA) as Schedule II substances due to a high potential of abuse, dependence, and neurotoxicity.

The non-medical use, or misuse, of stimulants is defined as taking stimulants without a valid prescription and/or use of stimulants other than as prescribed (UNODC 2011). Prescription stimulants are most commonly misused by young adults (aged 18-25 years) when compared to adolescents or older adults (SAMHSA 2021). In the United States, prescription stimulant misuse among young adults has gained popularity over the last decade, with amphetamine compounds becoming the second most commonly used illicit drug in college students (Hughes et al. 2016). There may be a high prevalence of stimulant abuse in college students because prescription stimulants are perceived to be relatively safe, easily accessible (Schulenberg et al. 2021) and improve academic performance and outcomes (Garnier-Dykstra et al. 2012; Hartung et al. 2013; Benson et al. 2015; Arria et al. 2018). Although though the primary reason for stimulant misuse is for cognitive enhancement, grade point average (GPA) was reported to be negatively correlated with stimulant misuse (Benson et al. 2015; Weyandt et al. 2013), even though college students perceived cognitive benefits (Ilieva et al. 2013). Therefore, the effects of amphetamines as cognitive enhancers may be a grossly exaggerated assumption and stimulants may be effective at correcting deficits rather than enhancing performance.

In adults without ADHD, there is some evidence that low, clinically relevant dosing of amphetamines can modestly improve specific domains of cognition, such as working memory

(Weyandt et al. 2016; Lakhan and Kirchgessner 2012). However, these studies utilized prescription stimulants well within the appropriate clinical dosing range for a short amount of time and exceeding this range can produce cognitive impairments (Ornstein et al. 2000; Chang et al. 2002). It is proposed that prescription stimulants exhibit dose-dependent effects, in which acute, low doses improve cognition and higher doses, or low doses used for an extended amount of time, induce cognitive impairments (Wood et al. 2013). When these drugs are used chronically, even at low doses that would be clinically prescribed, they can induce negative effects. Preclinical studies in rodents suggest long-term cognitive (Kamei et al. 2006; LeBlanc-Duchin and Taukulis 2009) and molecular (Hotchkiss and Gibb 1980) consequences of chronic, dosing within the range of what is clinically prescribed for humans of amphetamine derivatives. Furthermore, Ricuarte *et al*. reported that after 4 weeks of chronic treatment with an amphetamine compound similar to Adderall, adult non-human primates (NHPs) displayed marked dopaminergic dysregulation and potential neurotoxicity (Ricaurte et al. 2005).

The long-lasting consequences of chronic stimulant use in adults has not been systematically evaluated in controlled clinical trials. Currently there are few long-term (> 24 months) studies on stimulant use in the management of ADHD symptomology (Biederman et al. 2010; Ginsberg et al. 2014; Biederman et al. 2005) even though there is an increasing use of amphetamine stimulants as life-long maintenance medications. Adults are also prescribed higher doses and longer treatment durations compared to children even though adults have longer elimination half-lives, thus increasing the risk of adverse events (Berman et al. 2009). Lastly, the peak age for substance use disorder (SUD) from prescription stimulant misuse, is in the 30 to 44 years age range (McCabe et al. 2022) suggesting that middle adulthood could be a vulnerable age group that is often overlooked because of the short duration of most systemized studies.

Therefore, young adults are a potential at risk population of long-term behavioral deficits associated with prescription stimulant misuse. It is imperative to conduct long-term studies to better quantify the safety and efficacy of these widely used drugs.

1.2 Commonly used prescription amphetamine and methylphenidate compounds

Prescription stimulant compounds are chemically similar, containing a benzene ring with an ethylamine side chain and have a similar mechanism of action (Figure 1.1). Chemical structural variations may contribute to differences in pharmacological effects and abuse potential (Heal et al. 2013). Some of the most common prescription psychostimulants used in preclinical and clinical studies are further described.

Dextroamphetamine (d-AMP) is the dextro-isomer of racemic amphetamine. It is recommended by the U.S. Food and Drug Administration (FDA) for treatment of ADHD and narcolepsy with a maximum recommended dosage of 60 mg/day in adults. D-amphetamine has more pronounced effects in the central nervous system (CNS), compared to the levo-isomer, although l-amphetamine (l-AMP) has greater cardiovascular and peripheral effects (Heikkila et al. 1975; Kuczenski et al. 1995; Schechter 1978).

Adderall is the mixture of the neutral sulfate salts of amphetamine and dextroamphetamine in a ratio of 1:3 and is indicated for treatment of ADHD and narcolepsy. Adderall can be prescribed in either an immediate-release (IR) or extended-release (ER) form with an FDA-recommended dosage for the treatment of ADHD in adults to be 5-40 mg/day (IR) or 20 mg (XR) a day. For the treatment of narcolepsy in adults, 5-60 mg/day is recommended. In 2020, the average prevalence rate of Adderall without medical supervision was approximately

9% in young adults (aged 19-26 years old) and less than 1/3 (< 29.6%) of people perceived taking Adderall once or twice was harmful (Schulenberg et al. 2021).

Methylphenidate is a racemic mixture of its l-and-d isomers and is an amphetamine-like stimulant indicated for the treatment of ADHD and narcolepsy, with immediate-release (Ritalin) and extended-release (Concerta) forms. The FDA-dosing ranges for both ADHD and narcolepsy treatment in adults, for 20-30mg/day (IR) or 20-60mg/day (ER). In 2020, the average prevalence rate of Ritalin was approximately 2% in young adults (aged 19-26 years old). Ritalin hit its peak from 2002-2006 but on average only had about a 2.5% annual prevalence, whereas in 2009, Adderall seemingly replaced Ritalin as the preferred first-line ADHD prescription.

Desoxyn is the commercial name for FDA-approved METH and has a usual effective dose of 20-25 mg/day for ADHD and 15 mg/day for short-term obesity treatment in adults.

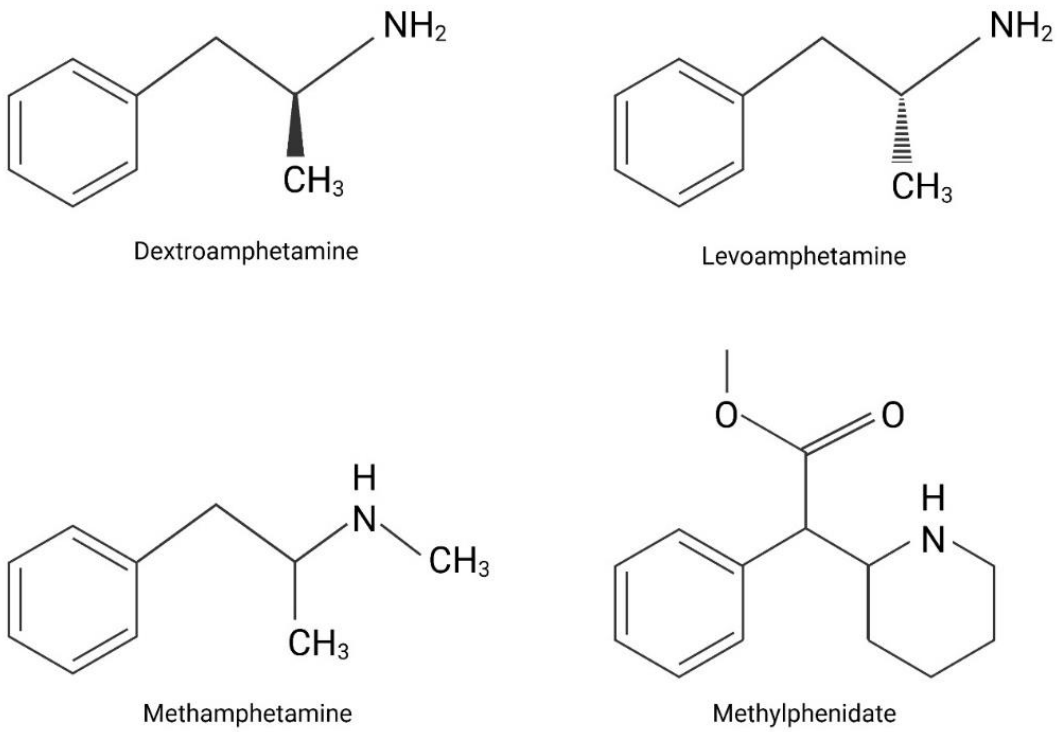

Figure 1.1 Chemical structures of commonly misused amphetamine-type stimulants. Made with Biorender.

1.3 Dopaminergic systems and neurotransmission

Amphetamine (AMP) and its derivatives mainly exert their reinforcing and rewarding effects through the elevation of extracellular dopamine. The primary mechanism of action of amphetamines is the increased the release of monoamine neurotransmitters, including dopamine (DA), norepinephrine (NE) and serotonin (5-HT), into the synapse through the reversal of monoamine transporters (Figure 1.2) (Miller 1989). Amphetamines have a similar chemical structure to catecholamine neurotransmitters and are readily taken up by their respective transporters into the presynaptic terminal as well as through passive diffusion. Amphetamines disrupt the function of the CNS proton-dependent vesicular monoamine transporter protein, VMAT2, causing a redistribution of monoamines from the synaptic vesicles to the cytosol (Cubells et al. 1994). Amphetamines can also inhibit monoamine oxidases enzymes, which decrease the breakdown of monoamines. Methylphenidate (MPH) is an amphetamine-like stimulant that has a similar mechanism of action to cocaine. Instead of using dopamine transporter (DAT) as a carrier (like amphetamines), MPH increases DA by blocking DAT (Han and Gu 2006). Unlike AMP, MPH does not target VMAT2 and therefore does not exhibit releasing actions (Freyberg et al. 2016).

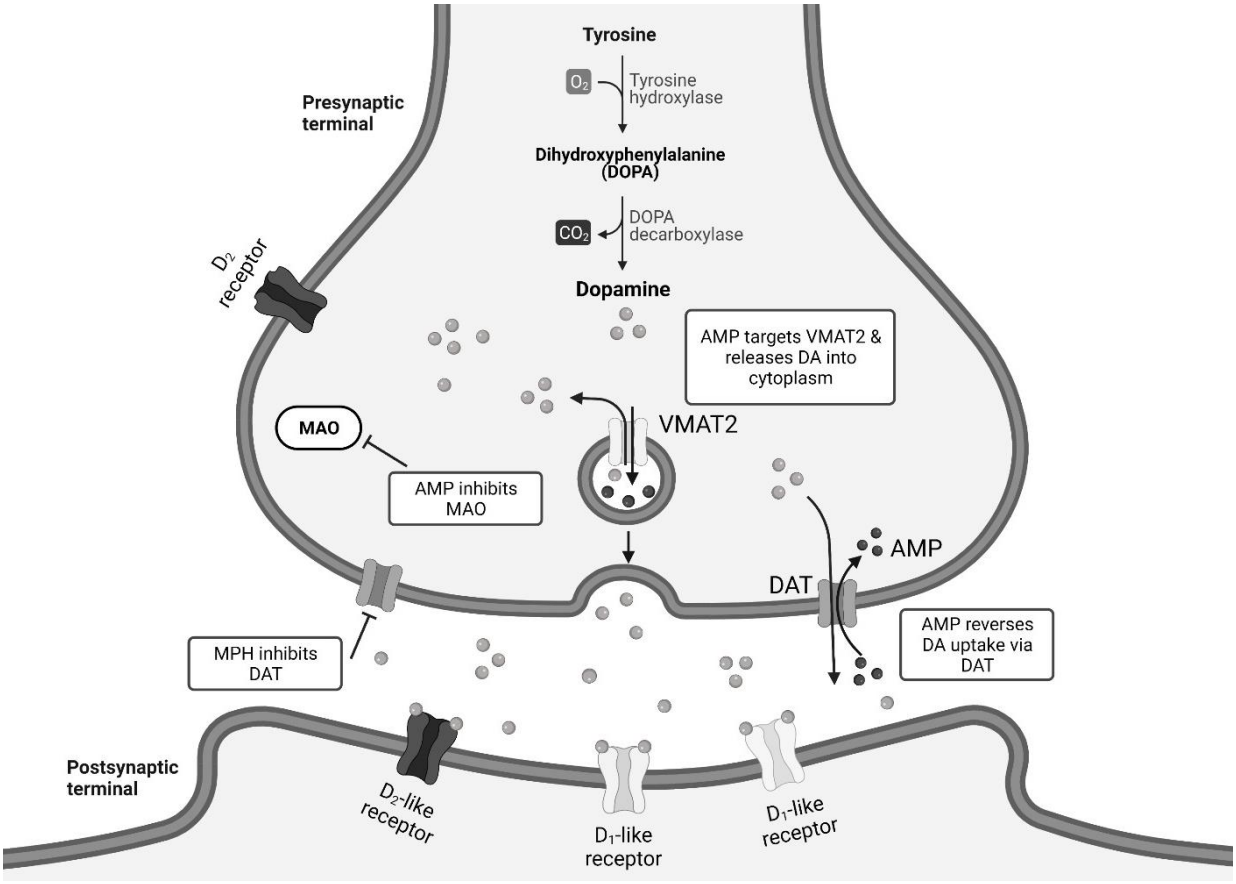

Figure 1.2 Mechanism of action of amphetamine (AMP) and methylphenidate (MPH) in a representative dopaminergic neuron. AMP is a substrate of dopamine transporter (DAT) and is taken up into the presynaptic terminal by DAT or passive diffusion. AMP interacts with synaptic membrane protein, vesicular monoamine transporter 2 (VMAT2), and forces dopamine (DA) efflux into the cytosol. Cytosolic DA is transported out of the presynaptic neuron to the synaptic cleft via DAT-mediated reverse transport. AMP also inhibits monoamine oxidase thereby decreasing DA metabolism. MPH is a DA reuptake inhibitor and binds to and blocks DAT, thus increasing extracellular DA. Adapted from (Miller 1989; Cubells et al. 1994; Han and Gu 2006). Made with Biorender.

The mesolimbic pathway, often referred to as the reward pathway, is a collection of dopaminergic neurons that originate in the ventral tegmental area (VTA) in the midbrain and project outward and primarily terminate onto the GABAergic medium spiny neurons (MSNs) of the nucleus accumbens (NAc) in the forebrain (Figure 1.3) (Swanson 1982). DA release into the NAc stimulates D_1-like (D_1, D_5) or D_2-like (D_2, D_3, D_4) receptors expressed on MSNs. Stimulation of D_1-like receptors activate G_{olfs} proteins, which in turn activates the effector protein, adenyl cyclase, increasing synthesis of cyclic adenosine monophosphate (cAMP). cAMP can go on to activate protein kinase A, which phosphorylates target proteins that modulate neuronal activity and gene expression (Cheng et al. 2011). D_2-like receptor stimulation activates $G_{i/o}$ proteins that inhibit adenylyl cyclase (Kebabian and Calne 1979; Missale et al. 1998). The mesolimbic pathway facilitates reinforcement and reward learning (Nicola et al. 2005). All drugs of abuse activate the mesolimbic dopamine system and this pathway is crucial for the acute rewarding effects of psychostimulants (Di Chiara and Imperato 1988; Koob and Volkow 2010).VTA DA neurons also project to the amygdala and hippocampus, which mediate emotional and memory associations, and to the prefrontal cortex (PFC), which mediates self-regulation, all of which participate in reward processing (Tse et al. 2011; Degoulet et al. 2009; Lodge and Grace 2008). Projections from the VTA to the cortex have also been shown to modulate working memory and executive function (Martig et al. 2009).

The nigrostriatal pathway connects the substantia nigra pars compacta (SNpc) in the midbrain with the dorsal striatum (caudate and putamen) in the forebrain (Gerfen and Wilson 1996) (Figure 1.3). Although the main function of this pathway is voluntary movement, striatonigral MSNs are essential for reward response (Wise 2009; Faure et al. 2005). Unilateral lesions of the nigro-striatal dopamine (DA) system show that METH can induce profound motor

impairments and long-lasting degeneration of dopaminergic cell bodies in the SNpc and dopaminergic terminals in the striatum (Ares-Santos et al. 2014). Previous research demonstrates that METH has differential neurotoxic effects on different dopaminergic systems with the nigrostriatal dopaminergic pathway being more susceptible to neurotoxicity compared to the mesolimbic pathway (Granado et al., 2010).

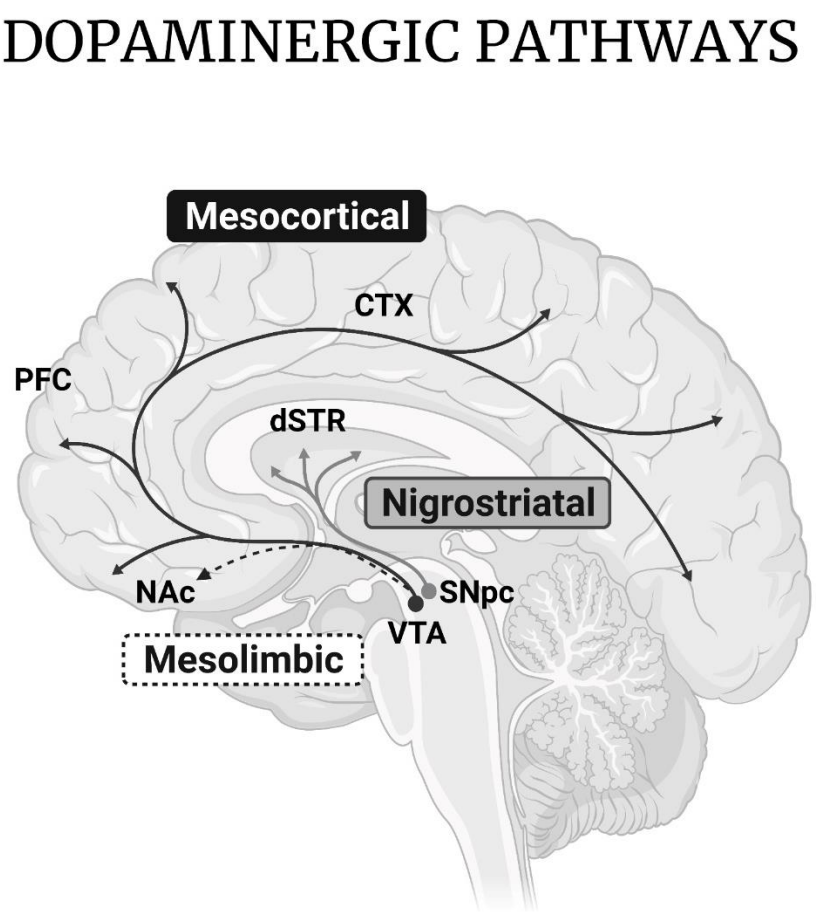

Figure 1.3. Midsagittal view of the brain depicting the mesocortical, mesolimbic and nigrostriatal dopaminergic pathways. CTX: cortex; PFC: prefrontal cortex; dSTR: dorsal striatum (caudate nucleus and putamen), Nac: nucleus acumbens; SNpc: substantia nigra pars compacta. Adapted from (Swanson 1982; Gerfen and Wilson 1996; Sesack and Pickel 1992). Made with Biorender.

1.4 Main mechanisms of amphetamine-induced neurotoxicity

Amphetamines and amphetamine-like stimulants have the potential to induce long-lasting damage to monoaminergic axon terminals (Seiden et al. 1976; Ricaurte et al. 1984). The biological mechanisms responsible for amphetamine-induced neurotoxicity have not been fully elucidated and involve complex interplays between oxidative stress and mitochondrial dysfunction, glutamate-related excitotoxicity and astroglia activation (Moratalla et al. 2017; Yang et al. 2018). More recently, epigenetics have come to the forefront of many fields, including substance use, and could play a crucial role in the addiction susceptibility (Nielsen et al. 2012).

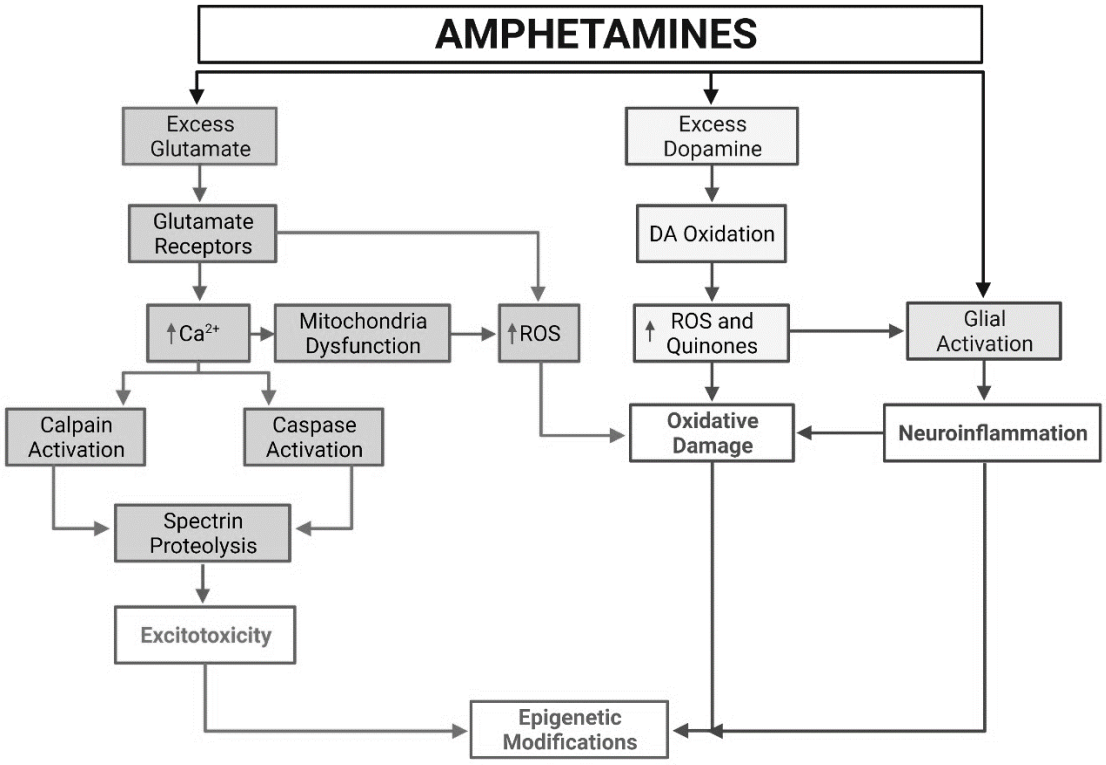

Figure 1.4. Proposed major molecular mechanisms of amphetamine neurotoxicity. ROS: reactive oxygen species; Ca^{2+}: calcium ions. Adapted from (Yamamoto and Raudensky 2008; Moratalla et al. 2017; Halpin, Collins, et al. 2014; Yang et al. 2018). Made with Biorender.

1.4.1 Oxidative stress

Chronic misuse of amphetamines can lead to long-lasting neuroadaptations that are mediated by oxidative stress. Amphetamines target VMAT2, causing a redistribution of monoamines from the reducing environment within synaptic vesicles to the cytosolic oxidized environment. In dopaminergic neurons, free DA accumulates within the presynaptic terminal and can undergo non-enzymatic autoxidation into reactive quinones and reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) and superoxide anions, that attack cytoplasmic proteins and lipids and can trigger the cell death cascade and cause long-lasting neurotoxicity (Brown and Yamamoto 2003; Cunha-Oliveira et al. 2013; Heikkila and Cohen 1973). Excessive oxidative stress produced by DA autooxidation also plays a role in mitochondrial dysfunction, by inhibiting key enzymes of the mitochondrial electron transport train (ETC) (Berman and Hastings 1999). Impaired mitochondria trigger caspase signaling and ROS can also damage DNA structures causing loss of genetic information leading to accelerated mitochondrial dysfunction by inhibition of the ETC (Vander Heiden et al. 1997; Delsite et al. 2003).

1.4.2 Glutamate excitotoxicity

Amphetamines have been previously shown to increase extracellular glutamate levels (Del Arco et al. 1999; Wolf et al. 2000) thus stimulating glutamate receptors, which are divided into metabotropic (mGLUR) and ionotropic (iGluR) glutamate receptors (Madden 2002). iGluRs, including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and N-methyl-D-aspartate receptor (NMDAR), can become overstimulated by amphetamines, and induce abnormal Ca^{2+} influx that increases free radical generation that can lead to neuronal damage (Yamamoto and Raudensky 2008; Lafon-Cazal et al. 1993; Sattler and Tymianski 2000).

1.4.3 Neuroinflammation

It is well-established in the literature that amphetamines induce a significant microglial response that results in the release of proinflammatory cytokines (Cunha-Oliveira et al. 2013). DA quinones may induce early activation of microglial cells and increase the expression of inflammatory signaling cascades (Loftis and Janowsky 2014). Activation of microglia and astrocytes enhance the production of proinflammatory cytokines (TNFα, IL-1, IL-6) and ROS, thus creating a self-sustaining cycle of neuroinflammation and oxidative stress (Nakajima et al. 2004; Tocharus et al. 2010; Taylor et al. 2013).

1.4.4 Epigenetic modifications

Amphetamines and amphetamine analogs have been shown to induce long-lasting post-translational changes in the expression of genes and proteins, particularly in brain regions implicated in the DA reward circuit (McCowan et al. 2015). One of the most well-established AMP-induced epigenetic modifications is that of c-fos, an immediate early gene (IEG) associated with conditioned drug behaviors (Cruz et al. 2015). Acute AMP treatment induces increased expression of the c-fos gene, with increased histone acetylation which is associated with active transcription, whereas chronic treatment represses c-fos expression and reduces histone acetylation (Hebbes et al. 1988; Renthal et al. 2008). Renthal *et al.* reported that chronic amphetamine (4/mg/kg/day for 7 days) exposure ameliorated c-fos expression and increased ΔFosB, a transcription factor implicated in addiction, at the c-fos promotor. ΔFosB expression can mediate stable transcriptional changes in the striatum (Renthal et al. 2008). Cadet *et al.* demonstrated that acute METH caused a global increase in gene expression, whereas chronic, escalating METH (1 mg/kg/day-12 mg/kg/day, for 2 weeks, i.p.) induced a global decrease

(Cadet et al. 2013). Overall, amphetamines can induce persistent changes in gene expression through epigenetic alterations.

1.5 Effects of amphetamine on behavioral outcomes

1.5.1 Cognitive function

Chronic misuse of amphetamines may lead to long-lasting changes in brain function and structure, and these changes have been implicated in cognitive alterations. Prescription stimulants are perceived, especially in a college population, to enhance cognitive functioning. However, these medications only produce some modest effects at enhancing cognition in young adults without ADHD (Repantis et al. 2010; Ilieva et al. 2013; Benson et al. 2015; Weyandt et al. 2016; Wilens et al. 2017; Weyandt et al. 2018) and grade point average (GPA) has actually been found to be negatively correlated with stimulant misuse among college students (Benson et al. 2015; Weyandt et al. 2013). The cognitive effects of amphetamines appear to be dependent on time course (acute vs. chronic use) and dose (low vs. high). In preclinical studies, when implemented as a single dose, amphetamines enhanced fear memory at low, clinically relevant doses, but impaired memory at high doses (Carmack et al. 2014; Wood and Anagnostaras 2009). Spatial learning and memory was reported to be improved by a single dose of MPH (1 mg/kg) but repeated doses (1 mg/kg, 6 days) impaired Morris water maze (MWM) performance (Carvallo et al. 2018). Chronic administration of amphetamines was also found to induce long-lasting impairments in spatial memory in male rats, with chronic d-AMP administration (2.5 mg/kg/day for 5 days) inducing spatial working memory deficits 7 days post injections (Basmadjian et al. 2021) and chronic METH administration (2 mg/kg/day for 5 days) inducing deficits in spatial working memory up to 30 days after injection cessation (Bigdeli et al. 2015).

These findings suggest that cognitive benefits are associated with acute low doses, but acute high doses and chronic low dosing may have adverse effects.

1.5.2 Motor function

Chronic use of amphetamines can cause neurotoxicity and long-lasting neurodegeneration of the nigrostriatal pathway, an important dopaminergic pathway that facilitates voluntary movement (Ares-Santos et al. 2014). Although it has been well-demonstrated that acute amphetamine administration increases locomotor activity (Smith 1965; Geyer et al. 1987), there is little research focusing on the long-term effects of repeated amphetamine exposure on motor function. In preclinical studies, repeated administration of amphetamines impaired motor coordination (Boroujeni et al. 2020; Huang et al. 2017; Pathak et al. 2015). For example, daily injections of d-AMP (1.8 mg/kg, s.c.) for 5 days elicited motor memory impairments on the rotorod task lasting up to 25 days after the end of injections (Pathak et al. 2015). Boroujeni *et al.* noted METH-induced (2 mg/kg/day for 3 days, 5 mg/kg/day for 4 days, i.p.) impairments in rotorod performance that lasted as long as 4 weeks after injection cessation (Boroujeni et al. 2020). It noteworthy that Bourounjeni *et al.* showed neuronal degradation in the cerebellum, a brain region important for motor learning and has been gaining traction for its potential role in addiction research (Miquel et al. 2016).

1.5.3 Addictive-like behavior

Addiction is defined by the National Institute on Drug Abuse (NIDA) as a chronic, relapsing disorder in which a substance is compulsively used despite its adverse health consequences and leads to changes in brain structure and function. Addiction is considered the most severe form of SUD, characterized by the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) (APA 2013). Prescription stimulants, such as Adderall and Ritalin, are

Schedule II controlled substances for high potential of abuse and dependence. It is noteworthy that research has found that children and adults using prescription stimulants for the management of ADHD were reported to have lower risks of developing SUD, however, ADHD in of itself is a risk factor for SUD development (Faraone 2018; Chang et al. 2014). Repeated use of psychostimulants has been proven to produce robust neurobiological alterations that contribute to addiction-related behaviors that can be modeled in preclinical models of drug addiction.

A phenomenon unique to psychostimulant drugs is "behavioral sensitization" in which repeated exposure to the drug leads to persistent behavioral and neurochemical sensitization to subsequent drug exposure (Boileau et al. 2006; Koob and Volkow 2010; Robinson and Becker 1986). After chronic exposure to amphetamines, animals may produce an augmented response during subsequent drug administration, indicating neuroadaptations in brain circuitry involved in drug-reward related behaviors (Cador et al. 1999; Anagnostaras and Robinson 1996). Conditioned place preference (CPP) is a non-contingent behavioral model used to evaluate drug-seeking behavior through the association of the drug with a specific context (Bardo and Bevins 2000) and amphetamine-like compounds produce significant place preference (Carmack et al. 2014). Lin *et al.* reported that previous, repeated METH treatment (1 mg/kg/day every other day for six sessions, i.p.) increased place preference scores in male rats, suggesting that prior METH administration increased drug reward behavior with subsequent exposure (Lin et al. 2007). In a self-administration model, previous exposure to d-AMP exacerbated drug-taking behavior in male rats and produced persistent enhancements in motivational incentive to obtain the drug (Lorrain et al. 2000).

1.6 Does chronic drug use induce an accelerated aging phenotype?

In the United States, it is estimated that over half (56%) of people aged 60 years will have engaged in the nonmedical use of amphetamines (Schulenberg et al. 2021). Accelerated aging is the phenomena of when a typical aging phenotype occurs at an earlier age than what is normally observed in a population (Margolick and Ferrucci 2015). There are several factors that can contribute to accelerated aging, of which substance abuse appears to affect the early onset of age-related disease and functional declines. This may be due in part to the many similarities in neurobiological mechanisms of aging and amphetamine neurotoxicity that could lead to potential interactions and further exacerbate integral processes (Bachi et al. 2017). Aging and chronic amphetamine use both lead to decreased functionality of dopamine and glutamatergic systems (Kaasinen and Rinne 2002; Gasiorowska et al. 2021; Peterson et al. 1991), decreased macroautophagy and apoptosis (Yu et al. 2017; Limanaqi et al. 2021), reactive astrogliosis (Clarke et al. 2018; Nichols et al. 1993; Frey, Andreazza, et al. 2006), epigenetic changes (Jones et al. 2015; McCowan et al. 2015) and increased oxidative stress and redox imbalances (Venkateshappa et al. 2012; Forster et al. 1996; Yamamoto and Raudensky 2008) that mediate functional decline (Colón-Emeric et al. 2013; Berman et al. 2009). Therefore, it is predicted that repeated amphetamine use will provide a “second hit” and exacerbate neurodegenerative sequalae in aging-vulnerable brain regions that will promote accelerated age-associated neurobehavioral impairments.

1.7 Specific biochemical and epigenetic alterations associated with amphetamine use

1.7.1 Markers of dopaminergic function

Dopamine nerve terminal function can be elucidated through the quantification of expression of dopaminergic neuron phenotypic markers including tyrosine hydroxylase (TH) and dopamine transporter (DAT). DAT regulates DA neurotransmission by modulating DA availability in the brain through reuptake mechanisms (Schmitt and Reith 2010; Giros et al. 1992). Tyrosine hydroxylase catalyzes the conversion of tyrosine to l-3,4-dihydroxyphenylalanine (l-DOPA) and is the rate limiting enzyme for the catecholamine synthesis pathway.

Acute administration of amphetamines increase extracellular dopamine, leading to increases in TH and DAT levels (Shepard et al. 2006; D'Arcy et al. 2016) as compared to repeated exposure, in which TH levels and activity and availability of DAT are reduced (Wilson et al. 1996; Ashok et al. 2017; O'Callaghan and Miller 1994).

Even though the reported studies were conducted using high, neurotoxic doses outside of the therapeutic dosing range, there is evidence in nonhuman primates (NHPs) that dosing within what is typically used in a clinical setting can produce long-lasting dopaminergic alterations. Ricuarte *et al.* reported that NHPS treated with 3:1 ratio of l-/d-amphetamine, at doses to mimic Adderall in human clinical treatment, had 30-50% reductions in striatal DA and DAT (Ricaurte et al. 2005). Additionally, extended access to METH self-administration (0.1mg/kg/infusion, i.v. during daily 15 h sessions for 5 –8 days) lead to persistent decreases in striatal and cortical DA, TH and DAT that lasted up to 14 days after the end of treatment (Krasnova et al. 2016). Moreover, there is evidence of some recovery of dopaminergic function in small studies of abstinent METH abusers (Wang et al. 2009; Boileau et al. 2016; Volkow et al. 2015)(Wang et al. 2009; Boileau et al. 2016; Volkow et al. 2015) and preclinical studies (Ricaurte et al. 1984; Melega et al. 1996; Friedman et al. 1998).

1.7.2 Markers of oxidative stress

The oxidative degradation of lipids by ROS (i.e., lipid peroxidation) of polyunsaturated fatty acids form highly reactive and unstable byproducts called thiobarbituric reactive substances (TBARS) including the reactive aldehyde, malondialdehyde (MDA), which can be measured by TBARS assay. MDA is a frequently used biomarker of oxidative stress (Janero 1990).

A majority of studies that report increased AMP-induced oxidative stress are in chronic abusers of METH or in animals that received neurotoxic doses (Moszczynska et al. 2004; Fitzmaurice et al. 2006; da-Rosa et al. 2012). However, there is evidence that repeated low dosing of AMP can also produce oxidative stress in the brain as well. For example, Frey *et al.* found that chronic d-AMP (2 mg/kg/day for 7 days, i.p.) treatment in adult male Wistar rats increased MDA in the hippocampus and PFC (Frey, Valvassori, et al. 2006). In a comparative study evaluating oxidative stress measure in various doses of METH (0.25, 0.5, 1 or 2 mg/kg) vs. d-AMP (2 mg/kg), adult male Wistar rats were chronically administered METH or d-AMP once a day for 14 days and displayed increased TBARS in the PFC, amygdala, hippocampus and striatum (da-Rosa et al. 2012). Male Wistar rats that were chronically exposed to MPH (21 days, i.p.) exhibited elevated MDA in higher doses (10mg/kg and 20 mg/kg) but not lower doses (2 mg/kg and 5 mg/kg) in hippocampal mitochondria (Motaghinejad et al. 2016).

To counteract AMP-induced ROS, antioxidant defenses detoxify harmful reactive species through different enzymatic (superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)) as well as non-enzymatic molecules, such as the abundant thiol glutathione (GSH) (Michiels et al. 1994; Dringen 2000). An *in vitro* study reported that METH treatment (2mM, 12 hours) decreased GSH levels in N27 rat dopaminergic cells (Chandramani Shivalingappa et al. 2012). In male Wistar rats, chronic MPH (21 days, i.p.) reduced glutathione

at higher doses (10mg/kg and 20 mg/kg) but not lower doses (2 mg/kg and 5 mg/kg) in hippocampal mitochondria (Motaghinejad et al. 2016).

1.7.3 Markers of Autophagy

Microtubule-associated protein light chain 3 (LC3) is expressed as 3 splice variants, LC3A, LC3B, LC3C. Specifically, LC3B plays a prominent role in the membrane structure of autophagosomes and can be considered a marker of autophagy (Wu et al. 2006). METH, even at low doses, impairs autophagy processes that can result in DA dysregulation and neurotoxicity (Limanaqi et al. 2021). *In vitro*, autophagy is rapidly upregulated in response to low-dose METH (1 μM) (Castino et al. 2008). Additionally, immortalized rat mesencephalic dopaminergic cells (N27 cells) treated with METH (2mM; 12 hours) were reported to have elevated LC3-II levels (Chandramani Shivalingappa et al. 2012).

1.7.4 Markers of Astrogliosis

Reactive astrogliosis is a response of astrocyte activation and considered a ubiquitous reaction to CNS injury that can be used as a sensitive marker of neuronal damage (Pekny and Pekna 2014). Glial fibrillary acidic protein (GFAP) is a protein that is upregulated in reactive astrogliosis and is commonly used as a marker of astrocytes (Yang and Wang 2015). Amphetamines have been shown to induce a significant microglial response that leads to the release of proinflammatory cytokines and activation of inflammatory signaling cascades (Cunha-Oliveira et al. 2013) that may be regulated by DA quinones (Loftis and Janowsky 2014; Thomas et al. 2008). Acute neurotoxic dosing of amphetamines was reported to upregulate GFAP levels in the striatum of male rodents (O'Callaghan and Miller 1994; Halpin, Northrop, et al. 2014). These effects can be long-lasting, as heightened GFAP levels can persist at least 30 days after the end of an acute neurotoxic METH binge treatment (10 mg/kg, s.c.) in young male Sprague-

Dawley rats (Friend and Keefe 2013) and after 2 years of abstinence in adults with chronic abuse of METH (Sekine et al. 2008). Repeated use of amphetamines can also induce astrogliosis, as rats that underwent METH self-administration (0.1 mg/kg/infusion, daily 15 h sessions for 5-8 days) exhibited increased GFAP levels in the striatum and cortex that lasted up to 7 days after drug cessation (Krasnova et al. 2016). Basmadjian *et al.* noted that chronic d-AMP administration in adult male Wistar rats increased GFAP expression in the prelimbic PFC that persisted up to 21 days after injections (Basmadjian et al. 2021).

1.7.5 Spectrin breakdown products

The cleavage of alpha-spectrin by calpain and caspase-3 yield breakdown products (SBDP) with the molecular weights of 150 and 145 kDa and 120kDa, respectively. These breakdown products are typically associated with excitotoxic cell death (Yan et al. 2012). Calpains are a calcium (Ca^{2+})-dependent non-lysosomal cysteine protease enzymes that are activated in the context of excitotoxicity in response to significant increases in intracellular Ca^{2+}. METH induces Ca^{2+}-dependent activation of proteases, such as calpains, to mediate long-term structural damage to dopaminergic terminals (Staszewski and Yamamoto 2006). A neurotoxic binge regimen (10 mg/kg every 2 hours, 4X, i.p.) of METH in male Sprague Dawley rats METH produced a substantial increase in calpain-specific spectrin breakdown (Halpin, Northrop, et al. 2014). Cysteine-aspartic proteases, or caspases, are protease enzymes that play in important role in apoptosis (Porter and Jänicke 1999). Caspase-3 is an executioner caspase that is essential in cell apoptosis (Wang et al. 1998) and *in vitro*, have been demonstrated to be robustly increased after METH treatment (Warren et al. 2007).

1.7.6 Epigenetic modifications

Repeated use of amphetamines can elicit epigenetic alterations that regulate gene transcription and have been found to play a role in learning, memory and reward processing (Kalda et al. 2007; Day and Sweatt 2010). DNA methylation and histone modifications are important epigenetic components that are implicated in transcriptional silencing (Bird and Wolffe 1999). Histone methylation can cause readily reversible changes in heterochromatin, whereas DNA methylation exhibits long-lasting gene repression, making it a stable epigenetic marker (Cedar and Bergman 2009). Mitochondrial DNA (mtDNA) copy number is a measure of the number of mitochondrial genomes per cell and is a marker of mitochondrial biogenesis and overall health (Phillips et al. 2014; Longchamps et al. 2020). mtDNA copy number has been associated with neurodegenerative diseases, aging and age-related disorders (Zhang et al. 2017; Pyle et al. 2016). In a mouse model in which mtDNA levels were genetically manipulated, the upregulation of mtDNA copy number was able to improve mitochondrial bioenergetics (Filograna et al. 2019).

In vitro, METH treatment (1.65 mM; 48 h) lowered mtDNA copy numbers (Wu et al. 2007). Additionally, male Long Evans rats that received chronic d-AMP treatment (1 mg/kg/day for 14 days), had increased global DNA methylation that persisted at least two weeks after injection cessation (Mychasiuk et al. 2013). KDM6A, a histone lysine demethylase, has been implicated in aging and aging-related disease, however little research has been conducted in evaluating its role in chronic psychostimulant exposure (Davis et al. 2020). Interestingly, Sadakierska-Chudy *et al.* discovered that male rats that underwent a cocaine self-administration paradigm had increased KDM6A levels (Sadakierska-Chudy et al. 2017).

1.8 Does aging affect these specific biochemical and epigenetic markers?

Certain areas of the brain, such as the cortex, striatum, cerebellum and hippocampus are particularly vulnerable to aging mechanisms and are associated with progressive functional decline (Feng et al. 2020; Lee et al. 2019). In humans, healthy aging is accompanied with a decline in DA signaling as evidenced by decreased DA receptors, DAT and TH, a reduction in DA release and loss of dopaminergic neurons, particularly in brain areas vulnerable to neurodegeneration, including the substantia nigra and striatum (Kaasinen and Rinne 2002; Gasiorowska et al. 2021). Aging changes Ca^{2+} homeostasis regulation and excessive calcium influx from glutamate-induced excitotoxicity can increase spectrin proteolysis and spectrin breakdown products that can lead to cell degeneration (Kaiser et al. 2005; Peterson et al. 1991). The aging striatum and hippocampus are particularly susceptible to reactive astrogliosis and age-related increases GFAP has been reported in humans and rodents (Nichols et al. 1993; Clarke et al. 2018). Additionally, there are age-associated decreases in macroautophagy which can exacerbate cellular impairments (Aman et al. 2021; Yu et al. 2017). Aging is also associated with increased oxidative stress, as the accumulation of oxidative damage induces a pro-oxidant shift in cellular reduction-oxidation (redox) state (Sohal and Orr 2012; Sohal and Forster 2014). Age-induced oxidative stress exacerbates lipid peroxidation level and as a result there is greater TBARS formation and lower antioxidant levels (Balkan et al. 2002; Venkateshappa et al. 2012).

There is evidence of age-associated impairments in epigenetic mechanisms as aging has been shown to correlate with mtDNA copy number, in which a progressive reduction in mtDNA copy number is observed (Filograna et al. 2021; Jones et al. 2015). DNA methylation is significantly correlated with aging and exhibits global hypomethylation (Xiao et al. 2016).

Overall, aging cause prominent alterations in several different biological processes that can have detrimental effects on health outcomes.

1.9 Rationale for use of METH in our studies

For our studies, we chose to use methamphetamine (METH) as a prototypical psychostimulant because of its structural similarity to amphetamine and the extensive research conducted on its neurotoxic effects *in vitro* and *in vivo* (Wallace et al. 1999; Yamamoto and Raudensky 2008; Halpin, Collins, et al. 2014). Although clinical use is limited, METH is legally available as the FDA-approved drug, Desoxyn, for the treatment of ADHD and obesity. METH has similar pharmacokinetics to amphetamine, however the addition of an N-methyl group to the amphetamine structure increases its lipophilicity and may cause it to cross the blood brain barrier more readily compared to amphetamine, contributing to its higher potency. Regardless of route of administration, the plasma half-life of METH is approximately 10 hours and ~70% of METH is excreted by the urine in 24 hours, with 30-50% as METH and 35-44% as unchanged amphetamine (Poklis et al. 1998). Between 30-54% of an oral dose is excreted in urine as unchanged methamphetamine and 10-23% as unchanged amphetamine. Following an intravenous dose, 45% is excreted as unchanged parent drug and 7% amphetamine. The biological half-life has been reported in the range of 4 to 5 hours. Approximately 70% of a methamphetamine dose is excreted in the urine within 24 hours: 30–50% as methamphetamine, up to 15% as 4-hydroxymethamphetamine and 10% as amphetamine. The terminal plasma half-life of methamphetamine of approximately 10 hours is similar across administration routes, but with substantial inter-individual variability. Acute effects can persist for up to 8 hours following a single moderate dose of 30 mg (Cruickshank and Dyer 2009).

1.10 Goals of current research

Previous research suggest that long-term abuse of amphetamine and amphetamine-type stimulants can elicit neuroadaptations that can impair dopaminergic systems and lead to adverse consequences in functional outcomes as well as increased vulnerability to addiction. It is hypothesized that amphetamine neurotoxicity involves a complex interplay of biological mechanisms, including oxidative stress, excitotoxicity, neuroinflammation and epigenetic changes. It is postulated that healthy aging acts on similar mechanisms to METH-induced neurotoxicity and therefore may exacerbate and lead to an accelerated aging phenotype. The current studies used an *in vivo* rodent model involving chronic dosing of METH, at doses designed to emulate the therapeutic dosing range of Desoxyn, that underwent behavioral testing for cognitive, motor and affective function as well as abuse potential at approximately 2 weeks or 5 months after the end of METH treatment. Biochemical tests were performed after behavioral testing, and brain concentrations of markers of dopaminergic function (DAT, TH), glutamate-mediated excitotoxicity (SBDP145, SBDP120), oxidative stress (TBARS, tGSH), astrogliosis (GFAP), macroautophagy (LC3B), and epigenetics (DNA methylation, mtDNA copy number, KDM6A) were measured. The goal of the current study was to address the possibility that even at low, clinically relevant doses not expected to produce neurotoxicity, chronic METH exposure could lead to age-associated functional losses and increased susceptibility to potential abuse.

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CHAPTER 2

SEX DIFFERENCES IN NEUROBEHAVIORAL CONSEQUENCES OF METHAMPHETAMINE EXPOSURE IN ADULT MICE

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Accepted to Psychopharmacology; 2022 Mar 26 https://doi.org/10.1007/s00213-022-06122-8

The authors declare that they have no conflict of interest.

Acknowledgements: This work was supported by National Institutes of Health/National Institute on Aging T32 AG020494 and a seed grant from University of North Texas Health Science Center Research Office.

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2.1 Abstract

Recreational and medical use of stimulants is increasing, and their use may increase susceptibility to aging and promote neurobehavioral impairments. The long-term consequences of these psychostimulants and how they interact with age have not been fully studied.

Our study investigated whether chronic exposure to the prototypical psychostimulant, methamphetamine (METH), at doses designed to emulate human therapeutic dosing, would confer a pro-oxidizing redox shift promoting long-lasting neurobehavioral impairments.

Groups of 4-month-old male and female C57BL/6J mice were administered non-contingent intraperitoneal injections of either saline or METH (1.4 mg/kg) twice a day for 4 weeks. Mice were randomly assigned to one experimental group: (i) short-term cognitive assessments (at 5 months), (ii) long-term cognitive assessments (at 9.5 months), and (ii) longitudinal motor assessments (at 5, 7 and 9 months). Brain regions were assessed for neurotoxicity after the behavior testing. Chronic METH exposure induced short-term effects on associative memory, gait speed, dopamine (DA) signaling, astrogliosis in females and spatial learning and memory, balance, DA signaling, and excitotoxicity in males. There were no long-term effects of chronic METH on cognition, however, it decreased markers of excitotoxicity in the striatum and exacerbated age-associated motor impairments in males.

In conclusion, cognitive and motor function were differentially and sex-dependently affected by METH exposure, and oxidative stress did not seem to play a role in the observed behavioral outcomes. Future studies are necessary to continue exploring the long-term neurobehavioral consequences of drug use in both sexes and the relationship between aging and drugs.

2.2 Introduction

There is a significant rise in the misuse of licit amphetamine type stimulants such as Adderall (l- and d-amphetamine mixed salts) or Ritalin (methylphenidate) (UNODC 2021; Weyandt et al. 2016)Although amphetamine stimulants are approved by the Food and Drug Administration (FDA) for medical use to treat ADHD or narcolepsy, and have proven to be safe and efficacious in children and adults when used as prescribed (Goodman et al. 2005; Spencer et al. 2007; Buitelaar et al. 2009), they can have significant adverse side effects and are classified as Schedule II drugs for a high potential of recreational abuse liability, dependence, and neurotoxicity. Amphetamine use is highest among young adults compared to other age groups (SAMHSA 2019) and in the last decade, recreational amphetamines have become the second most used drug by American college students and these numbers are potentially adding to the future at-risk population (Johnston et al. 2020).

While there is some evidence that acute use of low-dose amphetamine type stimulants leads to low or modest cognitive improvement, the reported cognitive outcomes of chronic use of stimulant medication are inconsistent (Repantis et al. 2010; Ilieva et al. 2013; Benson et al. 2015; Weyandt et al. 2016; Wilens et al. 2017; Weyandt et al. 2018). The long-term use of these drugs has not been systematically evaluated in controlled clinical trials, and preclinical studies in rodents suggest long-term cognitive consequences (Kamei et al. 2006; LeBlanc-Duchin and Taukulis 2009) and molecular changes (Hotchkiss and Gibb 1980) in moderate doses not expected to produce neurotoxic effects. Adults can be prescribed higher doses of prescription stimulants compared to children, even though the elimination half-life of amphetamines is much longer in adults and could increase the likelihood of adverse events (Berman and Hastings 1999). Unfortunately, there is very little research examining the long-term effects of chronic use of

amphetamines. For example, for Adderall, the FDA has stated that there have been no controlled trials longer than 4 weeks in adults. Therefore, it is imperative to conduct research on prolonged stimulant exposure in adults.

The abuse liability and arousal/attention-enhancing actions of psychostimulants are related to their ability to activate limbic and cortical brain reward pathways involving dopamine, serotonin, and glutamate. These systems produce oxidative stress via several mechanisms, most notably through dopamine accumulation which can undergo autooxidation into harmful quinones and reactive oxygen species (ROS) and metabolism (Graham 1978; Slivka and Cohen 1985; Stokes et al. 1999). Certain areas of the brain, such as the cortex, striatum, cerebellum and hippocampus are particularly vulnerable to oxidative insults and aging (Dubey et al. 1996; Rebrin et al. 2007; Andersen et al. 2003). Psychostimulants could provide a "second hit" to exacerbate and accelerate neurodegeneration linked to oxidative stress and functional aging. Chronic exposure to clinically relevant doses of d-amphetamine have been shown to have collateral effects outside of brain reinforcement systems in generating ROS in other aging-vulnerable regions such as the hippocampus and striatum (Frey et al. 2006; da-Rosa et al. 2012). These collateral actions are thought to play a significant role in the long-lasting psychomotor and neuropsychological impairments associated with amphetamine neurotoxicity (Yamamoto and Raudensky 2008). Based on these observations, our hypothesis is that chronic psychostimulant use may confer a pro-oxidizing redox shift in brain areas susceptible to aging, therefore promoting neurobehavioral impairments and increasing risk for functional decline. For this study, we chose to use methamphetamine (METH) as a prototypical psychostimulant because of its structural similarity to amphetamine and the extensive research conducted on its neurotoxic effects *in vitro* and *in vivo* (Wallace et al. 1999; Yamamoto and Raudensky 2008; Halpin et al. 2014).

Furthermore, studies of abstinent METH users show impairments on neuropsychological tests of executive function, learning and memory (Chang et al. 2002; McCann et al. 2008; Cherner et al. 2010) indicating that METH-induced functional impairments can persist even after they have stopped actively using METH. Additionally, abstinent METH abusers also have significantly increased biochemical markers of plasma oxidative stress (Huang et al. 2013). Along with oxidative stress, other markers of METH neurotoxicity also associated with aging may be affected by METH exposure. For example, dopaminergic systems can be affected during aging as well as during METH exposure (Kaasinen and Rinne 2002; Gasiorowska et al. 2021). Aging is also associated with increases in reactive astrocyte phenotype (Clarke et al. 2018; Nichols et al. 1993), decreased macroautophagy (Yu et al. 2017), calcium homeostasis dysregulation and increased spectrin breakdown products (He et al. 2013), and changes in mtDNA copy numbers and DNA methylation (Jones et al. 2015).

The aim of the current study was to evaluate whether chronic exposure to METH during adulthood leads to short- and long-term functional and molecular consequences. We treated 4-month-old mice with chronic, low-dose METH for 4 weeks and after cessation of treatment, we performed three experiments to evaluate the short- and long-term cognitive effects as well as the longitudinal motor effects. A variety of independent behavioral tasks were employed to study the expected accelerated aging phenotype in several domains of cognitive, motoric and affective function. At the end of behavioral testing, we evaluated markers of dopaminergic function, excitotoxicity, calcium homeostasis, astrocyte activation, epigenetics, and autophagy to study the potential molecular changes associated with behavioral outcomes.

2.3 Materials and Methods

Animals

A total of 224 C57BL/6J male and female mice were purchased from Jackson Laboratories at 15 weeks of age and maintained in the UNT HSC Vivarium. The studies were approved by the Institutional Care and Use Committee and were in accordance with NIH guidelines for the Care and Use of Laboratory Animals. The mice were subcutaneously tagged with Rf ID for individual identification and group housed (3-4 mice/cage based on Sex and treatment) at 23 ± 1° C, under a 12-h light/dark cycle starting at 0700 and given ad libitum access to food (LabDiet-5LL2) and water. At approximately 3.5 months of age, the mice were randomly assigned to either the saline (SAL) or methamphetamine (METH) treatment group. The animals received intraperitoneal injections of METH (1.4 mg/kg) or 0.9 % NaCl twice daily for 5 days a week. Based on body surface area calculation (Human Equivalent Dose in mg/kg = animal dose in mg/kg x 3/37) (Reagan-Shaw et al. 2008), the human equivalent dose would equate to 0.23 mg/kg or 14 mg/day for a 60kg adult. This dose is within dosage range use for Desoxyn (FDA-approved ADHD prescription containing METH; 5-25 mg/day with 20-25mg/day as the effective dose for ADHD and 5-15mg/day for narcolepsy) or amphetamines recommended dosage around 12.5 mg/day. The mice were randomly assigned to one of 3 experiments and body weights were taken weekly (Figure, 1). **Experiment I** (short-term): Cognitive assessments were performed 12 days after injection cessation, when the mice were 5 months old (n = 12/treatment/Sex). **Experiment II** (long-term): Cognitive assessments were performed 6 months after injection cessation, when the mice were 9.5 months of age (n = 20/treatment/Sex, however 1 SAL-treated male and 2 METH-treated females died during the study). **Experiment III** (longitudinal motor assessments): Motor assessments were performed 2 weeks after injection cessation, and every two months afterwards

(13 days, 2.3 months, and 4.4 months after injection cessation), when the mice were 5, 7 and 9 months of age (n = 24/treatment /group, however 1 SAL-treated female and 1 METH-treated female died during the study). We were unable to use these mice for the final cognitive assessments due to COVID and the shutdown of our laboratories.

Behavioral Testing

Spatial Learning and Memory- During initial training, the mice learned the motor component of swimming and climbing onto a platform (data not shown), using a plastic tank (diameter: 120 cm, height: 60 cm) filled with 34 cm of water opacified with nontoxic Crayola paint, maintained at 24±1°C and covered with a black curtain. The mice had to reach the hidden platform at the end of a straight alley (10x65 cm) within 60 s. Two sessions of 5 trials (Inter-Trial Interval (ITI) of 5 min) each were conducted on Friday then Monday. For testing, the curtain was removed, and the mice had to find a platform (10x10 cm) submerged 1.5 cm below the surface of the water. They had 4 starting locations and given 90 s to find the platform. Once a mouse reached the platform, it remained on the platform for 30 s before being put back into the carrier, for a 90 s ITI. Each session consisted of 5 platform trials, with daily sessions conducted over 4 days. During the probe trial (5^{th} trial of sessions 2 and 4) conducted over 30s, the platform was inaccessible to the mice. At the end of the trial the platform was raised back up and the mouse was given a maximum of 60 s to find the platform. A computerized tracking system (ANY-maze, Stoelting, Chicago, IL) was used to record path length, speed, and time spent within the 40 cm diameter annuli surrounding the platform location.

Discriminated Avoidance- The T-maze was constructed of acrylic black sides and a clear top that rests on a grid floor wired to distribute 0.69 mA of scrambled shock to the feet. On the first trial of the first session, the start box door was opened and the mouse received a shock when it

entered an arm until it ran to the opposite arm, which was then designated the correct arm for the remainder of the session. On subsequent trials, shock was initiated 5s after the opening of the start door if the mouse did not enter the correct goal arm, or immediately upon entry into the incorrect arm (60 s max time). Trials continued at 1 min intervals until the mouse met the criterion of a correct avoidance (running directly to the correct arm within 5 s) on four of the last five training trials. The second session was a reversal task in which the mice were required to run to the goal arm opposite than that which they were previously trained on. The number of trials to reach avoidance criterion was used as a learning measure.

Fear Conditioning- The apparatus was constructed of plexiglass walls (19 x 18 x 24 cm) that rested on a grid floor wired to distribute a 0.69 mA shock to the feet. The test cage was enclosed in a dimly-lit (35 lux), sound-attenuated chamber with a fan that provided background noise. A camera was mounted on the ceiling of the apparatus and tracked freezing behavior through a software program (ANY-maze, Stoelting, Chicago, IL). Day 1 was the conditioning test (session 1) in which the mouse was placed in an unfamiliar environment (conditioning context) consisting of a grid floor and white walls with black, vertical stripes. In a 5-min session, the mouse was presented with 2 pairings of a loud sound (conditioned stimulus; 2000 Hz) and a brief foot shock (unconditioned stimulus; 0.69 mA for 2 s). 24 hours later was the context conditioning test (session 2) in which the mice returned to the now familiar context in a 5-min session with no conditioned or unconditioned stimulus. One hour after the context conditioning test, was the novel context test (session 3) in which the mouse was placed in a novel environment consisting of a grey, smooth floor and grey walls for 3-min. The conditioned stimulus test (session 4) occurred immediately after the context conditioning test in which the conditioned stimulus was

presented for 3 min. Percentage of time freezing in each session was recorded by ANY-maze (Stoelting, IL) for analysis.

Gait Analysis- Testing was performed in a dark room using the CatWalk XT apparatus (Noldus Information Technology, the Netherlands). The walkway for data recording was set to an area of 8 cm by 32 cm that allowed for approximately 4 full step cycles and was calibrated before use. A run was defined as a crossing of the platform and only the compliant runs were used for data analysis with less than 10% speed variation between runs and less than 60% speed variation in under 5 s within a run. Five gait variables were analyzed: gait speed, swing speed, stride length, base of support and step cycle duration.

Spontaneous Locomotor Activity- Each mouse was placed in a clear acrylic test cage (40.5 × 40.5 × 30.5 cm) enclosed by a metal frame lined with photocells (Digiscan apparatus, Omnitech Electronics) under dim lights (23 lux) and with background noise (80dB) provided by a fan in a sound-attenuating chamber. During a 16-min period, movements in the horizontal and vertical planes were recorded by the photocells and processed by a software program (Fusion v5.5 Superflex Edition, Omnitech Electronics, Columbus, OH) to produce different variables of distance, vertical, and spatial components for analysis.

Bridge Walking- Balance was measured using a clear acrylic bridge (length: 60 cm; four different bridges 2 and 1 cm square and 2 and 1 cm round) that was suspended horizontally 50 cm above a foam pad between two platforms. The mouse was placed on one of the platforms for 5 s and then gently dragged to the middle of the bridge. Latency to fall (up to a maximum of 60 s) from the bridge was recorded for three trials and averaged across bridges.

Coordinated Running- Motor coordination was measured using a motor-driven accelerating rotorod (Accuscan Instruments, Model # AIO501RRT527M; AIO411RRT525M) with a nylon

cylinder (length: 45 cm, diameter: 3.2 cm) mounted horizontally at a height of 35.5 cm above a padded surface, with an acceleration of 0 to 75 rpm in 150 seconds. Each session consisted of four trials with 10 min ITI and a minimum of two hours between the two daily sessions. The mice were tested until they reached a stability criterion (three consecutive sessions by which the sessions mean latency to fall did not differ by more than 15%) which was calculated after the 7th session for the first time point, and after the 3rd session for the other two time points.

Biochemical Measurements

After behavioral testing, brains were dissected into brain regions (cerebral cortex, striatum, cerebellum, midbrain and hippocampus), snap frozen in liquid nitrogen, and saved at -80°C until further processing. Tissues were homogenized in antioxidant buffer (10 mM sodium phosphate, 0.9% sodium chloride, 200 uM DTPA and 1 mM BHT) containing protease inhibitor cocktail (Cell Signaling Technology 5872) and were centrifuged at 10,000 g for 10 min at 4°C. The supernatant was extracted, aliquoted and stored for future use. Protein concentration was determined using a BCA Assay Kit (Thermo Scientific 3225) and read at 562 nm using a Tecan Plate Reader F200.

Western Blotting- 25 µg of protein were loaded into 4-20% SDS-polyacrylamide gels (Biorad 4561096) and transferred to 0.45 µm nitrocellulose membranes (Biorad 162-0115). The blots were blocked at room temperature for 1 hr with 5% non-fat dry milk (Biorad 1706404) in TBS-T (1X TBS and 0.05% Tween-20) and probed with the primary antibodies overnight at 4°C: anti-GAPDH (1:5000; Cell Signaling Technology 97166); anti-GFAP (1:10000; Abcam ab7260), anti-Spectrin (1:1000; Abcam MAB1622), anti-DAT (1:1000; Sigma Aldrich AB2231), anti-LC3B (1:1000; Cell Signaling Technology 2775), anti-TH (1:1000; Cell Signaling Technology 13106). After three washes in TBS-T and the membranes were incubated with rabbit (1:5000;

Cell Signaling Technology 7074) or mouse (1:5000; Jackson ImmunoResearch 115-035-003) HRP-linked secondary antibody (1:5000; Cell Signaling Technology) for 1 hr at room temperature. Proteins levels on the membrane were visualized by enhanced chemiluminescent (ECL) detection with either West Pico (Thermo Scientific 34579) or West Femto (Thermo Scientific 34096) substrate and imaged using a BioSpectrum 500 UVP imaging system. ImageJ was used to quantify protein densities and results were normalized to the expression of GAPDH in the samples.

Oxidative stress measurements- Thiobarbituric acid-reactive substances (TBARS) were quantified using a TBARS Assay Kit (Cayman Chemical 10009055) read at 540 nm, according to manufacturer's instructions. Glutathione was quantified using an Assay Kit (Cayman Chemical 703002) read at 414 nm using the kinetic method where the plate was read every 5 min for 30 min, according to manufacturer's instructions.

mtDNA copy number- Mitochondrial DNA (mtDNA) copy number was assessed via quantitative PCR (qPCR) analysis. First, DNA was extracted from brain tissue samples using a Qiagen DNeasy Blood and Tissue Kit (Qiagen 69506), according to manufacturer's instructions, and DNA extracts were stored at -80°C. DNA samples were quantified using the Qubit BR Assay Kit (ThermoFisher Scientific Q32853) and normalized to a final concentration of 10 ng/µL with TE buffer. Each sample was assayed for mtDNA copy number via the ND1 gene and nuclear DNA (nuDNA) copy number via the HK2 gene. QPCR was performed in duplicate for each target using 2 µL of DNA per well. DNA samples were added into the wells of a 96 well plate (ThermoFisher Scientific 4326659) along with 12.5 µL of SYBR Green Master Mix (2X) (ThermoFisher Scientific 4309155), 8 µL of molecular grade water, and 1.25 µL each of the respective forward and reverse primer (ND1 F: 5'-CTAGCAGAAACAAACCGGGC-3' and

ND1 R: 5’-CCGGCTGCGTATTCTACGTT-3’) (HK2 F:5’-GCCAGCCTCTCCTGATTTTAGTGT-3’ and HK2 R: 5’-GGGAACACAAAAGACCTCTTCTGG-3’) (Quiros et al. 2017). QPCR amplification was performed using a 7500 Real Time PCR System (Applied Biosystems). Analysis of qPCR data was performed via the ΔΔCt quantification method to obtain mtDNA fold change relative to nuDNA copy number.

DNA methylation- DNA Methylation of the samples were quantified using MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric) (Eigentek P-1030), according to the manufacture’s instruction. The input DNA amount was 100 ng for each assay. The methylated fraction of DNA was quantified colorimetrically by reading the absorbance in a microplate spectrophotometer at 450 nm. Optical density (OD) intensity values read were proportional to the amount of methylated DNA. A standard curve was generated using methylated DNA standards provided in the kit. After confirming the negative control readings, the value (% DNA methylation) for each sample was determined as a ratio of the sample's OD relative to the standard's OD.

Statistical analyses

Data is presented in the form of mean ± standard error mean (SEM). Performance on behavioral (except rotorod and body weights) and biochemical tests was assessed using two-way analyses of variance (ANOVA) with the between-group factors of Sex and Treatment (Tx). Measures of body weight and rotorod were evaluated with three-way ANOVAs (with Weeks (Wk) or Sessions (Sess) as repeated measures). Significant main effects or interaction were assessed followed by individual degree-of-freedom F tests, in which the overall ANOVA error term was involved, to assess individual comparisons (symbols on graphs). The α level was set at 0.05 for

all analyses. The software used for the analyses was Systat 13 (Systat Software Inc., San Jose, CA, USA).

2.4 Results

Body Weights

The effects of chronic METH treatment and Sex on body weights are presented in Figure 2. Overall, females weighed less than males (Exp. I: $F(1,44)_{Sex} = 530.397$, $p < 0.001$; Exp. II: $F(1,74)_{Sex} = 332.573$, $p < 0.001$; Exp. III: ($F(1,89)_{Sex} = 435.01$, $p < 0.001$) and all mice gained weight as they aged (Exp. I: $F(8,352)_{Wk} = 99.648$, $p < 0.001$; Exp. II: $F(29,2146)_{Wk} = 463.012$, $p < 0.001$) and Exp. III: ($F(22,1958)_{Wk} = 365.593$, $p < 0.001$). Of note, there were significant interactions in Experiment I: $F(1,44)_{Sex \times Tx} = 6.136$, $p < 0.017$; $F(8,352)_{Wk \times Sex} = 5.49$, $p < 0.001$; $F(8,352)_{Wk \times Tx} = 2.588$, $p = 0.009$; Experiment II: $F(29,2146)_{Wk \times Sex} = 23.016$, $p < 0.001$ and Experiment III: ($F(22,1958)_{Wk \times Sex} = 4.987$, $p < 0.001$; $F(22,1958)_{Wk \times Tx} = 2.742$, $p < 0.001$. In all experiments, there were minor effects of the treatment on body weights that were supported by interactions between Weeks, Sex and Treatment in all experiments (Exp I: $F(8,352)_{Wk \times Sex \times Tx} = 2.318$, $p = 0.020$; Exp. II: $F(29,2146)_{Wk \times Sex \times Tx} = 2.549$, $p < 0.001$; Exp. III: $F(22,1958)_{Wk \times Sex \times Tx} = 1.609$, $p = 0.036$).

Morris Water Maze

Experiment I. The short-term effects of METH on measures of spatial learning and memory are presented in Figure 3 (left panels). During initial learning (average of Sessions 1 and 2), there was no effect of sex or treatment on latency (all Fs(1,44) > 0.100, all ps > 0.230) or path length (all $Fs(1,44) > 0.040$, all ps > 0.450). During the maximum performance phase (average of sessions 3 and 4), the METH groups took longer latencies and path length to reach the platform,

supported by main effects of Treatment ($F(1,44)_{Tx} = 6.97$, $p = 0.011$; $F(1,44)_{Tx} = 7.666$, $p = 0.008$), especially in the males (all ps < 0.04 post hoc). However, there was no significant interaction for either measure ($F(1,44)_{Sex\ x\ Tx}=0.141$, $p = 0.709$, $p = 0.309$; $F(1,44)_{Sex\ x\ Tx}=0.227$, $p = 0.636$). During initial learning, there was a main effect of Sex on speed ($F(1,44)_{Sex} = 15.392$, $p < 0.001$) in which males (regardless of treatment) swam faster than females, but no main effect of treatment or interaction ($F(1,44)_{Tx} = 0.821$, $p = 0.370$; $F(1,44)_{Sex\ x\ Tx}=0.031$, $p = 0.861$). This effect was not present during maximum performance ($F(1,44)_{Sex} = 1.262$, $p = 0.267$). There was no effect of Sex or Treatment on spatial bias (Table 1) during either sessions (all Fs(1,44) > 0.010, all ps > 0.400).

Experiment II. The long-term effects of METH on measures of spatial learning and memory are presented in Figure 3 (right panels). During initial learning, females had higher latencies and longer path length than males, supported by main effects ($F(1,74)_{Sex} = 20.767$, $p < 0.001$; $F(1,74)_{Sex} = 9.307$, $p = 0.003$ respectively), and there was no main effects of treatment or interactions ($F(1,74)_{Tx}=0.001$, $p = 0.980$; $F(1,74)_{Sex\ x\ Tx}=0.486$, $p = 0.488$; $F(1,74)_{Tx}=0.501$, $p = 0.481$; $F(1,74)_{Sex\ x\ Tx}=0.692$, $p = 0.408$). During maximum performance, the effect of Sex disappeared ($F(1,74)_{Sex}=0.031$, $p = 0.860$; $F(1,74)_{Sex}=0.877$, $p = 0.352$). Overall female swam faster than males during maximum performance only, supported by a main effect ($F(1,74)_{Sex}=10.091$, $p = 0.002$), especially within the METH group ($p = 0.012$ post hoc). There was no effect of sex or treatment on spatial bias (Table 1) during either sessions (all $Fs(1,44)>0.001$, all ps >0.190).

Active Avoidance

Experiment I. The short-term effects of METH on active avoidance performance are presented in Figure 4 (left panel). Sex or treatment did not have any effect on acquisition (all *Fs*(1,44) > 0.600, all *ps* > 0.110) or reversal (all *Fs*(1,44) > 0.015, all *ps* > 0.086).

Experiment II. The long-term effects of METH on active avoidance performance are presented in Figure 4 (right panel). There was no effect of sex or treatment during acquisition (all *Fs*(1,74) >0.110, all *ps* > 0.110) or reversal (all *Fs*(1,74) > 0.020, all *ps* > 0.590).

Fear Conditioning

Experiment I. The short-term effects of METH on freezing response are presented in Figure 5 (left panel). During the novel context (NC) session, there was no effect of sex or treatment on freezing behavior (all *Fs*(1,44) > 0.500, all *ps* > 0.210). In the novel context and conditioned stimulus (NC&CS) session, METH females spent 23% less time freezing compared to SAL females ($p = 0.014$ post hoc) while there was no difference in the males. These findings were supported by a significant interaction between Sex and Treatment ($F(1,44)_{\text{Sex x Tx}} = 4.553$, $p = 0.038$).

Experiment II. The long-term effects of METH on freezing response are presented in Figure 5 (right panel). During the NC session, females froze longer than males, supported by a main effect of Sex ($F(1,73)_{\text{Sex}}=6.693$, $p = 0.012$), especially SAL females spent 43% more time freezing compared to SAL males ($p = 0.005$ post hoc). There was no main effect of Treatment or interaction ($F(1,73)_{\text{Tx}} = 0.003$, $p = 0.958$; $F(1,73)_{\text{Sex x Tx}}=2.208$, $p = 0.142$). During the NC&CS session, there was no effect of sex or treatment on time spent freezing (all *Fs*(1,73) > 0.030, all *ps* >0.100).

Experiment III

Coordinated Running. The effects of METH on coordinated running performance are presented in Figure 6A. Overall females had longer latencies to fall than the males, regardless of treatment, supported by significant main effects of Sex (5 months: $F(1,92)_{Sex} = 8.914$, $p = 0.004$; 7 months: $F(1,91)_{Sex} = 11.108$, $p = 0.004$; 9 months: $F(1,89)_{Sex} = 13.505$, $p < 0.001$). At 5 and 7 months, there were no main effects of Treatment or interactions between any of the factors including sessions. However, at 9 months, males treated with METH fell off the rotating rod faster than SAL males and these observations were supported a significant main effect of Treatment ($F(1,89)_{Tx} = 4.511$, $p = 0.036$) followed by post hoc analyses.

Bridge Walking. The effects of METH on balance are presented in Figure 6B. Overall, females were able to stay on the balance beam longer than males, regardless of treatment, supported by main effects of Sex at 5 and 9 months but not at 7 months (5 months: $F(1,92)_{Sex} = 44.760$, $p < 0.001$; 7 months: $F(1,92)_{Sex} = 1.394$, $p = 0.241$; 9 months: $F(1,89)_{Sex} = 55.222$, $p < 0.001$). There were no main effects of Treatment or interactions at any of the timepoints (all $Fs(1,92)_{Tx} > 0.090$, all $ps > 0.150$; all $Fs(1,92)_{Sex \times Tx} > 0.020$, all $ps > 0.150$). At 5 months, METH males had 12% shorter latencies than SAL males ($p = 0.046$ post hoc).

Gait Measurements. The effects of METH on gait speed are presented in Figure 6C. At 5 months, METH females had 16% higher speed than SAL females and this was supported by a main effect of Treatment ($F(1,91)_{Tx} = 4.969$, $p = 0.028$) and followed by post hoc analyses ($p = 0.014$). This treatment effect was not observed at 7 ($F(1,91)_{Tx} = 3.408$, $p = 0.068$) or 9 months ($F(1,89)_{Tx} = 0.791$, $p = 0.376$). There were no main effect of Sex or interactions at any of the 3 time points (all Fs > 0.010, all ps > 0.180). Other measures of gait are presented in Supplementary-Table 2. At 5 months, main effects of Sex were observed for base of support

(BOS; front/hind) and stride length (front/hind) (all Fs(1,91) > 4.500, all ps < 0.040). At 5 months, effects of Sex were observed for BOS front ($F(1,91)_{Sex} = 4.734$, $p = 0.032$), stride length (front/hind) (all Fs(1,91) > 4.600, all ps < 0.035), swing speed (hind) ($F(1,91)_{Sex} = 17.407$, $p < 0.001$) and main effects of Treatment were observed for stride length (hind/front) (all Fs(1,91) > 4.500, all ps < 0.035), and swing speed (front) ($F(1,91)_{Tx} = 5.590$, $p = 0.02$). At 9 months, main effects of Sex were observed for BOS (hind) and stride length (front/hind) (all Fs(1,89) > 5.500, all ps < 0.020) and an interaction between Sex and Treatment for BOS (front) ($F(1,89)_{Sex \times Tx} = 6.108$, $p = 0.015$).

Locomotor Activity. The effects of METH on different measures of locomotor activity are presented in Figure 6D&E. At each time point, females had higher distances travelled compared to males (22-29%), supported by main effects of Sex (5 months: $F(1,92)_{Sex} = 11.061$, $p = 0.001$, 7 months: $F(1,92)_{Sex} = 4.334$, $p = 0.040$, 9 months: $F(1,89)_{Sex} = 14.574$, $p < 0.001$). Post-hoc analyses revealed that METH treated females traveled 22-29% more than METH males (all ps = 0.003) and at 9 months, SAL females traveled 21% more compared to SAL males ($p = 0.020$). There was no effect of Treatment or an interaction on distance (all Fs > 0.095, all ps > 0.300). There was no effect of sex, treatment or any interaction at any of the time points on rearing activity (all Fs > 0.010, all ps > 0.250)

Biochemical Measurements

Markers of Dopaminergic Function. The effects of METH on expression of DAT and TH are presented in Figure 7. In Experiment I, DAT expression in the striatum was increased in METH males compared to SAL males, but not in females. This observation was only supported by a main effect of treatment ($F(1,20)_{Tx} = 4.882$, $p = 0.039$) followed by post hoc analyses ($p =$

0.021), but not by an interaction ($F(1,20)_{Sex\ x\ Tx} = 1.785$, $p = 0.197$). In the hippocampus, METH males and SAL females had higher expression than SAL males. Only the main effect of Sex was significant ($F(1,20)_{Sex} = 4.891$, $p = 0.039$), though Treatment neared significance ($F(1,20)_{Tx} = 3.956$, $p = 0.061$; $F(1,20)_{Sex\ x\ Tx} = 2.731$, $p = 0.114$). Post hoc analyses revealed a significant difference for METH males ($p = 0.018$) and SAL females ($p = 0.013$) compared to SAL males. In the midbrain, SAL females had lower DAT expression than SAL males and METH females. This was supported by a main effect of Sex and an interaction ($F(1,20)_{Sex} = 5.197$, $p = 0.034$; $F(1,20)_{Sex\ x\ Tx} = 8.405$, $p = 0.009$). There was no effect of sex or treatment in any of the regions on TH expression (Striatum and Hippocampus, all Fs(1,19) < 1.400, all ps > 0.250; Cerebellum and Midbrain, all Fs(1,20) < 1.550, all ps > 0.230). While it seemed that treatment affected males and females differently in the cortex, it did not reach significance ($F(1,20)_{Sex} = 2.882$, $p = 0.105$; $F(1,20)_{Tx} = 3.554$, $p = 0.074$; $F(1,20)_{Sex\ x\ Tx} = 3.392$, $p = 0.080$).

In Experiment II, DAT expression was higher in females than males in the striatum and this was supported by a main effect of Sex ($F(1,20)_{Sex} = 4.366$, $p = 0.050$). In the cerebellum, METH treatment increased DAT expression by 26% in males and decreased it by 17% in females, supported by a significant interaction ($F(1,20)_{Sex\ x\ Tx} = 15.012$, $p = 0.001$) and post hoc analyses (all ps < 0.020). There was no effect of sex or treatment on TH expression in any of the regions (all Fs(1,20) < 2.500, all ps > 0.120).

Markers of Astrogliosis and Excitotoxicity. The effects of METH on astrogliosis, as measured by GFAP expression, and excitotoxicity, as measured by spectrin cleavage product 145 kDa, are presented in Figure 8. In Experiment I, females had decreased GFAP expression in the cortex compared to males ($F(1,20)_{Sex} = 6.813$, $p = 0.017$). A similar pattern was observed in the midbrain, but it failed to reach significance ($F(1,20)_{Sex\ x\ Tx} = 2.986$, $p = 0.099$). In the

hippocampus, METH females had lower GFAP expression than SAL females which was not observed in males ($F(1,20)_{Sex\ x\ Tx} = 5.120$, $p = 0.035$). There was no effect of sex or treatment on SBDP145 levels in any of the regions (all Fs (1,20) < 3.320, all ps > 0.080).

In Experiment II, females overall had lower GFAP expression than males in the cortex, hippocampus and cerebellum, which was supported by main effects of Treatment (all Fs(1,20) > 5.150, all ps < 0.040). Differential outcomes of the treatment were observed in males and females in the striatum, hippocampus and cerebellum (all Fs(1,20) > 4.500, all ps < 0.040). In these regions, SBDP145 expression was decreased in males and increased in females.

Marker of Apoptosis. The effects of METH on apoptosis (SBDP120 spectrin cleavage product) are presented in Supplementary-Table 3. SBDP120 levels were not affected by sex or treatment in any of the brain regions in Experiment I (all Fs(1,20) < 3.720, all ps > 0.060) or Experiment (all Fs(1,20) < 3.500, all ps > 0.070).

Marker of Autophagy. The effects of METH on autophagy (LC3B) are presented in Supplementary-Table 3. In Experiment I, there was no effect of sex or treatment on LC3B expression in any of the brain regions studied (all Fs(1,20) < 3.500, all ps > 0.060). In Experiment II, females had lower LC3B expression than males in the midbrain, which was supported by a main effect of Sex ($F(1,20)_{Sex} = 11.884$, $p = 0.003$) and METH treated males had lower expression of LC3B compared to SAL males in the cerebellum, with a main effect of Treatment that almost reached significance ($F(1,20)_{Tx} = 4.350$, $p = 0.050$).

Markers of Oxidative Stress. The effects of METH on total glutathione (tGSH) and TBARS are presented in Supplementary-Table 3. In Experiment I, a main effect of Sex, in which females had lower tGSH levels than males, was observed in the cortex ($F(1,20)_{Sex} = 18.807$, $p < 0.001$), especially in the SAL groups ($p = 0.001$ post hoc). In the striatum, an interaction approached

significance ($F(1,20)_{Sex\ x\ Tx} = 4.26$, $p = 0.052$) as METH treated females had higher tGSH levels compared to SAL females, an effect not seen in males. There was no effect of sex or treatment on TBARS in any of the regions (all Fs(1,20) < 1.910, all ps > 0.180). In Experiment II, there was a significant main effect of Sex in the cortex ($F(1,20)_{Sex} = 9.304$, $p = 0.006$) due to METH males having higher tGSH levels compared to METH females ($p = 0.003$ post hoc). There was no effect of sex or treatment on TBARS in any of the regions (all Fs(1,20) < 1.980, all ps > 0.170).

Epigenetic Markers. The effects of METH on mtDNA copy number and DNA methylation in all brain regions are presented in Figure 9 (striatum) and supplementary Table 4 (other regions). In the striatum, mtDNA copy numbers appeared higher in the METH treated mice than in the saline in Experiment I, however the main effect of Treatment did not reach significance ($F(1,19)_{Tx} = 3.827$, $p = 0.065$) (if we combine both sexes, ($F(1, 21)_{Tx} = 4.335$, $p = 0.050$) which suggests that METH treatment increased mtDNA copy number in the striatum. This effect was not observed in Experiment II ($F(1,20)_{Tx} = 0.008$, $p = 0.929$; $F(1,20)_{Sex\ x\ Tx} = 3.139$, $p = 0.092$). There was no effect of sex or treatment in any of the other regions in either experiment (all Fs < 2.650, all ps > 0.120).

Since the striatum was the only region where mtDNA seemed affected, DNA methylation was measured only in that region (Figure 9B). While females appeared to have lower levels of methylated DNA in the striatum in Experiment II, the main effect of Sex only approached significance ($F(1,20)_{Sex} = 3.873$, $p = 0.063$). And while it seemed that METH treated animals had higher DNA methylation levels, it was not supported by any statistics (all Fs(1,20) < 1.940, all ps > 0.170)

2.5 Discussion

The major findings were that chronic administration of low-dose METH in the short-term (1) impaired spatial learning and memory in males and associative learning in females, (2) increased gait speed in females and impaired balance in males, (3) increased DA signaling in the striatum, hippocampus and cortex of males and midbrain of females, (4) decreased astrogliosis in the hippocampus of females, (5) increased excitotoxicity in the hippocampus of males and (6) had no effect on oxidative stress. Chronic low-dose METH in the long-term (1) impaired motor coordination, (2) decreased excitotoxicity in the striatum of males and (3) had no effects on cognition, gliosis or oxidative stress.

In Experiment I, spatial learning and memory impairments were observed in males exposed to METH. Our findings are supported by previous literature in which male rats that received METH (2 mg/kg/day) for 5 days exhibited impairments in spatial reference memory up to 30 days after injection cessation (Bigdeli et al. 2015). However, the deficits observed in spatial learning and memory appear to be transient (Experiment II). Similarly, male rats treated with a binge METH dosing regimen (4 x 12.5mg/kg; 2 hr intervals, s.c.) exhibited impairments during Morris water maze acquisition 2.1 months post injection, but not 5.4 months post injection (Friedman et al. 1998), although they used a much higher dose and a different dosing regimen to ours. The lack of effect observed 4.5 months post injections may be due to the short-lived effect of chronic psychostimulant use. It is also likely that the anticipated aging acceleration is too subtle at the ages evaluated in this study to be detectable, as declines in water maze performance start at 16-18 months and in fear memory performance at 12 months (Yanai and Endo 2021; de Fiebre et al. 2006; Sumien et al. 2006).

Chronic use of amphetamines can cause neurotoxicity and long-lasting neurodegeneration of the nigrostriatal pathway, an important dopaminergic pathway that facilitates voluntary movement (Ares-Santos et al. 2014). In Experiment III, chronic, low-dose METH treatment impaired balance in males at 5 months of age, but the effects did not persist at 7 or 9 months of age. Daily injections of d-AMP (1.8 mg/kg, s.c.) for 5 days elicited motor memory impairments on the rotorod task starting 13 days after injection session and lasting up to 25 days into withdrawal in male CD1 mice (Pathak et al. 2015). We did not observe deficits in motor coordination until 9 months of age in METH-treated males, which may be due to differences in protocol or length/dose of amphetamine treatment. METH may have exacerbated age-related motor dysfunction in male mice, as the rotorod test performance gradually decreases with age (Sumien et al. 2009; Shoji et al. 2016).

While minor effects were observed from weeks to months after METH exposure, it was still important to determine if biochemical and molecular changes were present as they may precede behavioral changes. Furthermore, most studies were done using neurotoxic dosing, and while many studies have been done on acute outcomes, reports on outcomes of chronic METH exposure are sparse. Chronic, low-dose METH administration resulted in short-term increases in DAT and TH expression in the mesocorticolimbic system in Experiment I, which is supported by the literature (Shepard et al. 2006; D'Arcy et al. 2016). Although DAT downregulation and endocytosis is observed in longer treatment or high, neurotoxic dosing of METH (Wilson et al. 1996; Granado et al. 2010), there were no long-term changes in the expression of DAT in the mesocorticolimbic pathway in our study. Of note, chronic METH administration increased DAT expression in the cerebellum in males and but decreased it in females. There is evidence of some recovery of DAT in small studies of abstinent METH abusers (Wang et al. 2009; Boileau et al.

2016; Volkow et al. 2015) and preclinical studies (Ricaurte et al. 1984; Melega et al. 1996; Friedman et al. 1998).

One hypothesized mechanism involved in amphetamine-induced neurotoxicity is that increased oxidative stress promotes METH-association neurodegeneration, however, we did not find any major short or long-term changes in lipid peroxidation (TBARS) and redox status (tGSH) in the brains of METH-treated mice. Most studies that have found increased brain oxidative stress have been in chronic abusers of METH or in animals that received neurotoxic doses (Moszczynska et al. 2004; Fitzmaurice et al. 2006; da-Rosa et al. 2012). Most in vivo studies do not examine the effect of METH administration on oxidative stress parameters past 24 hours (McDonnell-Dowling and Kelly 2017). In our study, animals were euthanized approximately 2 weeks or 4.5 months after drug cessation and this may have given the animals enough time to recover. In studies that used an acute, neurotoxic dose of METH (10mg/kg) striatal TBARS, glutathione (GSH) glutathione disulfide (GSSG) and total glutathione (tGSH) were all increased, however all parameters normalized 24 hours after injection cessation (Harold et al. 2000; Flora et al. 2002). Of note, Flora et al. found acute increases in total glutathione in the frontal cortex and hippocampus that were still elevated up to 24 hours after METH administration. This could support our data, in which we found that males chronically injected with METH had increased tGSH in the hippocampus, 2 weeks after injection cessation. Increases in antioxidant activity may compensate for METH-induced oxidative stress and could potentially explain why we did not see increased oxidative stress in Experiment I. Frey et al. noted that SOD activity was increased in the hippocampus of male rats, even up to 7 days post injections (d-AMP; 1mg/kg/day for 7 days) (Frey et al. 2006) and antioxidant administration has been shown to

attenuate METH-induced oxidative stress (Huang et al. 2017; Hirata et al. 1998; Meng et al. 2020).

We examined other potential markers involved in cognitive and motor function, and neurodegeneration such as astrogliosis and calpain activation. GFAP expression was decreased in the hippocampus of females in the short-term cohort. This contrasts with previous literature of METH-induced GFAP activation (Friend and Keefe 2013; Shaerzadeh et al. 2018). One possible explanation could be that astrocytic estrogen (E2) regulated reactive gliosis in the brains of females. Estrogen has been shown to be neuroprotective and could mediate damage to the brain (Wang et al. 2020). This could also explain why GFAP levels were lower in the cortex and midbrain of females in both Experiment I and II as compared to males. The degradation of alpha-spectrin yields breakdown products (SBDP) with the molecular weights of 150 and145 kDa by calpain. These breakdown products are typically associated with excitotoxic cell death (Yan et al. 2012). We found that chronic METH administration induced persistent, long-lasting changes in calpain activity in the striatum of males. Calpain inhibition could act as a protective mechanism against METH and this is supported by the use of calpain inhibitors in TBI models to attenuate motor and learning impairments (Saatman et al. 2010). Short-term calpain overactivation was noted in the hippocampus of METH males, and excitotoxicity could explain the spatial learning deficits seen in males, but not females.

Epigenetic changes were also considered, but only an effect in the striatum was observed. mtDNA copy number is used as a marker of mitochondrial function and has been associated with neurodegenerative diseases (Pyle et al. 2016) and age-related disorders. In a mouse model in which mtDNA levels were genetically manipulated, the upregulation of mtDNA copy number was able to improve mitochondrial bioenergetics (Filograna et al. 2019). We found that in the

striatum in METH-treated males, there were short-term increases in mtDNA copy number, possibly as a result of a compensatory mechanism to METH-induced oxidative damage. In Experiment II, it appeared that METH decreased mtDNA copy number when males were approximately 9.5 months of age. Aging has been shown to correlate mtDNA copy number, in which a progressive reduction in mtDNA copy number is observed (Filograna et al. 2021) and METH may have exacerbated these age-associated epigenetic outcomes.

Our study found that behavioral and biochemical outcomes were different in male and female mice, especially in the short-term study. In humans, there are known sex differences in drug abuse, with women having lower rates of illicit drug use (Benson et al. 2015; Weyandt et al. 2016; Johnston et al. 2020), but using more of the drug, reaching dependence faster and having more adverse effects (Becker and Hu 2008). Preclinical animal models also exhibit sexual dimorphism in behavioral and biochemical responses to psychostimulant administration (Becker and Ramirez 1981; Bhatt and Dluzen 2005; Milesi-Hallé et al. 2007). In examining METH pharmacokinetics, female rats had lower clearance and increased excretion of METH and lower formation of the metabolite, amphetamine (Milesi-Hallé et al. 2005). Sex differences in pharmacokinetics may explain why female rats were more sensitive to stimulant effects of amphetamines (Castner et al. 1993; Simpson et al. 2012). Sex hormones may also play a role as 17β estradiol can modulate dopaminergic neurotransmission (Becker 1990; Fattore et al. 2008; Shams et al. 2016) and facilitate amphetamine-induced release of DA (Becker and Rudick 1999). There are basal sex differences in the dopaminergic system, with females having tighter regulation of DA release and clearance compared to males (Walker et al. 2000; Dluzen et al. 2008).

Although the focus of this study was on adult stimulant use, it is important to address that psychostimulant treatment to control ADD/ADHD are often started during adolescence. Studies comparing low-dose amphetamine like stimulants in young vs. adult rodents do suggest a greater sensitivity to low-dose psychostimulants in the younger animals at least in the short-term (Adriani et al. 1998; Martins et al. 2006). It is possible that if we had treated our mice at an earlier age that we would have seen more and longer-lasting impairments.

While this is a first study looking at the long-term effects of chronic, low-dose METH exposure on motor and cognitive function, it comes with a few caveats. As the effects observed were minor, the choice of dose may need to be revisited and increased. Furthermore, this is not a direct translation to studying widely prescribed ADHD medications, as they differ in lipophilicity and their bioavailability may impact neurobehavioral outcomes. The duration of the study could be expanded as we started observing accelerated motor impairments in males but not cognitive impairments which tend to appear later during normal aging. It would be of interest to also determine abuse liability to determine whether chronic exposure to METH in adulthood predispose the animals to have greater drug seeking behavior.

Chronic administration of the prototypical psychostimulant, METH, at low, clinically relevant doses induced short, but not long-term changes in cognition and neurodegenerative markers. It did induce long-term motor impairments. Chronic psychostimulant use did not confer a pro-oxidizing redox shift and may not play a role in promoting accelerated aging phenotype. Unfortunately, our knowledge of low-dose psychostimulants is limited and the literature on the interaction between aging and psychostimulant use is even more so. Further studies must be done on long-term consequences of these widely used amphetamine compounds in both Sexes to better understand safety in adults and an aging population.

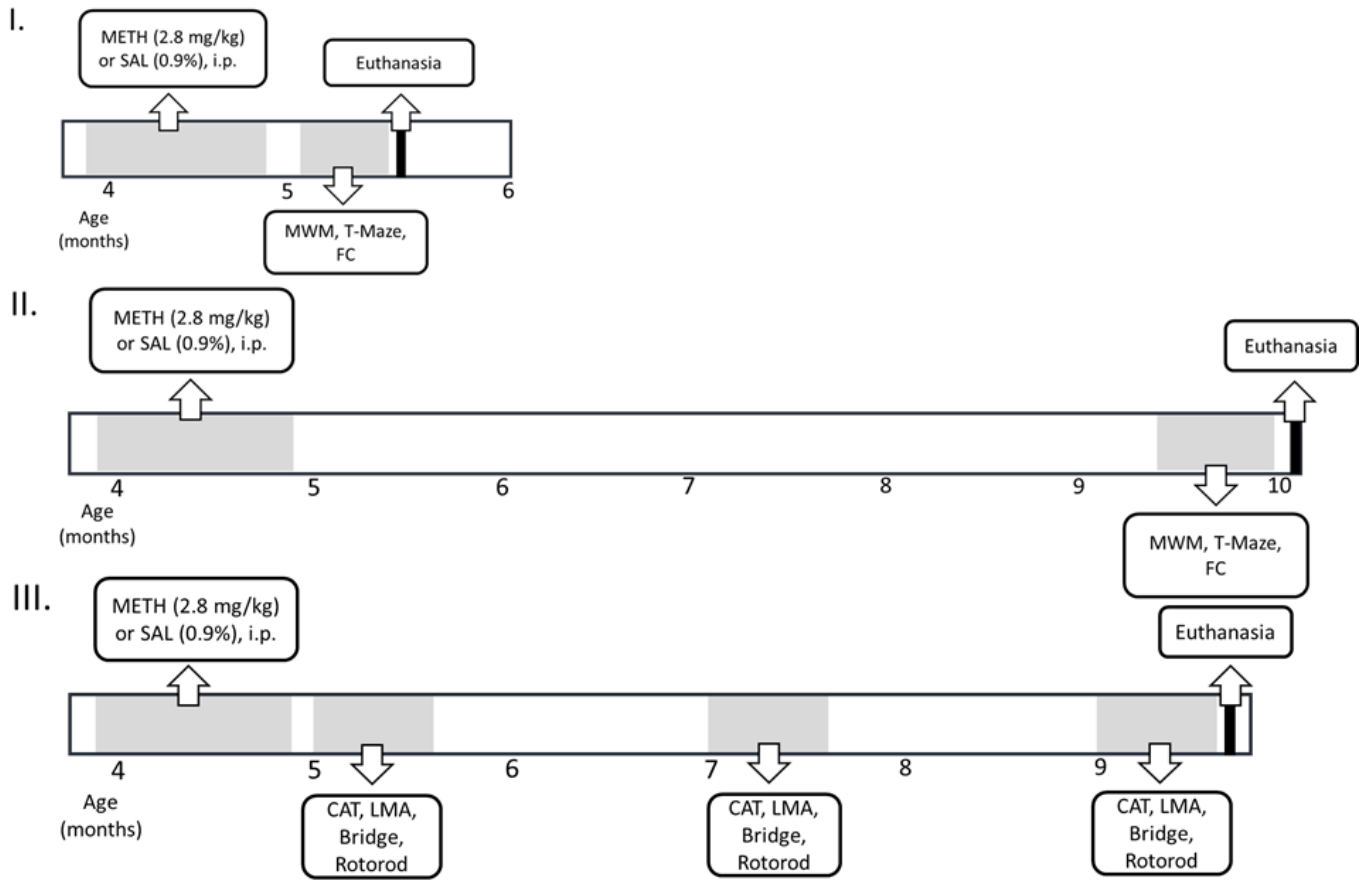

Figure 2.1 Experimental design for chronic METH administration in Experiment I (short-term), Experiment II (long-term) and Experiment III (longitudinal motor assessments). In all experiments, mice received non-contingent i.p. injections of either METH (2.8 mg/kg (+)-methamphetamine in 2 x 1.4 mg/kg injections) or SAL (0.9% saline) 5 days a week for 4 weeks In Experiment I, cognitive testing began at 5 months of age. In Experiment II, cognitive testing started at 9.5 months of age. In Experiment III**,** motor testing started at 5 months and occurred again at 7 and 9 months of age. MWM: Morris water maze, FC: Fear conditioning, CAT: Catwalk, LMA: locomotor activity.

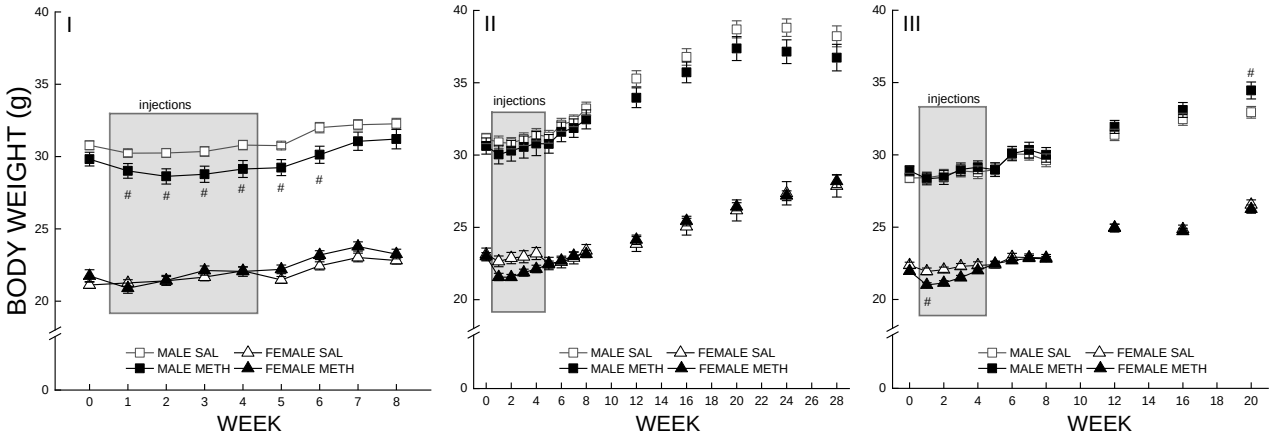

Figure 2.2 Effects of METH on body weights in male and female mice in Experiment I (short-term), Experiment II (long-term) and Experiment III (longitudinal motor assessments). Each value represents the mean ± SEM (Experiment I: n = 12; Experiment II: n = 19-20; Experiment III: n = 23-24). $^{\#}p < 0.05$ vs. sex-matched SAL.

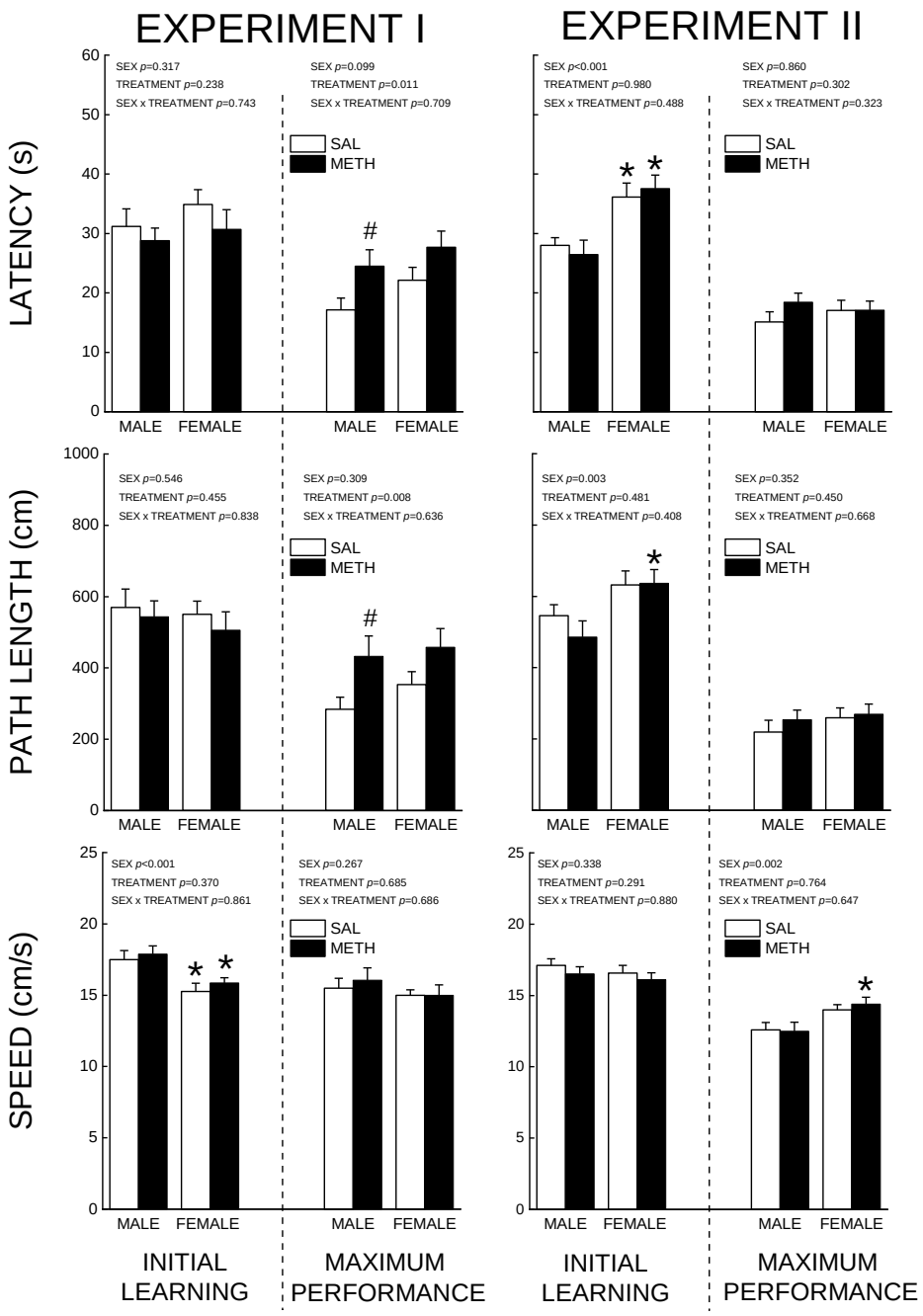

Figure 2.3 Effects of METH on spatial learning and memory in male and female mice as measured by latency and path length taken to reach the submerged platform and speed in Experiment I (short-term) and Experiment II (long-term). Each value represents the mean ± SEM (I: n = 12; II: n = 19-20). Post-hoc analyses: $^{\#}p < 0.05$ vs. sex-matched SAL; $^{*}p < 0.05$ vs. treatment-matched males.

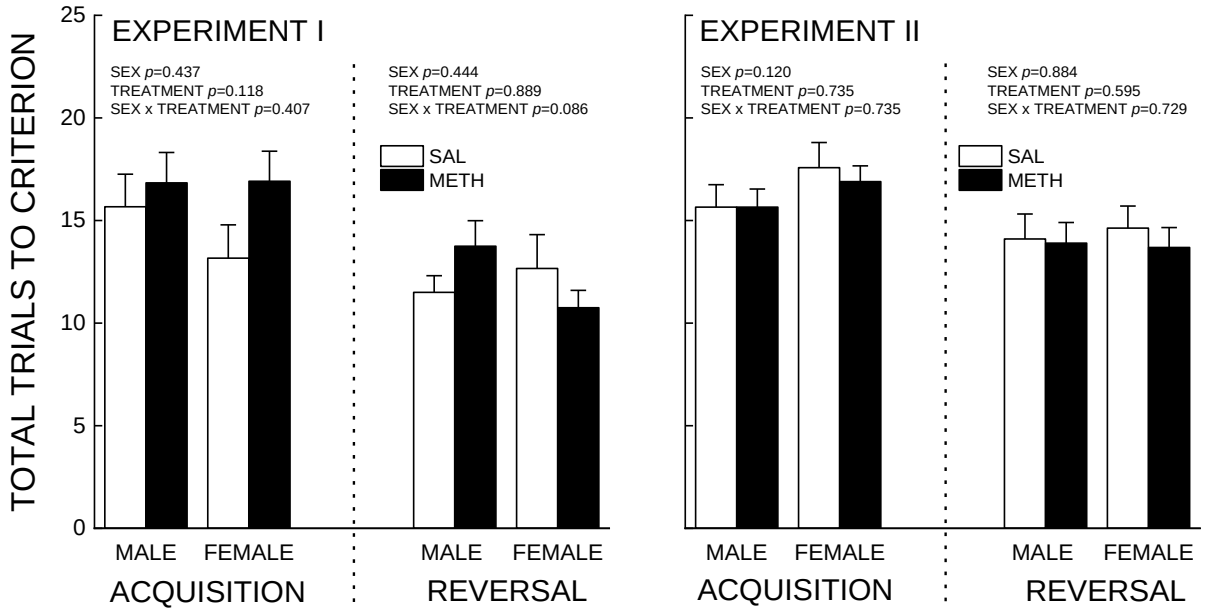

Figure 2.4 Effects of METH on learning (acquisition) and cognitive flexibility (reversal) as measured by the total trials to reach criterion in Experiment I (short-term) and Experiment II (long-term). Each value represents the mean ± SEM (I: n = 12; II: n = 19-20).

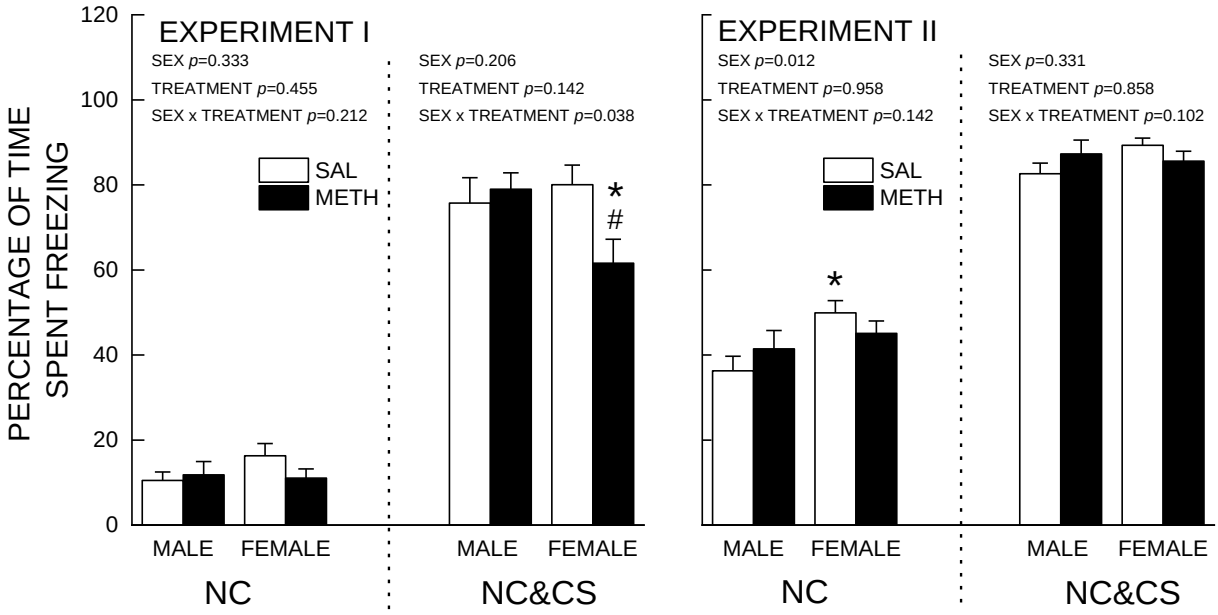

Figure 2.5 Effects of METH on freezing response during the novel context (NC) and the novel context and conditioned stimulus (NC&CS) sessions of the fear conditioning test in Experiment I (short-term) and Experiment II (long-term). Each value represents the mean ± SEM (I: n = 12; II: n = 19-20). Post-hoc analyses $^{\#}p < 0.05$ compared to sex-matched control. $*p < 0.05$ compared to treatment-matched males.

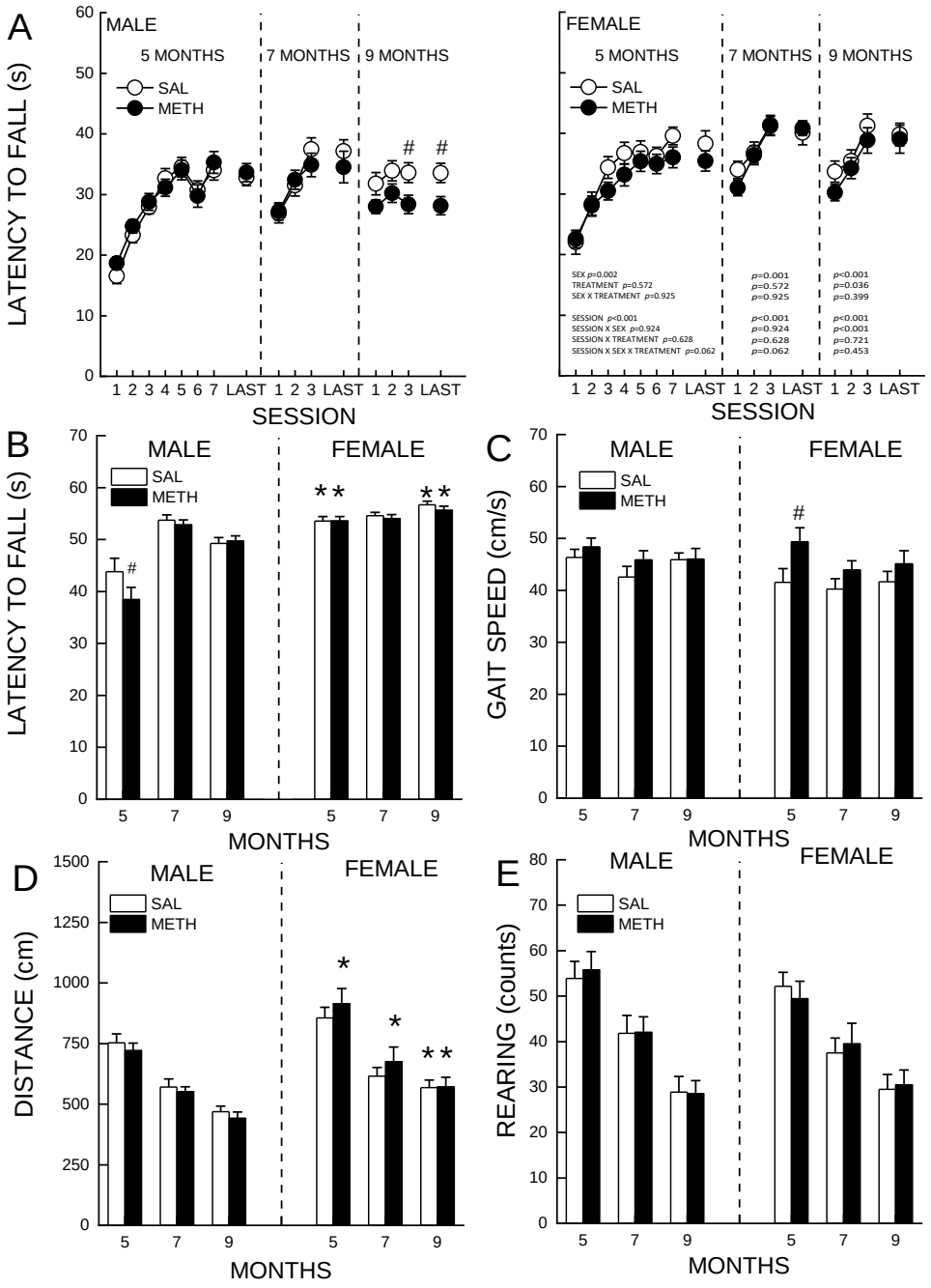

Figure 2.6 The effect of METH on coordinated running (A), balance (B), gait speed (C), distance travelled (D) and rearing (E) in male and female mice. Each value represents the mean ± SEM (n = 22-24). Post hoc analyses: $^{\#}p < 0.05$ compared to Sex-matched control. $^{*}p < 0.05$ compared to treatment-matched males.

Balance (C): 5 months: SEX $p<0.001$, TREATMENT $p=0.160$, Sex x TREATMENT $p=0.152$; 7 months: SEX $p=0.241$, TREATMENT $p=0.425$, Sex x TREATMENT $p=0.884$; 9 months: SEX $p<0.001$, TREATMENT p 0.757, Sex x TREATMENT $p=0.408$. Gait Speed (D): 5 months: SEX $p=0.396$, TREATMENT $p=0.028$, Sex x TREATMENT $p=0.186$; 7 months: SEX $p=0.263$, TREATMENT p 0.068, Sex x TREATMENT $p=0.919$; 9 months: SEX $p=0.199$, TREATMENT $p=0.376$, Sex x TREATMENT $p=0.414$. Distance (E): 5 months: SEX $p=0.001$, TREATMENT $p=0.756$, Sex x TREATMENT $p=0.316$; 7 months: SEX $p=0.040$, TREATMENT $p=0.608$, Sex x TREATMENT $p=0.333$; 9 months: SEX $p<0.001$, TREATMENT $p=0.707$, Sex x TREATMENT $p=0.631$. Rearing (F): 5 months: SEX $p=0.273$, TREATMENT $p=0.913$, Sex x TREATMENT $p=0.536$; 7 months: SEX p 0.368, TREATMENT $p=0.764$, Sex x TREATMENT $p=0.819$; 9 months: SEX $p=0.694$, TREATMENT $p=0.917$, Sex x TREATMENT $p=0.846$

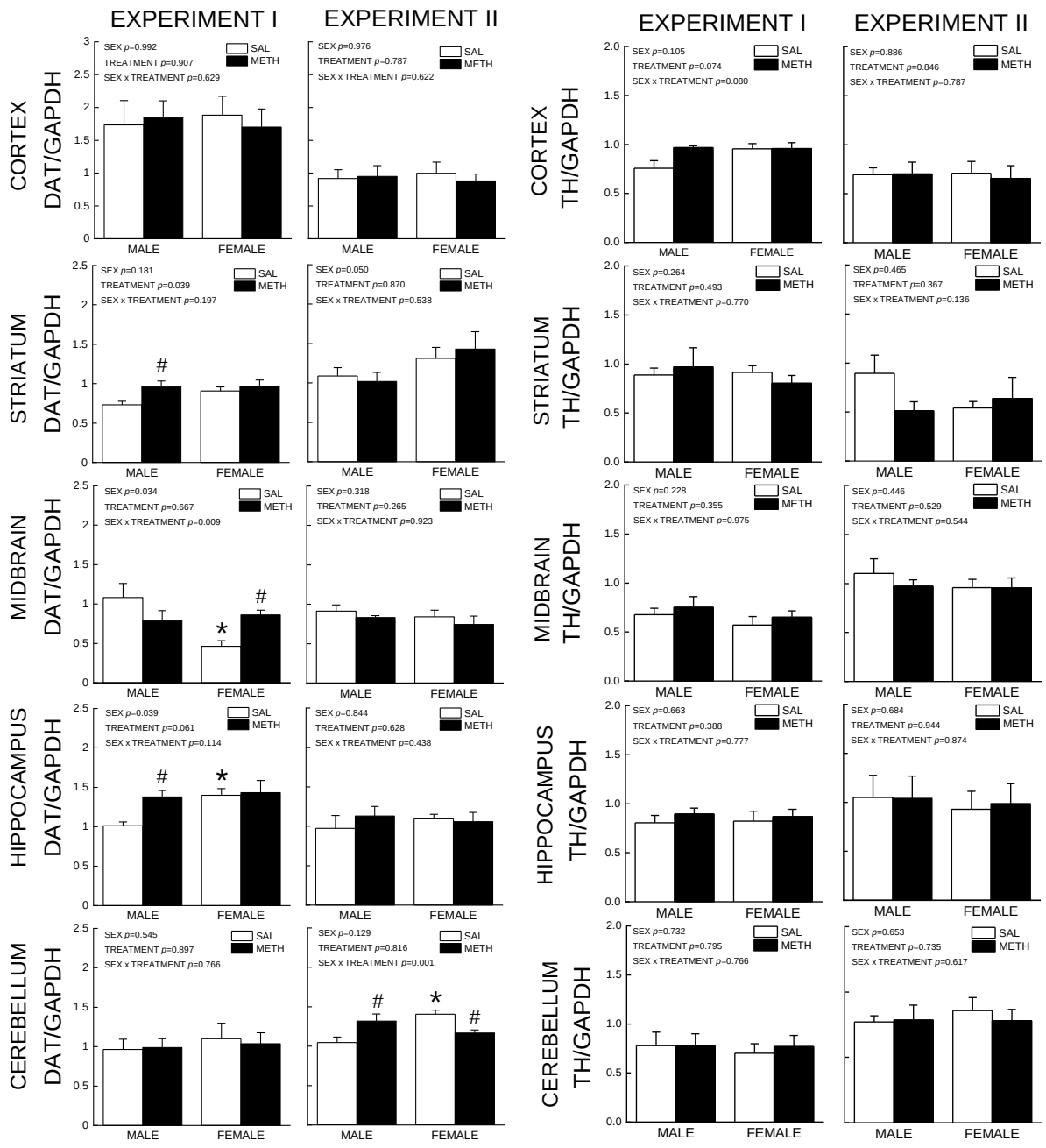

Figure 2.7 The effect of METH on dopamine transporter (DAT-left panels) and tyrosine hydroxylase (TH-right panels) expressions in 5 brain regions of male and female mice. Each value represents the mean ± SEM (n = 6; except 5 for female SAL Striatum). Post hoc analyses: $^{\#}p < 0.05$ compared to Sex-matched control. $^{*}p < 0.05$ compared to treatment-matched males.

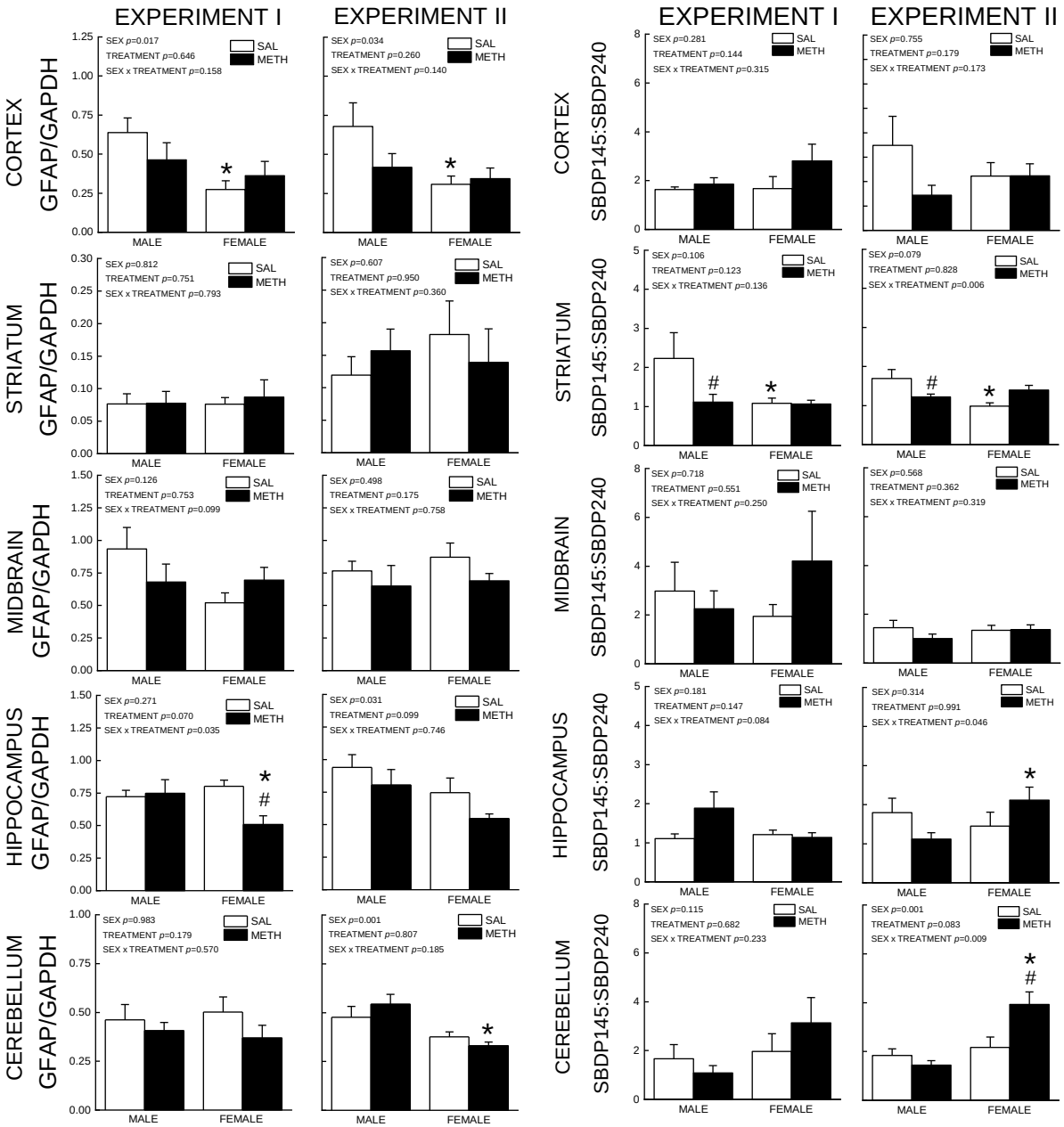

Figure 2.8 The effect of METH on glial fibrillary acidic protein (GFAP-left panels) and spectrin cleavage product (SBDP-right panels) expressions in 5 brain regions of male and female mice. Each value represents the mean ± SEM (n = 6). Post hoc analyses: $^{\#}p < 0.05$ compared to Sex-matched control. $*p < 0.05$ compared to treatment-matched males.

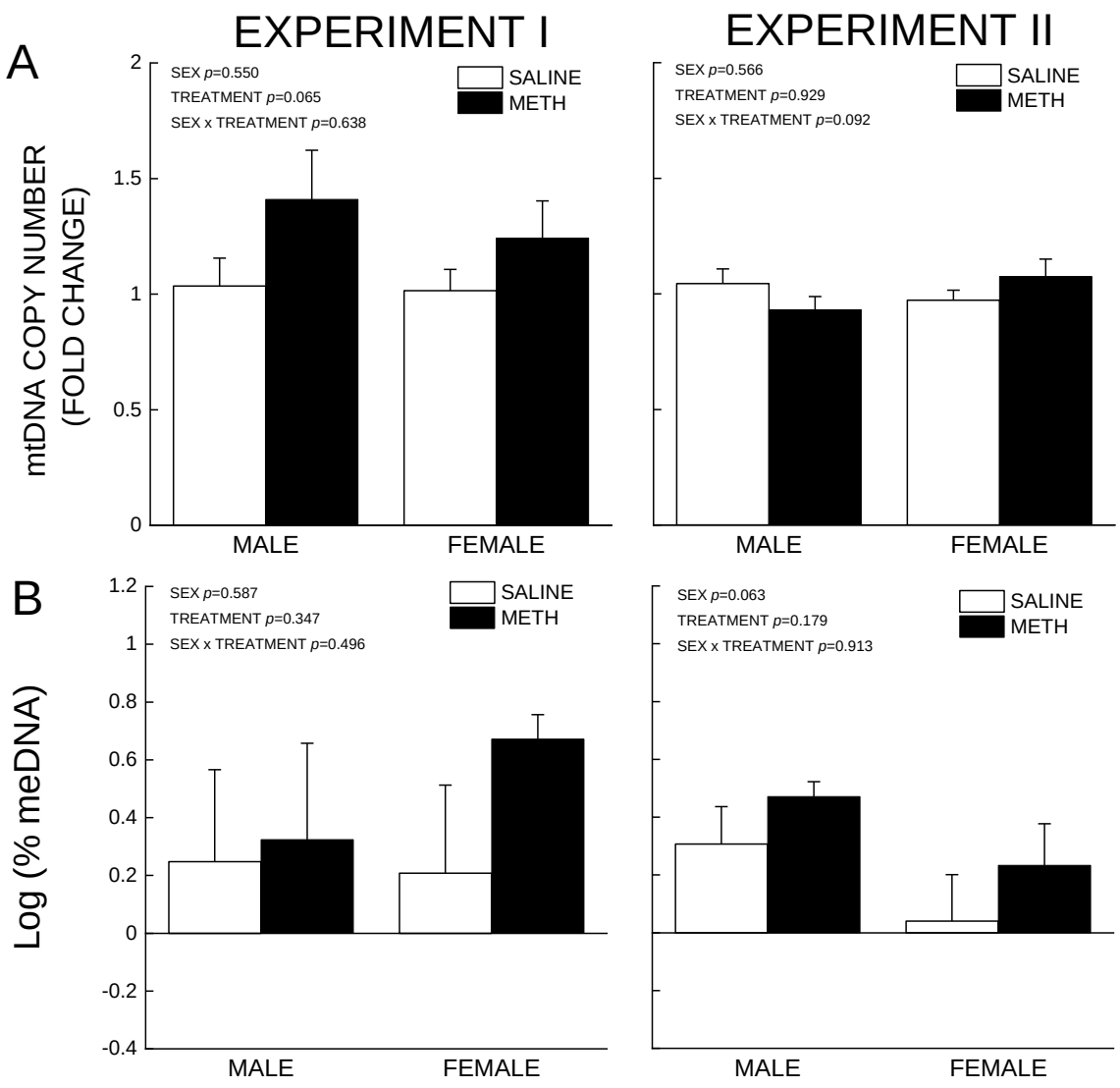

Figure 2.9 The effect of METH on mitochondrial copy number (A) and DNA methylation (B) in the striatum of male and female mice. Each value represents the mean ± SEM (n = 6; except n=5 for mtDNA copy number, Experiment I: female METH).

Table 2.1 Probe trial measurements during Morris water maze test for Experiments I and II

	Experiment I				Experiment II			
	MALE		FEMALE		MALE		FEMALE	
	SAL	METH	SAL	METH	SAL	METH	SAL	METH
% time in annulus 40 (initial learning)	30.6 ± 4.0	27.3 ± 3.0	24.7 ± 4.1	27.1 ± 4.3	28.6 ± 2.8	26.3 ± 2.9	26.5 ± 4.0	30.2 ± 4.2
% time in annulus 40 (maximum performance)	25.9 ± 4.2	24.1 ± 4.6	25.0 ± 3.4	19.6 ± 4.9	25.2 ± 3.5	21.9 ± 2.8	26.0 ± 2.8	29.3 ± 3.4

SAL: saline; METH: methamphetamine

Table 2.2 Gait measurements for Experiment III

	5 MONTHS				7 MONTHS				9 MONTHS			
	MALE		FEMALE		MALE		FEMALE		MALE		FEMALE	
	SAL	METH	SAL	METH	SAL	METH	SAL	METH	SAL	METH	SAL	METH
Base of Support (front; cm)	1.49 ± 0.03	1.53 ± 0.03	1.45 ± 0.04	1.39 ± 0.04*	1.55 ± 0.03	1.53 ± 0.04	1.47 ± 0.03	1.47 ± 0.03	1.34 ± 0.04	1.49 ± 0.03#	1.44 ± 0.04*	1.42 ± 0.03
Base of Support (hind; cm)	2.66 ± 0.06	2.62 ± 0.04	2.49 ± 0.05*	2.57 ± 0.04	2.37 ± 0.04	2.25 ± 0.04	2.37 ± 0.05	2.36 ± 0.05	2.70 ± 0.05*	2.62 ± 0.06	2.53 ± 0.04*	2.57 ± 0.04
Stride Length (front; cm)	8.58 ± 0.14	8.43 ± 0.16	7.78 ± 0.24*	8.26 ± 0.20	8.35 ± 0.17	8.62 ± 0.11	8.03 ± 0.12	8.38 ± 0.11	8.79 ± 0.12	8.70 ± 0.20	8.06 ± 0.18*	8.35 ± 0.16
Stride Length (hind; cm)	8.13 ± 0.25*	8.09 ± 0.15	7.35 ± 0.23*	7.95 ± 0.22	8.25 ± 0.17	8.53 ± 0.13	7.86 ± 0.14	8.23 ± 0.16	8.61 ± 0.14	8.43 ± 0.20	7.81 ± 0.23*	8.10 ± 0.23
Swing Speed (front; cm/s)	103.0 ± 4.7	102.4 ± 3.6	97.71 ± 5.6	101.3 ± 5.0	95.6 ± 4.5	106.07 ± 3.4	89.8 ± 3.1	97.6 ± 4.2	95.5 ± 3.2	95.9 ± 3.2	90.6 ± 3.7	93.0 ± 4.6
Swing Speed (hind; cm/s)	94.5 ± 3.5	96.6 ± 2.9	85.7 ± 4.7	93.8 ± 4.8	100.89 ± 3.7	107.41 ± 3.6	87.13 ± 2.8*	92.25 ± 3.6*	98.4 ± 2.2	99.6 ± 3.9	92.3 ± 3.9	93.6 ± 4.4
Step Cycle (front; s)	0.19 ± 0.01	0.19 ± 0.01	0.20 ± 0.01	0.18 ± 0.01	0.20 ± 0.01	0.19 ± 0.01	0.21 ± 0.01	0.20 ± 0.01	0.19 ± 0.01	0.19 ± 0.01	0.20 ± 0.01	0.20 ± 0.01
Step Cycle (hind; s)	0.19 ± 0.01	0.20 ± 0.01	0.20 ± 0.01	0.18 ± 0.011	0.20 ± 0.01	0.20 ± 0.01	0.21 ± 0.01	0.20 ± 0.01	0.19 ± 0.01	0.19 ± 0.01	0.20 ± 0.01	0.19 ± 0.01

SAL: saline; METH: methamphetamine

Post hoc analyses: *$p<0.05$ vs. treatment-matched males (within the same time point)

Post hoc analyses: # $p<0.05$ vs. sex-matched SAL (within the same time point)

Table 2.3 Biochemical measurements for Experiments I and II

	Experiment I				Experiment II			
	MALE		FEMALE		MALE		FEMALE	
	SAL	METH	SAL	METH	SAL	METH	SAL	METH
OXIDATIVE STRESS								
tGSH-CX	24.5 ± 1.8	20.6 ± 1.6	15.6 ± 1.0*****	16.1 ± 1.7	17.0 ± 1.2	18.1 ± 0.8	15.6 ± 1.6	12.8 ± 0.5*****
tGSH-ST	26.9 ± 1.2	26.6 ± 0.9	24.5 ± 2.4	31.1 ± 1.8	27.1 ± 0.9	27.0 ± 3.0	24.7 ± 1.6	26.0 ± 1.6
tGSH-MB	46.0 ± 3.4	51.0 ± 6.3	47.7 ± 5.9	52.3 ± 2.6	36.6 ± 10.3	38.3 ± 7.5	32.3 ± 6.9	25.5 ± 6.4
tGSH-HP	44.3 ± 1.8	39.4 ± 2.1	38.6 ± 3.6	42.3 ± 1.3	39.2 ± 3.6	39.4 ± 3.5	37.2 ± 7.2	47.0 ± 13.3
tGSH-CB	89.1 ± 12.8	89.5 ± 9.7	92.0 ± 15.8	86.0 ± 8.0	89.2 ± 8.8	89.1 ± 9.5	77.1 ± 11.9	84.8 ± 15.7
TBARS-CX	8.2 ± 0.5	8.6 ± 1.3	8.8 ± 0.6	9.2 ± 1.1	8.5 ± 1.5	9.5 ± 1.1	6.7 ± 1.2	8.0 ± 0.9
TBARS-ST	11.9 ± 1.0	10.4 ± 1.0	9.4 ± 1.3	9.9 ± 1.4	9.4 ± 0.6	9.4 ± 0.8	8.8 ± 0.5	10.3 ± 0.8
TBARS-MB	10.4 ± 1.2	8.3 ± 0.6	10.2 ± 1.5	9.6 ± 1.1	8.1 ± 1.0	9.1 ± 1.3	7.8 ± 0.7	8.8 ± 0.7
TBARS-HP	9.3 ± 0.3	9.4 ± 0.5	9.0 ± 0.5	9.6 ± 0.9	7.0 ± 0.9	7.8 ± 0.9	7.8 ± 0.8	8.8 ± 1.0
TBARS-CB	22.1 ± 4.1	20.2 ± 2.5	14.1 ± 2.6	22.6 ± 5.2	20.8 ± 2.7	17.0 ± 1.8	16.3 ± 1.4	16.9 ± 1.6
AUTOPHAGY								
LC3B-CX	0.77 ± 0.03	0.93 ± 0.07	0.65 ± 0.07	0.83 ± 0.19	0.81 ± 0.11	0.87 ± 0.17	0.68 ± 0.15	0.54 ± 0.13
LC3B-ST	0.93 ± 0.05	0.95 ± 0.11	0.93 ± 0.14	0.91 ± 0.12	0.77 ± 0.07	0.82 ± 0.12	0.79 ± 0.13	0.88 ± 0.13
LC3B-MB	1.31 ± 0.08	1.38 ± 0.33	0.93 ± 0.12	1.01 ± 0.13	1.11 ± 0.08	1.11 ± 0.11	0.77 ± 0.12*	0.73 ± 0.11*
LC3B-HP	0.610 ± 0.05	1.03 ± 0.22	1.06 ± 0.26	1.05 ± 0.22	0.99 ± 0.30	0.70 ± 0.17	0.58 ± 0.15	0.64 ± 0.15
LC3B-CB	0.80 ± 0.09	0.80 ± 0.23	0.82 ± 0.13	0.89 ± 0.17	0.78 ± 0.07	0.54 ± 0.06	0.85 ± 0.14	0.73 ± 0.06
APOPTOSIS								
SBDP120-CX	0.74 ± 0.13	0.53 ± 0.07	0.66 ± 0.33	1.58 ± 0.59	0.96 ± 0.40	0.45 ± 0.17	0.60 ± 0.14	0.73 ± 0.15
SBDP120-ST	1.06 ± 0.40	0.33 ± 0.08	0.30 ± 0.03	0.30 ± 0.04	0.58 ± 0.17	0.33 ± 0.03	0.37 ± 0.05	0.43 ± 0.6
SBDP120-MB	0.96 ± 0.22	0.70 ± 0.28	0.58 ± 0.16	1.50 ± 0.55	0.35 ± 0.06	0.22 ± 0.04	0.25 ± 0.02	0.26 ± 0.02
SBDP120-HP	0.37 ± 0.08	0.47 ± 0.12	0.43 ± 0.06	0.34 ± 0.04	0.53 ± 0.06	0.45 ± 0.05	0.46 ± 0.05	0.50 ± 0.09
SBDP120-CB	0.47 ± 0.15	0.28 ± 0.12	0.44 ± 0.15	0.77 ± 0.22	0.85 ± 0.21	0.79 ± 0.22	1.00 ± 0.21	1.05 ± 0.13

SAL: saline; METH: methamphetamine; CX: Cortex; ST: Striatum; MB: Midbrain; HP: Hippocampus; CB: Cerebellum; tGSH: Total Glutathione; TBARS: Thiobarbituric acid reactive substances;
n=6/group except for tGSH-CB male SAL.
tGSH measured in μM. TBARS measured as μM/μg of protein. Densitometry a.u. for LC3B/GAPDH and SBDP120
Post hoc analyses: *$p<0.05$ vs. treatment-matched males within same experiment

Table 2.4 Genetic measurements for Experiments I and II

	Experiment I				Experiment II			
	MALE		FEMALE		MALE		FEMALE	
	SAL	METH	SAL	METH	SAL	METH	SAL	METH
mtDNA-CX	0.97 ± 0.14	1.05 ± 0.12	1.16 ± 0.19	1.01 ± 0.07	1.06 ± 0.09	0.99 ± 0.11	1.00 ± 0.13	0.95 ± 0.09
mtDNA-MB	0.97 ± 0.11	1.11 ± 0.11	1.14 ± 0.23	1.15 ± 0.14	0.97 ± 0.09	1.04 ± 0.13	1.10 ± 0.15	1.36 ± 0.08
mtDNA-HP	1.01 ± 0.15	1.01 ± 0.22	1.10 ± 0.14	0.90 ± 0.13	1.06 ± 0.17	0.94 ± 0.22	1.06 ± 0.18	0.92 ± 0.06
mtDNA-CB	0.81 ± 0.08	0.94 ± 0.14	1.38 ± 0.32	1.45 ± 0.42	1.01 ± 0.11	1.01 ± 0.15	1.08 ± 0.15	1.02 ± 0.20

SAL: saline; METH: methamphetamine; CX: Cortex; MB: Midbrain; HP: Hippocampus; CB: Cerebellum; n=6/group except for: Experiment I CB male SAL and METH (n = 4), CB female SAL (n = 5), ST female METH (n = 5) and Experiment II HP male METH (n = 5).

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EFFECTS OF CHRONIC METHAMPHETAMINE EXPOSURE ON REWARDING BEHAVIOR AND NEURODENEGERATION IN ADULT MICE

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The authors declare that they have no conflict of interest.

Acknowledgements: This work was supported by National Institutes of Health/National Institute on Aging T32 AG020494 and a seed grant from University of North Texas Health Science Center Research Office.

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3.1 Abstract

Recreational and medical use of stimulants among young adults have gained popularity in the United States over the last decade and their use may increase vulnerability to brain biochemical changes and addictive behaviors. The long-term effects of chronic stimulant exposure in adulthood have not been fully elucidated.

Our study investigated whether chronic exposure to the prototypical psychostimulant, methamphetamine (METH), at a dose designed to emulate human therapeutic dosing, would promote neurotoxicity and increase susceptibility to addiction.

Groups of 4-month-old male and female C57BL/6J mice were administered non-contingent intraperitoneal injections of either saline or METH (1.4 mg/kg) twice a day for 4 weeks. METH (0.5 mg/kg)-induced conditioned place preference (CPP) was tested in mice to determine the rewarding effects of previous METH exposure. Mice were randomly assigned to either the short-term or long-term experiment group in which CPP testing was performed 13 days after the end of injections (short-term) or 5 months after the end of injections (long-term). Brain regions were assessed for neurotoxicity and dopaminergic function after behavioral testing.

Chronic METH exposure induced short-term changes to reward-related behaviors and dopamine (DA) signaling in males and apoptosis in females. There were no long-term biochemical changes in females, however 9.5-month-old females exhibited a diminished place preference response. Previous exposure to METH induced a heightened sensitivity to subsequent doses of METH especially in males and these observations were supported by alterations in the dopaminergic system in males. While the CPP response in females was smaller, it disappeared in the long-term suggesting tolerance may have occurred. In conclusion, future studies are necessary to continue exploring the long-term neurobehavioral consequences of drug use in both sexes.

3.2 Introduction

Drug addiction is a chronic relapsing disorder in which the compulsive use of a substance persists despite adverse health consequences and results in long-lasting changes to brain function and structure (APA 2013). Addiction is the most severe form of substance use disorder (SUD) and in the United States, 40.3 million people were found to have an SUD in 2020 (SAMHSA 2021). Psychostimulants are a broad class of psychoactive drugs that stimulate the central nervous system (CNS), causing arousal, wakefulness, and euphoria. Chronic use of these drugs can cause adverse health consequences such as psychosis, cardiovascular events, memory loss and addiction (Genova et al. 1997; Favrod-Coune and Broers 2010). The most frequently abused illicit psychostimulants include cocaine and amphetamines. According to the United Nations Office on Drug and Crime (UNODC), approximately 27 million people worldwide were past users of amphetamines, with the highest prevalence in North America, in which methamphetamine and non-medical prescription stimulants were most commonly used (UNODC 2021). The non-medical use/misuse of prescription stimulants is defined as the use of licit stimulants other than as prescribed and/or without a legitimate prescription (UNODC 2011). According to national surveys, 5.1 million people have misused prescription stimulants, with the highest levels of drug use among young adults (SAMHSA 2021). Recreational and medical use of stimulants among young adults have gained popularity in the United States over the last decade, with amphetamine (e.g. Adderall, dextroamphetamine) and methylphenidate (e.g. Ritalin, Concerta) compounds becoming the second most common drugs used among college students (Schulenberg et al. 2021). Although amphetamines and amphetamine-type stimulants are viewed as the first-line treatment for attention deficit hyperactivity disorder (ADHD), they are classified as Schedule II controlled substances due to high risk of adverse consequences, such

as neurotoxicity, abuse and dependence (DEA 2020). It is noteworthy that although young adults have a higher prevalence for prescription stimulant misuse, middle adulthood (30-44 years old) is actually the peak age range for the development of SUD symptoms from prescription stimulants misuse (McCabe et al. 2022). This suggests that young and middle adulthood are key age groups that may be the most vulnerable to the adverse effects of prescription stimulant misuse and it is imperative to conduct long-term studies to better understand the safety of these widely prescribed licit drugs.

Chronic use of amphetamines can induce neuroadaptations and promote long-lasting molecular and behavioral changes (Kamei et al. 2006; LeBlanc-Duchin and Taukulis 2009; Hotchkiss and Gibb 1980). Amphetamines activate the reward circuitry system of the brain and exhibit most of their rewarding effects through increased dopamine (DA) levels (Faraone 2018). The mesocorticolimbic dopaminergic pathway is a prominent component of the brain reward circuit that originates in the ventral tegmental area (VTA) of the midbrain and projects into the nucleus acumbens (NAc), amygdala, cortex, and hypothalamus (Everitt et al. 1999). The continued activation of DA reinforces drug use behaviors, leading to potential abuse and addiction. The behavioral transition from the occasional use of psychostimulants to drug addiction could involve a shift from the ventral striatum (NAc and olfactory tubercle) to the dorsal striatum (caudate nucleus and putamen), when drug use becomes habitual and compulsive (Everitt and Robbins 2005). The nigrostriatal dopaminergic pathway connects the substantia nigra (SNpc) in the midbrain to the dorsal striatum in the forebrain and has been mostly implicated in voluntary movement, however this system is also critical to habit learning and reward processing (Faure et al. 2005).

Recently, the rewarding effects of amphetamines and its derivatives have been implicated in prominent changes in gene expression. Amphetamines induce stable post-translational changes in gene and protein expression, particularly in brain regions associated in dopamine (DA) reward circuitry (McCowan et al. 2015). Several studies have shown the impact of repeated amphetamine use on epigenetic regulation mechanisms such as DNA methylation and histone modifications (Kalda et al. 2007; Mychasiuk et al. 2013; Renthal et al. 2008).

The adverse consequences of amphetamines are mainly related to their neurotoxic potential, which refers to the ability of amphetamines and their derivatives to produce substantial alterations to neurons that can cause reversible and irreversible damage (Seiden et al. 1976; Hotchkiss and Gibb 1980). Glutamate excitotoxicity is considered to be a prominent molecular mechanism of AMP-induced neurotoxicity and numerous studies support its role in mediating neurotoxic damage to DA neurons (Nash and Yamamoto 1992; Mark et al. 2004; Giorgetti et al. 2001). Amphetamines increase extracellular glutamate levels (Del Arco et al. 1999; Wolf et al. 2000) and stimulate glutamate receptors such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and N-methyl-D-aspartate receptor (NMDAR) (Madden 2002). Amphetamines can overstimulate AMPA and NDMA receptors which can lead to Ca^{2+} dysregulation, thus triggering free radical generation that results in neuronal damage (Lafon-Cazal et al. 1993; Sattler and Tymianski 2000).

The repeated use of amphetamines can result in a hypersensitivity to the rewarding effects of the drug that can persist for years and it is this increased sensitivity to subsequent dosing of the drug that is believed to be critical to the development of reward-associated behaviors (Boileau et al. 2006). In our study we used the behavioral model, conditioned place preference (CPP), a non-contingent drug administration paradigm used to test the abuse potential of drugs. This is a well-

established test of classical conditioning used to measure the enduring neurobiological changes induced by the drug by pairing a drug treatment with a specific environmental context to evaluate the rewarding or aversive effects of the drug (Kuhn et al. 2019). Repeated exposure to amphetamines can induce sensitization to the rewarding effects and potentiate place preference response, supporting the addictive properties of these drugs that create a progressively heightened reward-associated behaviors through subsequent doses (Lett 1989; Valenti et al. 2021).

Our study investigated the effects of chronic exposure to the prototypical stimulant, methamphetamine (METH), at a dose designed to emulate therapeutic dosing, on abuse potential and biochemical markers of dopaminergic function and neurodegeneration in male and female mice. METH was chosen for this experiment due to its well-established neurotoxicity (Ares-Santos et al. 2014; Friedman et al. 1998; Halpin et al. 2014; Yang et al. 2018), similarities in pharmacokinetic and structural properties to amphetamine (AMP) (Melega et al. 1995) and its use in treating ADHD symptomology as the FDA-approved drug, Desoxyn. We treated 4-month-old mice with chronic, low-dose METH for 4 weeks and then were tested for conditioned place preference 13 days or 5 months after injection cessation to evaluate drug seeking behavior. At the end of behavioral testing, we evaluated potential markers of METH neurotoxicity, epigenetic changes and dopaminergic dysregulation in brain regions important to reward circuitry. We hypothesized that mice that were previously administered chronic METH will be more sensitive to the rewarding effects of subsequent METH exposure as well as METH-induced neurotoxicity and dopaminergic dysregulation.

3.3 Materials and Methods

Animals

The studies were approved by the UNT HSC Institutional Care and Use Committee and adhered to NIH guidelines for the Care and Use of Laboratory Animals. Fifteen-week-old male and female C57BL/6J mice (n = 71) were purchased from Jackson Laboratories and maintained in the UNT HSC Vivarium. Mice were group housed (3-4 mice per cage based on sex and treatment) at 23 ± 1° C under a 12-h light/dark cycle starting at 0700 and had ad libitum access to water and food (LabDiet-5LL2).

At approximately 3.5 months of age, the mice were randomly assigned to either the saline (SAL) or methamphetamine (METH) treatment group. The animals received intraperitoneal injections of METH (1.4 mg/kg; total daily dose of 2.8 mg/kg) or saline (0.9 % NaCl) twice a day for 5 days a week. The dose chosen for METH is described previously (Davis et al. 2022). The mice (n=12/sex/treatment) were tested for conditioned place preference (CPP) either 13 days (short-term) or 5 months (long-term) after injection cessation (Figure 1). However, in our long-term group, 1 METH-treated female died during the study. Due to COVID-related shutdown of our laboratories, we were unable to complete the long-term male group. The mice were euthanized by cervical dislocation and then the striatum and midbrain were dissected and snap frozen at -80°C.

Behavioral Testing

Conditioned Place Preference

Mice were placed in a clear acrylic test chamber (40.5 × 40.5 × 30.5 cm) that was lined with photocells (Digiscan apparatus, Omnitech Electronics) under dim illumination (23 lux) with ambient noise (80 dB) provided by a fan within the sound-attenuating chamber. The chamber

had interchangeable hole (perforated) and grid (bar) steel floors (full: 30.5 cm × 15.2 cm and split: 15.2 × 15.2 cm). The dose of METH was chosen according to previous research in which 0.5 mg/kg METH was found to produce maximum conditioned place preference response (Shetty et al. 2017). Day 1 was the pre-test to examine initial floor bias in which mice were intraperitoneally injected with 0.9% saline and placed in the CPP chamber with free access to explore the floor types (grid and perforated) for 30 min. Days 2 and 3 were the conditioning sessions in which the mice were injected with the METH (0.5 mg/kg) in the morning and placed in the chamber with the METH-paired floor for 30 min before returning to their home cage. After 4 h, mice were injected with saline and placed in the chamber with the SAL-paired floor for 30 min before returning to their home cage. Day 4 was the final preference test in which mice received saline and were placed on the split floors (grid and perforated) and time spent on the METH-paired floor was measured. Preference scores were calculated by subtracting the pre-test time (Day 1) on the METH-paired floor from the post-time (Day 4) on the METH-paired floor.

Biochemical Measurements

Western blot analyses

Supernatants from tissue homogenates (1 mM BHT, 200 μM DTPA, 10 mM sodium phosphate, and 0.9% sodium chloride + protease inhibitor cocktail (Cell Signaling Technology 5872); 10,000 x g for 10 min at 4°C) were used for western blot analyses. Protein concentration was determined at 562nm using a BCA Assay Kit (Thermo Scientific 3225). Proteins (25 or 40 μg) were loaded onto a 4-20% SDS-polyacrylamide gel (Biorad 4561096) and transferred to a 0.45 μm nitrocellulose membrane (Biorad 162-0115) at 4°C overnight. Membranes were blocked at room temperature for 1 h in 5% non-fat dry milk (Biorad 1706404) in TBS-T (1X TBS and 0.05% Tween-20). Blots were incubated with the primary antibodies (anti-DAT (1:1000; Sigma

Aldrich AB2231), anti-αII spectrin (1:1000; Abcam MAB1622), anti-TH (1:1000; Cell Signaling Technology 13106) and anti-GAPDH (1:5000; Cell Signaling Technology 97166, anti-KDM6A (1:1000; Genetex GTX121246)) in TBS-T and 5% milk overnight at 4°C, washed three times (10 min each) in TBS-T, and finally incubated with mouse (1:5000; Jackson ImmunoResearch 115-035-003) or rabbit (1:5000; Cell Signaling Technology 7074) HRP-linked secondary antibody (1:5000; Cell Signaling Technology) at room temperature for 1 h. Membranes were washed with TBS-T (3 times, 10 min each) and then incubated with either West Femto or Pico (Thermo Scientific 34096, 34579) prior to being imaged using BioSpectrum 500 UVP imaging system. To quantify protein density, ImageJ was used and the data were normalized to GAPDH expression.

Statistical analyses

All data were presented as mean ± standard error mean (SEM). The short-term effects of Sex (Sx) and Treatment (Tx) were assessed using two-way analysis of variance (ANOVA) for CPP and biochemical measures. Body weights were evaluated in three-way ANOVAs (with Weeks (Wk) as repeated measures), followed by two-way ANOVAs at each week. The long-term effect of treatment in females was assessed using one-way ANOVA for CPP and biochemical measures. Body weight was evaluated by two-way ANOVA (with Weeks) as repeated measures). Individual comparisons between sexes and treatments were performed following significant main effects or interaction by using single degree-of-freedom F-tests with the overall ANOVA error term. For all analyses, the α level was set at < 0.05. The software used for analyses was Systat 13 (Systat Software Inc., San Jose, CA, USA).

3.4 Results

Body Weights

The short- and long-term effects of chronic METH administration on body weights are presented in Figure 2. Overall, mice gained weight as they aged (short-term: $F(6,264)_{Wk}=20.221$, $p<0.001$; long-term: $F(27,567)_{Wk}=62.384$, $p<0.001$). In the short-term experiment, males weighed more than females regardless of treatment, which was supported by a main effect of Sex ($F(1,44)_{Sx}=93.380$, $p<0.001$) and interactions ($F(6,264)_{Wk \times Sx}=4.699$, $p<0.001$; $F(6,264)_{Wk \times Sx \times Tx}=3.183$, $p=0.005$). Treatment did not significantly affect the body weights of males or females ($F(1,44)_{Tx}=0.509$, $p=0.479$; $F(1,44)_{Sx \times Tx}=2.794$, $p=0.102$; $F(6,264)_{Wk \times Tx}=1.593$, $p=0.149$). There was no effect of treatment in the long-term study ($F(27,567)_{Wk \times Tx}=1.170$, $p<0.255$).

Conditioned Place Preference

The effects of sex and treatment on conditioned place preference are presented in Figure 3. In the short-term study, it appeared that previous chronic METH exposure increased preference scores especially in males. However, this treatment effect did not reach significance ($F(1,44)_{Sx}=0.736$, $p=0.396$; $F(1,44)_{Tx}=3.173$, $p=0.082$; $F(1,44)_{Sx \times Tx}=0.299$, $p=0.588$). In the long-term study, females that were previously treated with chronic METH spent 93% less time on the METH-paired floor than mice that had previously treated with SAL, which was supported by a main effect ($F(1,21)_{Tx}=4.576$, $p=0.044$).

Biochemical Measurements

Dopaminergic Measures: Dopamine transporter (DAT) and tyrosine hydroxylase (TH). The effects of sex and treatment on DAT and TH are presented in Figure 4. There were no short-term effects of sex or treatment on DAT expression in the midbrain ($F(1,20)_{Sx}=0.102$, $p=0.753$; $F(1,20)_{Tx}=2.098$, $p=0.163$; $F(1,20)_{Sx \times Tx}=0.497$, $p=0.489$). In the striatum, males with previous chronic METH exposure had 34% lower DAT levels than the SAL group ($p<0.001$ post hoc) while females with previous chronic METH exposure had slightly higher expression compared to

their SAL group (and 45% higher than the male METH group; p=0.001 post hoc). These observations were supported by a main effect of Treatment and interaction with Sex ($F(1,20)_{Sx}$=1.639, p=0.215; $F(1,20)_{Tx}$=4.882, p=0.039; $F(1,20)_{Sx \times Tx}$=17.287, p<0.001).

The was no long-term effects of previous chronic METH exposure on DAT expression in the striatum ($F(1,10)_{Tx}$=0.005, p=0.944) or midbrain ($F(1,10)_{Tx}$=0.987, p=0.344) of females.

In the short-term study, there was no effect of treatment on TH expression in the midbrain ($F(1,20)_{Tx}$=2.495, p=0.130; $F(1,20)_{Sx \times Tx}$=1.056, p=0.316). In the striatum, saline-treated females had 41% lower TH levels compared to SAL males ($F(1,20)_{Sx}$=4.294, p=0.051; p=0.008 post hoc) and males with previous chronic METH exposure had 32% lower TH levels compared to the male SAL group (p=0.033 post hoc). This was supported by a significant interaction between Sex and Treatment (p=0.616; $F(1,20)_{Sx \times Tx}$=7.437, p=0.013). There was no long-term effect of previous chronic METH exposure on TH expression in the striatum ($F(1,10)_{Tx}$=0.518, p=0.488) or midbrain ($F(1,10)_{Tx}$=0.002, p=0.963) of females.

Neurodegeneration Measures: SBDP145 and SBDP120. The effects of sex and treatment on calcium dyshomeostasis (kDa145 spectrin cleavage product) and apoptosis (kDa120 spectrin cleavage product) are presented in Figure 5. There were no short-term effects of either sex or treatment on SBDP145 levels in the striatum or midbrain (all Fs(1,20)<2.635, all ps>0.120) and no long-term effect of treatment in the striatum ($F(1,10)_{Tx}$=0.101, p=0.757) or midbrain ($F(1,10)_{Tx}$=2.071, p=0.181) of females.

In the short-term study, female with previous chronic exposure to METH had 32% higher SBDP120 levels in the midbrain compared to SAL females (p=0.036 post hoc) and 65% higher SBDP120 levels compared to males treated with chronic METH (p=0.001 post hoc). These observations were supported by a main effect of Sex and an interaction ($F(1,20)_{Sx}$=7.727,

p=0.012; $F(1,20)_{Sx \times Tx}$=5.857, p=0.025). In the striatum, even though female exposed to chronic METH had higher expression of SBDP120 than the other groups, it was not supported by main effects or an interaction ($F(1,20)_{Sx}$=2.578, p=0.124; $F(1,20)_{Tx}$=1.745, p=0.201; $F(1,20)_{Sx \times Tx}$=3.973, p=0.060. There was no long-term effect of treatment on SBDP120 levels in the striatum ($F(1,10)_{Tx}$=0.666, p=0.423) or midbrain ($F(1,10)_{Tx}$=0.944, p=0.354) of females.

Epigenetic Modifications: KDM6A. The effects of sex and treatment on KDM6A expression are presented in Figure 6. There were no short-term effects of either sex or treatment on KDM6A levels in the striatum or midbrain (all Fs(1,20)<1.146, all ps>0.297). There was no long-term effect of treatment in females on KDM6A expression in either brain region (striatum: $F(1,10)_{Tx}$=1.668, p=0.226; midbrain: $F(1,10)_{Tx}$=0.196, p=0.667).

3.5 Discussion

The main findings were that previous administration of low-dose METH in the short-term (1) had a greater drug reward response to subsequent METH doses in males compared to females (2) decreased DA signaling in the striatum of males but not females and (3) increased apoptosis in the midbrain and striatum of only females. Previous administration of chronic, low-dose METH in females in the long-term (1) ameliorated drug reward response to subsequent METH dosing and (2) increased excitotoxicity in the midbrain and (3) had no effects on DA signaling or apoptosis in the striatum or midbrain.

In the short-term experiment, males with prior exposure to chronic, low-dose METH produced a greater CPP response than males without prior METH exposure. Our findings are supported by the literature in which prior exposure to chronic psychostimulants produces long-lasting behavioral and neural sensitization that can further amplify rewarding drug behaviors upon

subsequent exposure (Lorrain et al. 2000; Robinson and Becker 1986). Moreover, Lin *et al*. demonstrated that prior sensitization to chronic METH (1 mg/kg, every other day for 6 days, i.p.) further augmented subsequent CPP response in male Sprague Dawley rats (Lin et al. 2007). However, it is interesting to note that there was a much less pronounced CPP response in 5-month-old females that had been previous administered chronic METH. In comparison, 9.5-month-old females with prior exposure to chronic, low-dose METH actually produced a lower CPP response compared to females previously treated with SAL. This was an unexpected finding as most of the preclinical literature suggests increased susceptibility of females to psychostimulants (Anker and Carroll 2011; Van Swearingen et al. 2013). For example, it was reported that female rats were more vulnerable to METH (0.02 mg/kg) self-administration and exhibited increased motivation compared to males (Roth and Carroll 2004). A potential explanation may be that repeated exposure to low-dose METH produced neuroadaptations that ameliorated response to subsequent dosing. Strakowski *et al.* reported that women who received d-amphetamine (d-AMP; 0.25 mg/kg) once reported higher ratings of drug liking compared to women who were administered d-amphetamine three times (once every 48 hours, for 5 days, p.o.) (Strakowski et al. 2001). Although this study was performed in humans for a short amount of time and limited dosing, it does provide potential insights into the differential rewarding effects of acute vs. repeated dosing of amphetamines in a clinical setting. Unfortunately, very little is known about the neurobiological consequences of chronic exposure to drugs of abuse in females (Becker et al. 2012). In regards to our findings, although the literature states that females have a greater response to the behavioral effects of amphetamines (Milesi-Hallé et al. 2007), even when equivalent brain concentration of amphetamine is controlled for (Becker et al. 1982), it may be that repeated use dampens the rewarding effects of the drug through neuroadaptations

of the reward system. There are currently no studies examining conditioned place preference in older rodents or how the rewarding effects of drugs affect older individuals, despite age-related changes in pharmacokinetics and pharmacodynamics (Shi and Klotz 2011).

We observed in the short-term experiment that previous exposure to METH decreased DAT and TH in the striatum, but not midbrain of males. Neuroimaging studies of chronic exposure to amphetamines and amphetamine compounds report a downregulation of dopaminergic markers such as DAT, DA receptors, TH, and VMAT2 (Ashok et al. 2017; Proebstl et al. 2019). In an escalating d-AMP dose regimen (1-10 mg/kg, 2X a day for 6 weeks), a significant decrease in DA was observed in the caudate nucleus of male rats 3 days into withdrawal (Paulson and Robinson 1996). The same authors also reported no change in DA levels in the caudate of adult male Holtzman rats who received a challenge dose of d-AMP (0.5 mg/kg) after previous exposure to chronic, escalating d-AMP (Paulson and Robinson 1995). DAT undergoes downregulation with low DA levels, and a larger reduction from prior chronic METH exposure could mean a more sensitive dopaminergic response to subsequent dosing. We did not see dopaminergic changes in females at 5 or 9.5 months of age, and that may be due in part to the complex sex differences in the dopaminergic system that are mediated by different regulatory mechanisms. Females have a faster reuptake and release of dopamine and synaptic DA is more tightly regulated by DAT and D_2 receptors in females (Walker et al. 2006). A tighter regulation of dopaminergic transmission could in females could explain why there were changes in DAT/TH expression in only males.

Previous chronic, low-dose METH did not induce changes in in either the calpain (145kDa) or caspase-3 (120kDa) mediated spectrin breakdown product in males. However, in 5-month-old females, there was an increase in SBDP120 levels, suggesting apoptosis in the striatum and

midbrain. Interestingly, females appear more sensitive to caspase-mediated cell death, compared to cell death in males that is primarily caspase independent (Du et al. 2004). Neuronal apoptosis plays a critical role in METH neurotoxicity, and it is potentially concerning that chronic doses of METH well within the human therapeutic dosing range could potentially target vulnerable dopaminergic neurons in brain areas involved in the mesolimbic and nigrostriatal pathways. Although, these effects were not seen in 9.5-month-old females, this implies that the detection of neuronal apoptosis may be a more short-lived effect, or it could be more time dependent, as tissue was collected approximately 3 days after the last CPP METH injection (0.5 mg.kg) in the short-term experiment compared to 7 days after the last CPP injection in the long-term experiment.

In 9.5-month-old females, calpain-mediated degradation of spectrin was higher in the midbrain of females who had undergone chronic METH exposure. This effect was not seen in 5-month-old females, suggesting age-associated calcium dysregulation in the midbrain. These findings are supported by Bernath *et al*. who reported increased levels of SBDP145/150 fragments but not SBDP120 fragments in the brains of aged rodents (Bernath et al. 2006). Excitotoxicity has been posited as an important mechanism in amphetamine-induced toxicity to dopaminergic neurons (Yang et al. 2018) and long term treatment of METH (50 μM; 48 hours) was found to upregulate L-type Ca^{2+} channels and increase intracellular Ca^{2+} (Andres et al. 2015).

Repeated amphetamine administration can lead to addiction, and elucidating potential epigenetic changes is a recent, promising avenue to elucidate the transition from recreational use to addiction (Godino et al. 2015). Amphetamines can alter gene expression through histone methylation (Renthal et al. 2008; Ikegami et al. 2010). In one study, METH (2 mg/kg)-induced CPP induced histone methylation modifications in the NAc (Aguilar-Valles et al. 2014),

suggesting that the rewarding effects of amphetamines are modulated by changes in chromatin that may prime differential gene transcription to subsequent amphetamine stimulation. We chose to evaluate KDM6A, a histone demethylase, that belongs to the KDM6 family of histone H3 lysine 27 (H3K27) demethylases and promotes gene repression. KDM6A has been implicated in aging and neurodegenerative diseases and could be a potential marker for sex chromosome-related resiliency in these processes (Davis et al. 2020). Currently there are no studies evaluating chronic amphetamine exposure and KDM6A expression, although there is one study in which KDM6A expression was increased in male rats that underwent cocaine self-administration (Sadakierska-Chudy et al. 2017). Although we did not find changes in KDM6A levels, results may be different in males.

There are many challenges in the translational relevance of modeling chronic drug abuse/addiction using rodent models and there are several caveats to our studies. The use of METH as a prescription stimulant is limited and therefore our results cannot be directly generalized to more widely prescribed and clinically relevant psychostimulants. In CPP behavioral testing, only one METH training dose (0.5 mg/kg) was implemented, and future experiments could utilize a dosing range to determine maximum CPP response. In our experiments we only used one behavioral test to model addiction-related behaviors, however it may be of interest to examine a self-administration paradigm to evaluate the reinforcing effects of chronic METH exposure and behaviors that may be more representative of the transition from casual use to addiction in humans (Kuhn et al. 2019). Previous research has shown that previous exposure to amphetamines produced a long-lasting enhancement in the incentive motivation the animals exhibited in their effort to obtain the drug (Lorrain et al. 2000). Similar to our previous studies in which we observed subtle changes in biochemical and functional outcomes as a result

of chronic, low-dose METH administration (Davis et al. 2022), we only observed minor effects in our experiments and thus the METH dosage may need to be increased in future experiments. In conclusion, previous exposure to chronic, low-dose METH differentially affected males and females and induced short-term molecular and behavioral changes. It is important to understand addiction susceptibility and the underlying molecular changes. This research gives way to broader implications of psychostimulant abuse and the potential risk factors of the short-and-long term effects of therapeutic amphetamines on addiction.

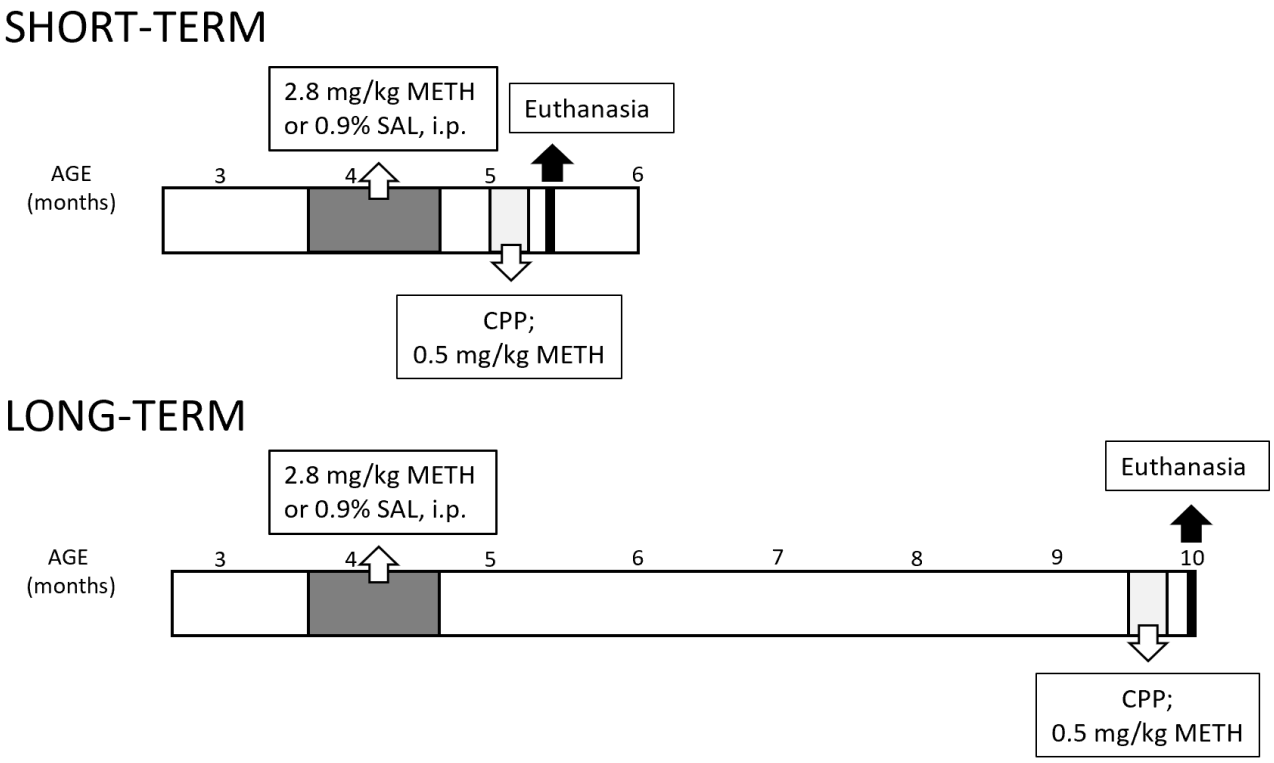

Figure 3.1 Experimental design of the short-term and long-term studies of METH exposure.

In both experiments, mice received non-contingent i.p. injections of either METH (total daily 2.8 mg/kg (+) methamphetamine) or SAL (0.9% saline) 5 days a week for 4 weeks. In the short-term, CPP testing began at 5 months of age. In the long-term, CPP testing started at 9.5 months of age. On Days 2 and 3 of the CPP test, mice received non-contingent i.p. injections of METH (0.5 mg/kg).

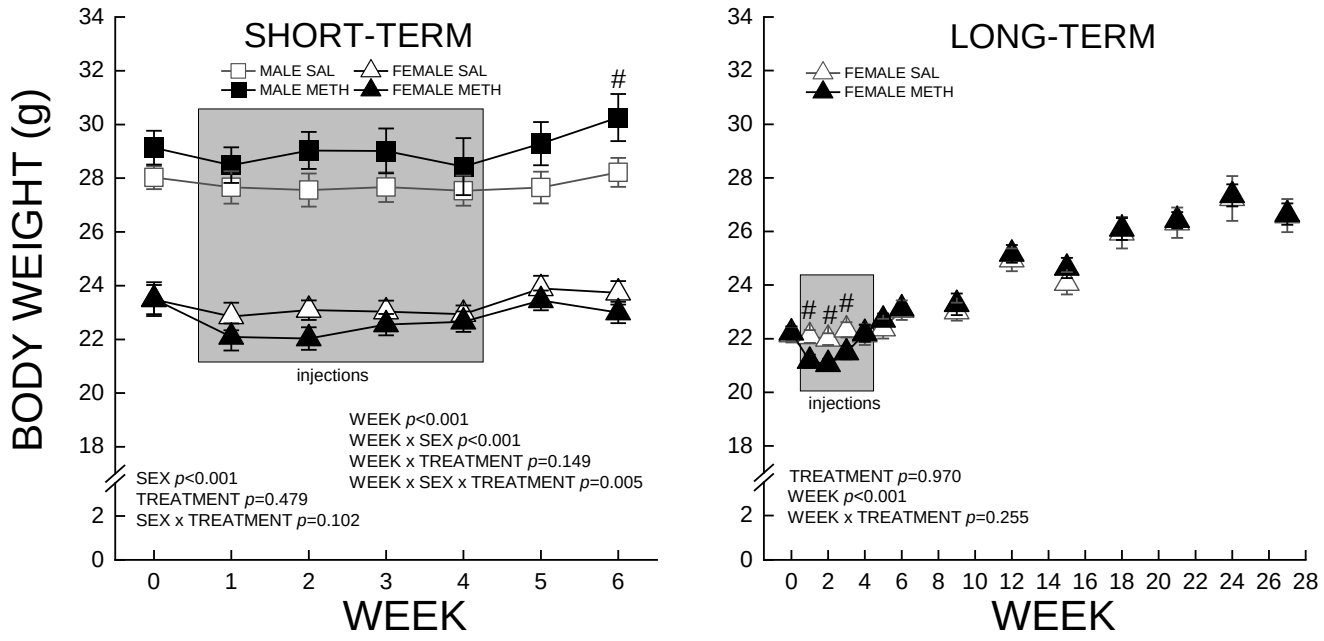

Figure 3.2 Short-term and long-term effects of METH on body weights in male and female mice. Each value represents the mean ± SEM (Short-term: all n = 12; long-term: SAL: n = 12, METH: n = 11). #$p < 0.05$ vs. sex-matched SAL.

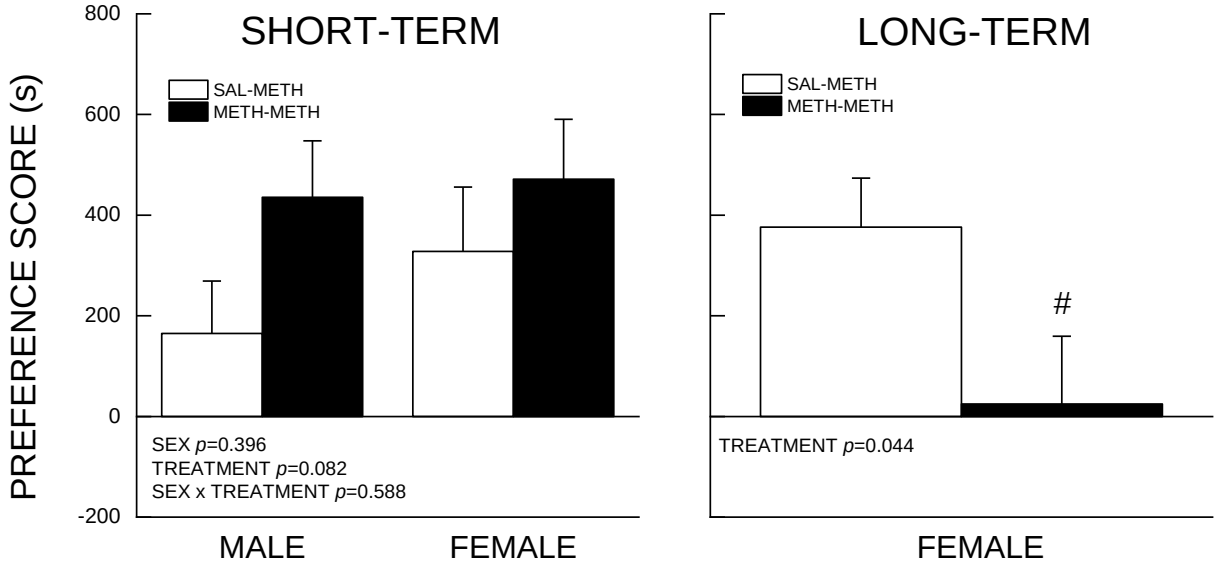

Figure 3.3 Short-term and long-term effects of METH on conditioned place preference (CPP) in male and female mice. Each value represents the mean ± SEM (short-term: all n = 12; long-term: SAL: n = 12, METH: n = 11). #$p < 0.05$ vs. SAL-METH group.

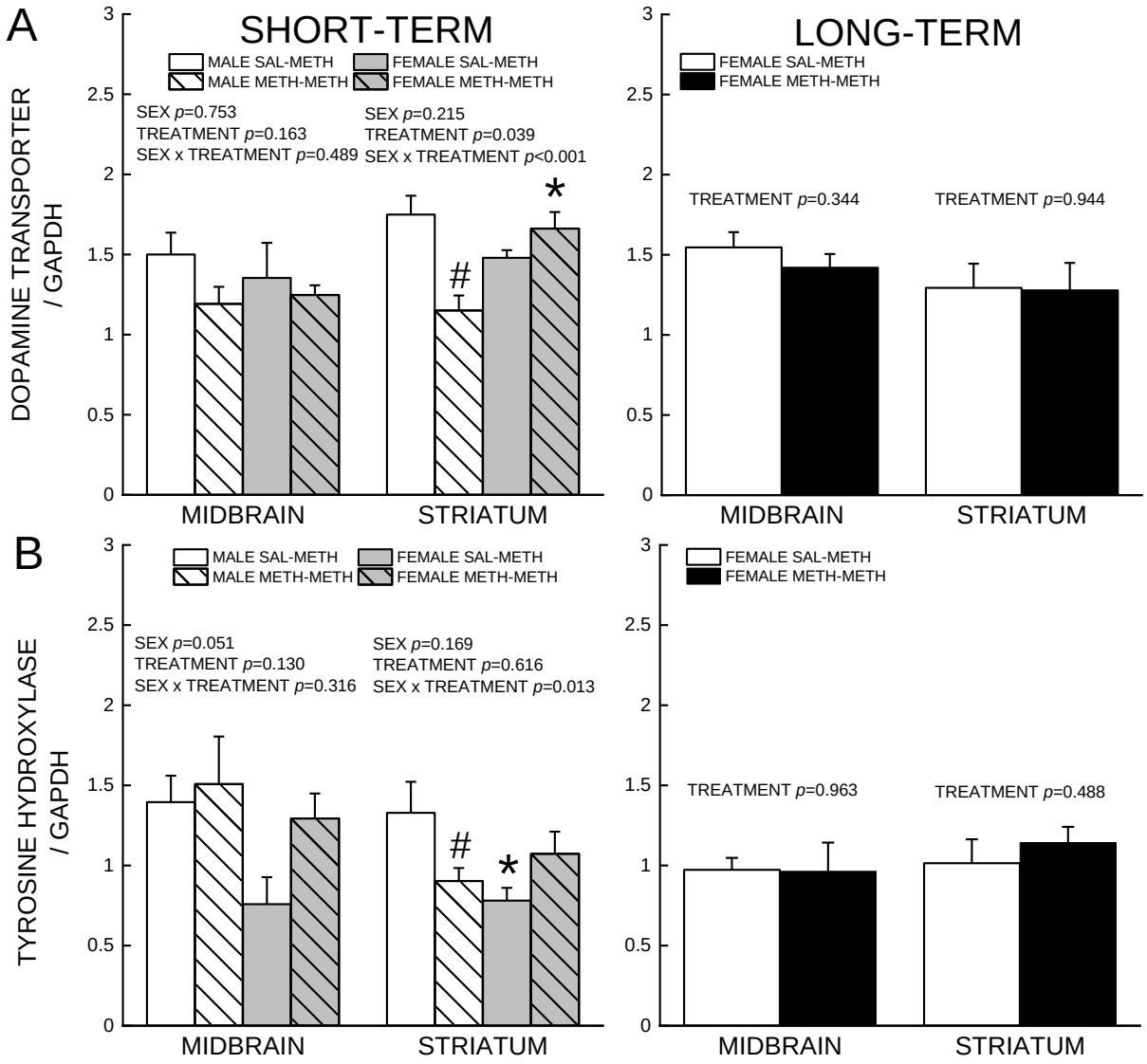

Figure 3.4 Short-term and long-term effects of METH on dopamine transporter (A) and tyrosine hydroxylase (B) expression in the midbrain and striatum of male and female mice. Each value represents the mean ± SEM (n = 6). #$p < 0.05$ vs. sex-matched SAL-METH group. *$p < 0.05$ vs. treatment-matched males.

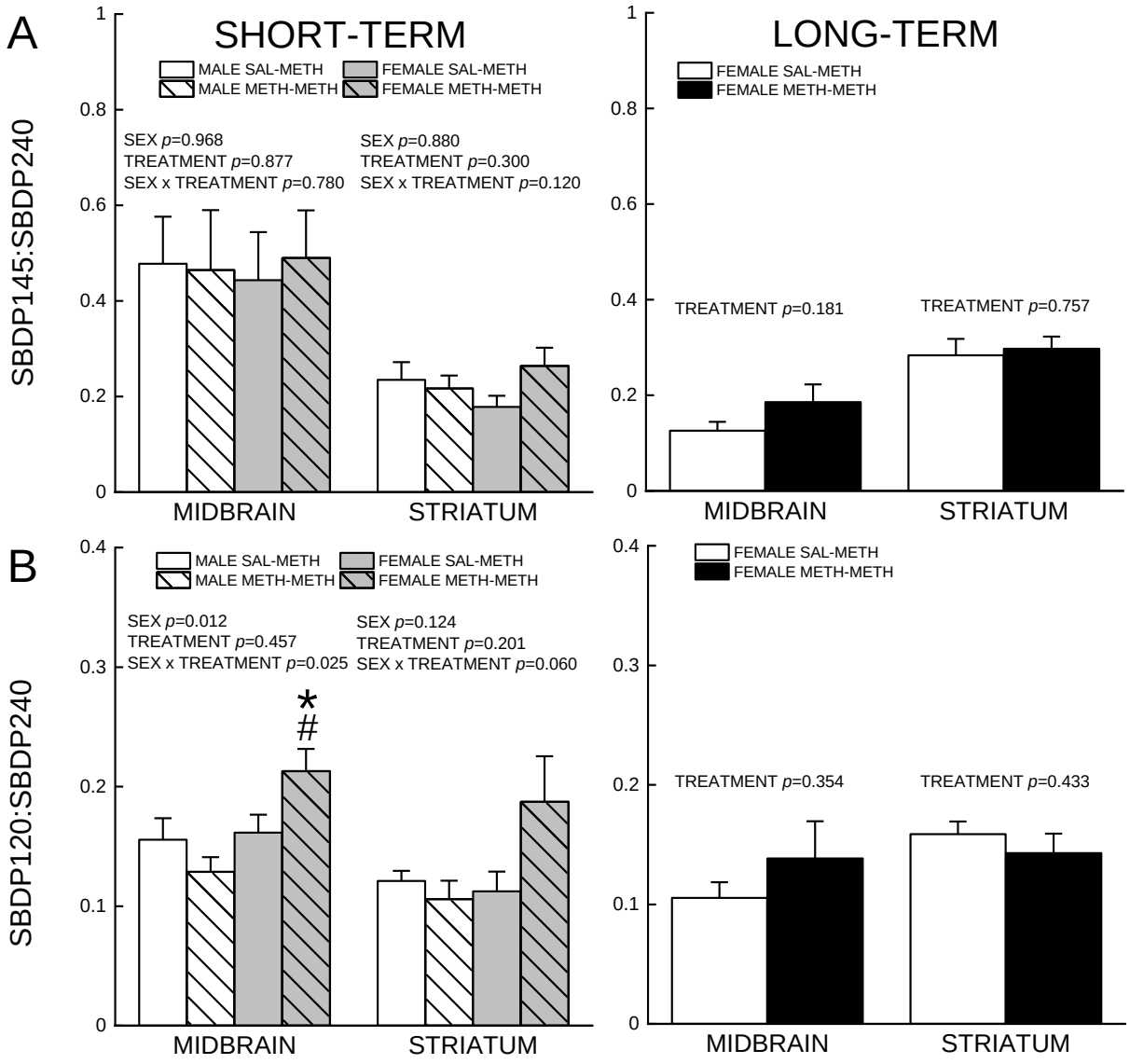

Figure 3.5 Short-term and long-term effects of METH on calcium dyshomeostasis (A) and apoptosis (B) in the midbrain and striatum of male and female mice. Each value represents the mean ± SEM (n = 6). #$p < 0.05$ vs. sex-matched SAL-METH group. *$p < 0.05$ vs. treatment-matched males.

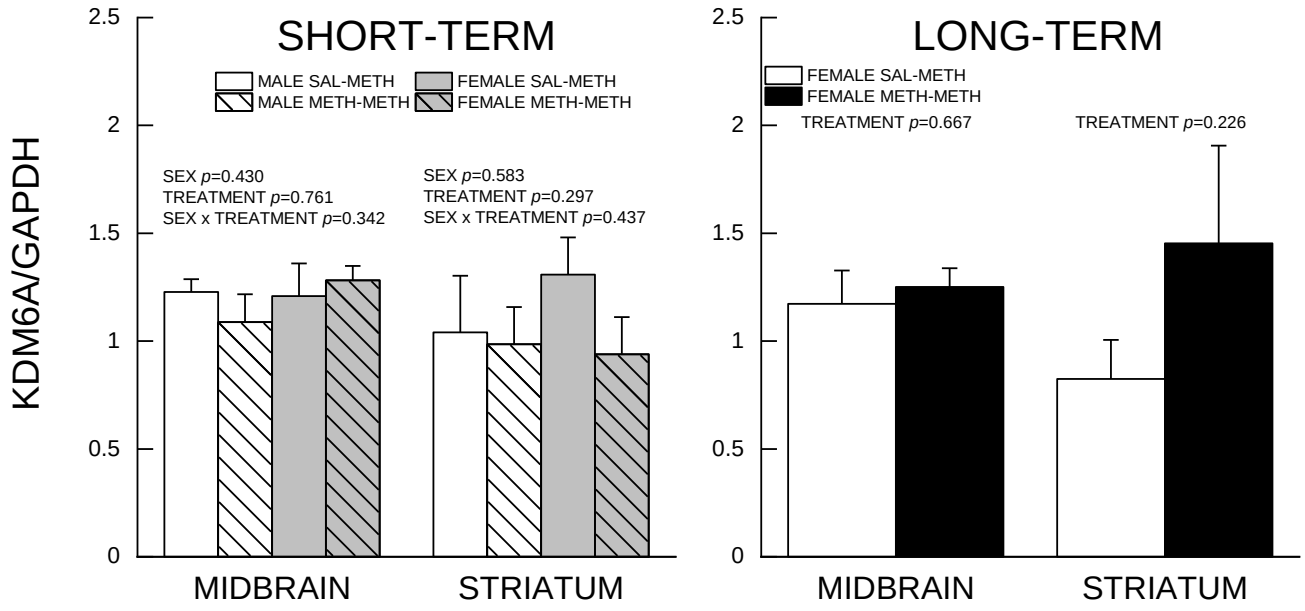

Figure 3.6 Short-term and long-term effects of METH on KDM6A expression in the midbrain and striatum of male and female mice. Each value represents the mean ± SEM (n = 6).

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CHAPTER 4

GENERAL DISCUSSION

Prescription stimulant misuse has become a major public health problem due to its increasing prevalence and adverse consequences particularly among young adults. The overall objective of this dissertation was to elucidate the effects of chronic methamphetamine (METH), at doses lower than what is expected to produce neurotoxicity, on brain function and behavior in an *in vivo* rodent model. Based on the body of literature, I chose to use METH as a prototypical psychostimulant to study the effects of chronic exposure in adult mice to create a model of chronic psychostimulant abuse that could be further applied to more clinically relevant prescription stimulant drugs, such as Ritalin and Adderall.

4.1 Summary of results

The major findings of research, summarized in Tables 4.1, 4.2, 4.3, 4.4 and 4.5, included several short-term changes in behavior, with increased gait speed and fear memory deficits in females, and impairments in balance and spatial memory, but increased drug-seeking behavior in males. Behavioral alterations were supported by short-term, sex-dependent changes in biochemistry, as males displayed increased dopaminergic markers and decreased excitotoxicity, whereas females displayed increased excitotoxicity. When animals received 2 doses of METH (0.5 mg/kg) for the conditioned place preference test, males previously exposed to chronic METH treatment were more sensitive to dopaminergic changes and exhibited a downregulation of TH and DAT in the striatum and midbrain, however there was an opposite effect in females

treated with chronic METH, with an upregulation of dopaminergic markers. Previous chronic METH also upregulated the expression of excitotoxicity markers in the striatum of females, but not males. Overall, biochemical changes were region dependent as males primarily had most changes occur in the striatum, but females had less changes in biochemical markers, but more brain areas affected, including the cortex, striatum, and midbrain.

There were few long-term effects on behavior in mice chronically treated with low dose METH. There were no long-lasting effects in cognition in mice chronically treated with METH. There were progressive changes in motor function, in the longitudinal analysis of motor coordination, however only in males. Interestingly, 9.5-month-old females with previous METH exposure exhibited a dampened drug-seeking response in the CPP test compared to females that has only received METH doses (0.5 mg/kg/day for 2 days) for CPP testing. This suggests that acute dosing of METH may produce a greater rewarding effect and that chronic METH may have produced tolerance to subsequent dosing. These observations were supported by the lack of changes in dopaminergic markers in the striatum and midbrain, key brain structures in the rewarding effects of drugs of abuse. Overall, there were minor biochemical alterations in dopaminergic function, oxidative stress, autophagy, astrogliosis and DNA methylation in 9.5-month-old males and females. Another significant finding was that excitotoxicity, characterized by spectrin proteolysis, was downregulated in most brain regions in males, but had increased expression in the striatum and cerebellum of females. This may suggest that chronic METH may induce compensatory mechanisms in males, but increased susceptibility to excitotoxicity in females. However, in CPP testing, acute doses of METH did not greatly affect excitotoxicity markers in 9.5-month-old females that had previously been administered chronic METH.

In conclusion, chronic METH, administered at a low-doses designed to emulate therapeutic dosing in a clinical setting, did not induce significant impairments in cognition or neurodegenerative markers although motor coordination was revealed to be significantly impaired in males. Females treated with chronic METH exhibited lower drug-seeking behavior at older, but not younger ages, suggesting an interplay between aging and addiction.
The hypothesis of the studies was that chronic, low-dose METH treatment would (i) produce long-lasting behavioral impairments (ii) lead to neuroadaptive changes in dopaminergic function (iii) exacerbate age-associated increases in astrogliosis, autophagy and oxidative stress and (iv) induce epigenetic modifications.

Ultimately, our results did not fully support our hypothesis as most the METH-induced changes in biochemistry and behavior observed in 5-month-old mice did not persist in the 9.5-month-old mice. Notably, there were no changes in oxidative stress measures or cognitive function. Although the results were unexpected, we propose that low-dose stimulants administered chronically through an interplay of different biochemical mechanisms such as Ca^{2+}-dependent excitotoxicity and neuroinflammation can induce short-term changes in motor and cognitive function, drug-seeking behavioral, and specific biochemical markers that may change throughout the lifespan.

4.2 Study caveats

It was hypothesized that chronic METH would induce an accelerated aging phenotype, based on the overlap of common biological mechanisms, including oxidative stress, excitotoxicity and neuroinflammation. However, anticipated age-associated impairments were not detected for cognitive performance, but were observed for coordinated running in 9-month-

old males. It is likely that the mice were not old enough to produce robust deficits in cognitive performance, as different behavioral traits exhibit different age-related patterns and physical function tends to progressively decline at as early as 6 months of age, whereas cognition decline occurs much later (Yanai and Endo 2021). For example, performance on the Morris water maze starts to decline from 16-18 months, with considerable impairments at 22 months, and fear memory impairments emerge at 12 months of age (de Fiebre et al. 2006; Sumien et al. 2006; Yanai and Endo 2021; Shoji et al. 2016).

There are many challenges in the translational relevance of modeling chronic drug abuse/addiction using rodent models. For our studies, we chose to utilize non-contingent intraperitoneal injections of METH, however, the most common route of administration of prescription stimulant misuse in adults is through oral administration, although non-oral routes have been reported, albeit to a lesser extent (e.g. snorting, smoking, injection) (Butler et al. 2021; Cassidy et al. 2015). There are marked differences in the drug half-life between humans and rodents, for example METH has an elimination half-life of approximately one hour in rats (Rivière et al. 2000) and 10 hours in humans (Cruickshank and Dyer 2009).

Although amphetamine and METH are similar psychostimulant drugs in terms of molecular structure and pharmacokinetics, they do differ in a few different properties. METH is a more potent CNS stimulant and the addition of a methyl group increases lipophilicity and makes it easier to cross the blood brain barrier compared to amphetamines, which may contribute to its addictive potential. Compared to d-amphetamine, METH has stronger DAT-mediated effects, lower glutamate release in the NAc, and greater protein and lipid oxidative damage (Goodwin et al. 2009; Shoblock et al. 2003; da-Rosa et al. 2012). Therefore, it is important not to

generalize findings in one amphetamine type stimulant to another as biochemical differences may lead to different outcomes.

4.3 Future directions

These studies are a first exploration into the long-lasting functional and biochemical consequences of chronic METH exposure, at a dose within the clinical dosing range for stimulant treatment of ADHD, in adult mice. To further examine the interplay between psychostimulants and healthy aging, another an aged group of animals (>18 months) could be added to detect cognitive changes that were not observed in our middle-aged groups. It is possible that there will be exacerbated functional impairments that prove our hypothesis of an amphetamine-induced accelerated aging phenotype. There are very few studies conducted in rodents that evaluate the effects of long-term use of prescription stimulants that more closely resemble human use patterns of misuse. To increase translational significance, more relevant prescription stimulant drugs (e.g. mixed l-and-d amphetamine salts to mimic Adderall use, or methylphenidate for Ritalin) could be used in additional long-term studies. Finally, a microdialysis study could be conducted to determine plasma and regional brain concentrations of methamphetamine and its active metabolites for additional pharmacokinetic data. Unfortunately, due to COVID-19 and the shutdown of our laboratories, we were unable to perform the long-term CPP study in our male mice, and it is important to finish out these studies in males, to compare susceptibility to potential abuse between sexes. In our studies we reported that chronic METH exposure reduced drug-seeking behavior in 9.5-month-old females. We expect that males treated with chronic amphetamine will display no differences in place preference response based on the findings of Strakowski *et al.* in which men, regardless of if they had received one or three

doses of d-amphetamine (0.25 mg/kg) did not report higher ratings of drug liking (Strakowski et al. 2001).

4.4 Conclusion

The data presented in this dissertation provides evidence of short-and-long term functional and biochemical alterations as a result of chronic METH treatment in adult mice. Our current knowledge on clinically relevant psychostimulants is limited and research examining the long-term consequences of use is even more so. It is important to understand how prescription stimulant misuse in young adults may affect long-term functional outcomes and if these individuals display increased vulnerability to aging processes.

Table 4.1 Overview of short-and-long-term biochemical outcomes of chronic METH exposure in different brain regions in males.

MALE	SHORT-TERM									
	DA Function		Oxidative Stress		Astrogliosis	Excitotoxicity		Autophagy	Epigenetics	
BRAIN REGION	DAT	TH	TBARS	tGSH	GFAP	SBDP145	SBDP120	LC3B	DNA Methylation	mtDNA copy #
CORTEX	-	↑	-	↓	-	-	-	-	n.	-
STRIATUM	↑	-	-	-	-	↓	↓	-	-	↑
MIDBRAIN	↓	-	-	-	-	-	-	-	n.	-
HIPPOCAMPUS	↑	-	-	↓	-	↑	-	-	n.	-
CEREBELLUM	-	-	-	-	-	-	-	-	n.	-
MALE	LONG-TERM									
	DA Function		Oxidative Stress		Astrogliosis	Excitotoxicity		Autophagy	Epigenetics	
BRAIN REGION	DAT	TH	TBARS	tGSH	GFAP	SBDP145	SBDP120	LC3B	DNA Methylation	mtDNA copy #
CORTEX	-	-	-	-	↓	↓	-	-	n.	-
STRIATUM	-	↓	-	-	-	↓	↓	-	-	-
MIDBRAIN	-	-	-	-	-	↓	↓	-	n.	-
HIPPOCAMPUS	-	-	-	-	-	↓	-	-	n.	-
CEREBELLUM	↑	-	-	-	-	-	-	↓	n.	-

n. refers to not measured; DAT: dopamine transporter; TH: tyrosine hydroxylase; tGSH: Total Glutathione; TBARS: Thiobarbituric acid reactive substances; GFAP: glial fibrillary acidic protein; SBDP145: spectrin breakdown product at 145kDa; SBDP120: spectrin breakdown product at 120kDa.

Table 4.2 Overview of short-and-long-term biochemical outcomes of chronic METH exposure in different brain regions in females.

FEMALE	SHORT-TERM									
	DA Function		Oxidative Stress		Astrogliosis	Excitotoxicity		Autophagy	Epigenetics	
	DAT	TH	TBARS	tGSH	GFAP	SBDP145	SBDP120	LC3B	DNA Methylation	mtDNA copy #
CORTEX	-	-	-	-	-	↑	↑	-	n.	-
STRIATUM	-	-	-	↑	-	-	-	-	↑	-
MIDBRAIN	↑	-	-	-	-	-	↑	-	n.	-
HIPPOCAMPUS	-	-	-	-	↓	-	-	-	n.	-
CEREBELLUM	-	-	↑	-	-	-	-	-	n.	-
FEMALE	LONG-TERM									
	DA Function		Oxidative Stress		Astrogliosis	Excitotoxicity		Autophagy	Epigenetics	
	DAT	TH	TBARS	tGSH	GFAP	SBDP145	SBDP120	LC3B	DNA Methylation	mtDNA copy #
CORTEX	-	-	-	↓	-	-	-	-	n.	-
STRIATUM	-	-	↑	-	-	↑	-	-	-	-
MIDBRAIN	-	-	-	-	-	-	-	-	n.	↑
HIPPOCAMPUS	-	-	-	-	↓	-	-	-	n.	-
CEREBELLUM	↓	-	-	-	-	↑	-	-	n.	-

n. refers to not measured; DAT: dopamine transporter; TH: tyrosine hydroxylase; tGSH: Total Glutathione; TBARS: Thiobarbituric acid reactive substances; GFAP: glial fibrillary acidic protein; SBDP145: spectrin breakdown product at 145kDa; SBDP120: spectrin breakdown product at 120kDa.

Table 4.3 Summary of the effects of chronic METH exposure on weight and longitudinal motor outcomes in male and female mice.

	5 MONTHS		7 MONTHS		9 MONTHS	
	MALE	FEMALE	MALE	FEMALE	MALE	FEMALE
BODY WEIGHT	-	-	-	-	-	-
LOCOMOTOR ACTIVITY	-	-	-	-	-	-
GAIT SPEED	-	↑	-	-	-	-
BRIDGE WALKING	↓	-	-	-	-	-
COORDINATED RUNNING	-	-	-	-	↓	-

Table 4.4 Summary of the effects of chronic METH exposure on short-and-long-term cognitive outcomes in male and female mice.

	SHORT-TERM		LONG-TERM	
	MALE	FEMALE	MALE	FEMALE
SPATIAL LEARNING	↓	-	-	-
SWIM SPEED	-	-	-	-
ACTIVE AVOIDANCE	-	-	-	-
FEAR CONDITIONING	-	↓	-	-
PLACE PREFERENCE	↑	-	n.	↓

n. refers to not measured.

Table 4.5 Overview of short-and-long-term biochemical outcomes of previous chronic METH exposure on acute dosing of METH in the conditioned place preference test.

	SHORT-TERM				LONG-TERM			
	MALE		FEMALE		MALE		FEMALE	
	STRIATUM	MIDBRAIN	STRIATUM	MIDBRAIN	STRIATUM	MIDBRAIN	STRIATUM	MIDBRAIN
DAT	↓	↓	↑	-	n.	n.	-	-
TH	↓	-	↑	↑	n.	n.	-	-
SBDP145	-	-	↑	-	n.	n.	-	↑
SBDP120	-	-	↑	↑	n.	n.	-	-
KDM6A	-	-	-	-	n.	n.	-	-

n. refers to not measured; DAT: dopamine transporter; TH: tyrosine hydroxylase; SBDP145: spectrin breakdown product at 145kDa; SBDP120: spectrin breakdown product at 120kDa.

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