UNIVERSITY OF CALIFORNIA SAN DIEGO

Cognitive-Behavioral Effects of Monoamine Transporter Inhibitors and Reversers

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Experimental Psychology

by

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The dissertation of Madeline Marie Pantoni is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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DEDICATION

I dedicate this dissertation to Flounder and Marley.

EPIGRAPH

It’ll all even out in the wash.

Stephan Anagnostaras

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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Chapter 3, in full, is a reprint of the material as it appears in Cognitive effects of MDMA in laboratory animals: a systematic review focusing on dose. *Pharmacological Reviews,* 71, 413–449. Pantoni, M. M., and Anagnostaras, S. G. (2019). DOI: 10.1124/pr.118.017087. Reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics. All rights reserved. The dissertation author was the primary investigator and author of this paper.

Chapter 4, in full, is currently being prepared for submission for publication of the material. Pantoni, M. M., Kim, J. L., Van Alstyne, K. R., and Anagnostaras, S. G. The dissertation author was the primary investigator and author of this paper.

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ABSTRACT OF THE DISSERTATION

Cognitive-Behavioral Effects of Monoamine Transporter Inhibitors and Reversers

by

Madeline Marie Pantoni

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Professor Stephan Anagnostaras, Chair

The classical monoamine neurotransmitters — dopamine, norepinephrine, and serotonin — are critically involved in a range of brain functions and their transporters (DAT, NET, and SERT) are targets for many psychoactive drugs. Some of the oldest and most widely prescribed psychotherapeutics (e.g., antidepressants, psychostimulants) are monoaminergic drugs, but their mechanisms remain poorly understood and many pose serious safety or efficacy challenges to patients. Still, there have been few meaningful

advances in neuropsychiatric drug development over the last three decades. Growing evidence suggests that MDMA and other psychedelics may transform care for an array of poorly treated conditions. The purpose of my dissertation is to elucidate the mechanisms underlying the behavioral effects of monoaminergic drugs to inform the development of novel, optimized drugs that retain or lack specific therapeutic or adverse effects. We have taken a systematic approach to examining monoamine transporter inhibitors and reversers at both low and high doses on various behavioral outcomes in mice. In Chapter 2, we explore whether existing drugs may mimic the therapeutic, memory-enhancing effects of low-dose psychostimulants but lack the adverse, reinforcing effects of high-dose psychostimulants. Bupropion (a low affinity DAT inhibitor) and atomoxetine (a high affinity NET inhibitor) produced these desired effects in combination but not alone. In Chapter 3, we systematically analyze all preclinical findings on the cognitive effects of MDMA with a critical focus on dose. We found no evidence that low, clinically relevant doses (< 3 mg/kg MDMA) produce cognitive impairments. In Chapter 4, we further analyze the potential adverse effects of MDMA across a wide range of doses. High doses (≥ 3 mg/kg MDMA) produced memory impairments and some evidence of an addictive potential while low, clinically relevant doses (≤ 1 mg/kg MDMA) did not. In Chapter 5, we present a novel method for assessing prepulse inhibition of acoustic startle in rodents, which may be especially useful for antipsychotic drug screening. In Chapter 6, we discuss our findings in the context of the current "psychedelic renaissance" and provide a roadmap for systematically analyzing classical and novel monoaminergic compounds to advance drug development for the most critical unmet medical needs in neuropsychiatry.

CHAPTER 1

Introduction

The classical monoamine neurotransmitters — dopamine, norepinephrine, epinephrine, and serotonin — are critically involved in range of functions, including motor control, cognition, emotion, memory processing, vascular regulation, and endocrine modulation (Kandel et al., 2000). Monoaminergic dysfunction is also implicated in various neuropsychiatric disorders, such as addiction, anorexia nervosa, anxiety, attention deficit hyperactivity disorder (ADHD), depression, Huntington's disease, Parkinson's disease, schizophrenia, and Tourette syndrome (Lucki, 1998; Beaulieu and Gainetdinov, 2011; Borodovitsyna et al., 2017). A major mechanism by which extracellular monoamine levels are regulated are via the dopamine (DAT), norepinephrine (NET), and serotonin (SERT) transporters. Specifically, these plasma membrane proteins transport released neurotransmitters back into the presynaptic terminal (Torres et al., 2003; Kristensen et al., 2011).

Many psychoactive drugs, both therapeutic and recreational, target the monoamine transporters, including psychostimulants, antidepressants, and mixed stimulant-psychedelics like MDMA (±3,4-methylenedioxymethamphetamine) (Tatsumi et al., 1997; Rothman and Baumann, 2003; Gether et al., 2006; Iversen, 2006). These drugs differ in their binding affinities to DAT, NET, and/or SERT as well as in their actions as transporter inhibitors versus transporter reversers. Transporter inhibitors bind to the transporter and block the reuptake of transmitter into the presynaptic terminal, while transporter reversers are transported into the presynaptic terminal and promote the release of transmitter into the extracellular space (Fleckenstein et al., 2000; Torres et al., 2003; Gether et al., 2006; Kristensen et al., 2011). Typically, reversers are more effective than inhibitors in increasing

extracellular monoamine levels; this is also affinity- and dose-dependent (Rothman and Baumann, 2003; Howell and Negus, 2014). Since these changes in extracellular monoamine levels underlie functional changes (Carlsson, 1964), the dose, affinity, and action of drugs that target the monoamine transporters critically mediate their functional effects. However, in many cases, the specific mechanisms underlying specific behavioral effects are poorly understood. A greater understanding of these patterns could facilitate the development of novel psychotherapeutics for disorders that have no current drug treatments (e.g., autism; Ghosh et al., 2013) or that have drug treatments with inconsistent safety (e.g., psychostimulants; Lakhan and Kirchgessner, 2012) or efficacy (e.g., antidepressants; Fava, 2003).

Ongoing research in our lab is aimed at exploring the behavioral effects of drugs that target the monoamine transporters. **Table 1.1** includes many of these monoaminergic drugs that our lab has studied in previous and the present experiments. This list comprises of various drug classes, including stimulants (e.g., methylphenidate, amphetamine, cocaine), non-stimulants (e.g., atomoxetine), antidepressants (e.g., bupropion, citalopram), and mixed stimulant-psychedelics (e.g., MDMA). For each drug, their action (inhibit or reverse) and binding affinity (K_i values) at DAT, NET, and SERT, as well as their behavioral effects at low and high doses, are specified. We are specifically interested in drug effects on locomotion, reinforcement, memory, and depression, which together, encompass a few of the most salient behaviors modulated across this broad class of drugs. For drug effects not reported in their U.S. Food and Drug Administration (FDA) approved labeling, we investigated using preclinical models of behavior that are widely used and

demonstrate high predictive validity. Specifically, we assessed the behavioral effects of these drugs in mice using the following tests: open field (locomotion; Walsh and Cummins, 1976), conditioned place preference (reinforcement; Tzschentke, 2007), Pavlovian fear conditioning (memory; Maren, 2001), and the Porsolt forced swim test (depression; Porsolt et al., 1977). Together, these findings will further clarify the mechanisms (i.e., dose, affinity, and action) that underlie the therapeutic (e.g., memory-enhancing, antidepressant) versus adverse (e.g., memory-impairing, reinforcing) behavioral effects of drugs that target the monoamine transporters.

Psychostimulants are a large class of monoaminergic drugs that are used therapeutically and also abused. The psychostimulants amphetamine (e.g., Adderall) and methylphenidate (e.g., Ritalin) are highly effective cognitive enhancers and first-line treatments for ADHD, the most common neuropsychiatric disorder of childhood (Rowland et al., 2002; Daughton and Kratochvil, 2009; Caye et al., 2019). However, these same compounds, along with other psychostimulants like cocaine, are also drugs of abuse that can lead to addiction and a myriad of neurocognitive problems (Rogers and Robbins, 2001; Wood et al., 2014). Accumulating evidence suggests that *dose* is the critical factor that dissociates the therapeutic versus adverse effects of psychostimulants (Arnsten, 2006; Wood et al., 2014). At low doses, psychostimulants enhance cognition and have a low abuse potential, whereas at high doses, they impair cognition and have a high abuse potential (Wood et al., 2007; Shuman et al., 2009, 2012; Wood and Anagnostaras, 2009; Carmack et al., 2014). Because of their abuse potential at high doses, psychostimulants are regulated under the strictest conditions for medically approved drugs by the United States

Controlled Substances Act (21 USC § 812, 2002) and similar laws in most other countries. Patients requiring these controlled medications face a major public health deficit due to poor access to psychiatrists and other health providers as well as complex and expensive procedures for obtaining refills (Saxena et al., 2007; Burke-Shyne et al., 2017). Non-controlled medications such as atomoxetine (Strattera), bupropion (Wellbutrin), clonidine (Catapres), and guanfacine (Intuniv) are also used to treat ADHD but are less effective than psychostimulants (Faraone, 2009; Faraone and Buitelaar, 2010; Catalá-López et al., 2017). Thus, there is an imperative need to develop a cognitive enhancer with low abuse liability but similar efficacy to that of psychostimulants (Childress et al., 2020). In Chapter 2, we explore whether combinations of existing, non-controlled drugs may mimic the procognitive effects of psychostimulants but lack a significant addictive potential. We hypothesized that the combination of bupropion (Wellbutrin or Zyban, a low affinity DAT inhibitor; Richelson and Pfenning, 1984) and atomoxetine (Strattera, a high affinity NET inhibitor; Wong et al., 1982) would enhance the cognitive processes of short- and long-term memory but not elicit the addiction-related behaviors of locomotor stimulation or reinforcement in mice. Indeed, while atomoxetine alone enhanced short-term memory, the addition of bupropion was required to enhance long-term memory. Additionally, combined atomoxetine and bupropion did not elevate locomotor activity or produce reinforcement. These findings suggest that this drug combination or a drug with a similar mechanism could be developed as a novel, non-addictive cognitive enhancer.

Scientific interest in the potential therapeutic value of psychedelic drugs [e.g., lysergic acid diethylamide (LSD), psilocybin, MDMA, ketamine] is currently booming

(Rucker et al., 2018; Nutt, 2019; Andersen et al., 2020; Nutt and Carhart-Harris, 2020; Vollenweider and Preller, 2020; Inserra et al., 2021). Although psychedelics are generally associated with recreational use (UNODC, 2020), growing evidence suggests that these drugs may potentially be effective psychotherapeutic agents for various treatment-resistant and untreated neuropsychiatric disorders (Belouin and Henningfield, 2018). MDMA is of particular interest because of its seemingly unique prosocial effects, including the ability to increase empathy, trust, extroversion, and sociality (i.e., empathogen-entactogen effects; Kamilar-Britt and Bedi, 2015; Liechti, 2015; Sessa and Nutt, 2015; Bershad et al., 2016; Heifets and Malenka, 2016; Feduccia et al., 2018). Data from Phase 2 clinical studies indicates that MDMA augments and enhances the effectiveness of psychotherapy for treatment-resistant post-traumatic stress disorder (Bouso et al., 2008; Mithoefer et al., 2011, 2013, 2018; Oehen et al., 2013; Ot'alora et al., 2018) and may even outperform approved stand-alone pharmacotherapies (e.g., paroxetine and sertraline) in terms of efficacy (Feduccia et al., 2019). MDMA also shows promise as a primary treatment for the social deficits that currently stand untreated in a wide range of neuropsychiatric disorders (Ghosh et al., 2013; Danforth et al., 2016; Heifets and Malenka, 2016). Despite the many possible therapeutic applications of MDMA, there is some concern regarding its adverse effects, including its potential to produce addiction, neurotoxicity, cognitive and emotional dysfunction, and even acute adverse cardiovascular and hepatic events (Schenk and Newcombe, 2018). We hypothesized that like psychostimulants (Wood et al., 2014), the therapeutic and adverse effects of MDMA may be dissociable by dose, and that the adverse effects may only arise at high doses or in heavy users. Prior to studying MDMA and these

effects in our own lab, we turned to the existing literature to better understand its therapeutic viability. In Chapter 3, we provide a comprehensive review of MDMA, including its history, pharmacology, and neurotoxic and cognitive effects in humans and animals. The central aim of this review was to systematically analyze all findings on the cognitive effects of MDMA in laboratory animals with a critical focus on dose. In all, we found no preclinical evidence that low, clinically relevant doses of MDMA (< 3 mg/kg) produces neurotoxicity or cognitive impairments. These findings support the therapeutic viability and further investigation of low-dose MDMA.

MDMA is considered to produce relatively unique behavioral effects as the prototypical empathogen-entactogen drug (i.e., generating a state of empathy and interpersonal closeness; Nichols, 1986; Liechti, 2015). However, MDMA is chemically similar to psychostimulants in many regards, including that it is a phenethylamine amphetamine derivative, targets the monoamine reuptake transporters (specifically, is a reverser like amphetamine), and has some stimulant-like effects (Richelson and Pfenning, 1984; Gold and Koob, 1989; Rothman and Baumann, 2003; Torres et al., 2003; Hill and Thomas, 2011). MDMA is also similar to selective serotonin reuptake inhibitor (SSRI) antidepressants such as citalopram and tryptamine hallucinogens such as N,N-dimethyltryptamine (DMT) that preferentially influence the serotonergic system (Shulgin, 1986; Battaglia et al., 1988; Rothman et al., 2001; Halberstadt and Nichols, 2010). It is possible that some of the behavioral effects of MDMA are not as unique as previously believed, but rather that when dose is taken into consideration, MDMA and other monoaminergic drugs may similarly influence behavior. Moreover, the safety profile of

low, clinically relevant doses of MDMA may not drastically differ from that of psychostimulants and antidepressants. In Chapter 4, we investigate the effects of MDMA across a wide range of doses (0.01–10 mg/kg) in mice on the behaviors that our lab has used to study other monoaminergic drugs, including locomotion, reinforcement, memory, and depression. Low doses of MDMA (≤ 1 mg/kg) had no effect on memory, addiction-related behaviors, or depressive-like behavior, while high doses of MDMA (≥ 3 mg/kg) produced memory impairments, some evidence of an addictive potential, and antidepressant effects. Together, these findings suggest that low- to moderate-dose MDMA, which has been administered in recent clinical studies (approximately 1–2 mg/kg; Feduccia et al., 2018), poses little risk of neural and behavioral toxicity.

There have been only a few meaningful advances in neuropsychiatric drug development over the last 30 years (Hyman, 2013). This, in part, is due to a high attrition rate of drug candidates from the preclinical stages through to the clinical stages (Kola and Landis, 2004; Paul et al., 2010). Translational success in neuropsychiatric research requires valid and reliable animal models of human behavior, as opposed to many other diseases that can rely on *in vitro* models (Becker and Greig, 2010; Bale et al., 2019). As such, the development of tools to assess animal behavior is critical to the development of novel neuropsychiatric drugs (Garner, 2014). In Chapter 5, we present a novel method for assessing prepulse inhibition of the acoustic startle reflex in rodents. Prepulse inhibition is a translational model of sensorimotor gating (Swerdlow et al., 2008), deficits of which are a core feature of schizophrenia and other neuropsychiatric disorders such as Huntington's disease, obsessive compulsive disorder, and Tourette syndrome (Kohl et al., 2013). Ergo,

prepulse inhibition has been the main model used to develop antipsychotic drugs (Geyer et al., 2001). The rodent startle reflex is typically assessed in small stabilimeter chambers that constrain animal movement, which can be stressful and unpleasant for animals and requires extensive habituation and calming procedures (Geyer and Swerdlow, 1998; Geyer and Dulawa, 2003). We consider that our novel method, which uses standard video to quantify the acoustic startle reflex in freely moving mice, may be especially useful to screen for potential antipsychotic drugs.

In Chapter 6, we discuss our findings in the context of the current neuropsychiatric drug development crisis. We demonstrate how the systematic analysis of existing drugs can inform the development of novel, optimized drugs that retain or lack specific therapeutic or adverse effects. As such, we examine the use of psychedelics, entactogens, and stimulants in neuropsychiatry and provide a roadmap for their systematic analysis. A broad effort to systematically analyze both classical and novel monoaminergic compounds will significantly advance drug development for some of the most critical unmet medical needs in neuropsychiatry.

Table 1.1 Binding affinities and behavioral effects of monoamine transporter inhibitors and reversers.

[a]Actions of methylphenidate, cocaine, atomoxetine, bupropion, and citalopram as transporter inhibitors and of d-amphetamine and MDMA as transporter reversers are previously reviewed (Kristensen et al., 2011).

[b]Published K_i values are shown for methylphenidate, d-amphetamine, cocaine, bupropion, citalopram (Richelson and Pfenning, 1984), atomoxetine (Wong et al., 1982), and MDMA (Rothman et al., 2001) in the rat brain. Please note low K_i values indicate high affinity. Binding affinities of combined atomoxetine/bupropion are represented symbolically: (+) low affinity, (++) high affinity, (–) negligible affinity.

[c](↑) The drug elevates locomotor activity at the specified dose; (↓) the drug decreases locomotor activity; (–) no effect; (?) the drug effect is not known; (*) the drug effect will be investigated in the present experiments.

[d](↑) The drug increases addictive potential at the specified dose; (–) no known addictive potential; (?) the drug effect is not known; (*) the drug effect will be investigated in the present experiments.

[e](↑) The drug enhances memory at the specified dose; (↓) the drug impairs memory; (–) no effect; (?) the drug effect is not known; (*) the drug effect will be investigated in the present experiments.

[f](↓) The drug has antidepressant efficacy at the specified dose; (?) the drug effect is not known; (*) the drug effect will be investigated in the present experiments.

[g]Methylphenidate's locomotor, reinforcing, and memory effects are previously published (Figs. 1 and 2 in Carmack et al., 2014).

[h]d-Amphetamine's locomotor, reinforcing, and memory effects are previously published (Figs. 1 and 3 in Wood and Anagnostaras, 2009; Fig. 4 in Carmack et al., 2014).

[i]Cocaine's locomotor, reinforcing, and memory effects are previously published (Figs. 1 and 3 in Wood et al., 2007; Fig. 4 in Carmack et al., 2014).

[j]Atomoxetine's locomotor, reinforcing, and memory effects are previously published (Figs. S1 and S2 in Carmack et al., 2014).

[k]Bupropion's locomotor and memory effects are previously published (Fig. S1 in Carmack et al., 2014); its reinforcing and antidepressant effects are reported in Wellbutrin's FDA approved labeling (GlaxoSmithKline, 2011).

[l]Citalopram's locomotor and memory effects are previously published (Fig. S1 in Carmack et al., 2014); its reinforcing and antidepressant effects are reported in Celexa's FDA approved labeling (Forest Laboratories, 2011).

[m]Combined atomoxetine/bupropion's locomotor, reinforcing, and memory effects will be investigated in the present experiments (Chapter 2).

[n]MDMA's locomotor, reinforcing, memory, and antidepressant effects will be investigated in the present experiments (Chapters 3 and 4).

		Binding affinity (K_i)[b]				Behavior			
Drug	Action[a]	DAT (nM)	NET (nM)	SERT (nM)	Dose	Locomotion[c]	Reinforcement[d]	Memory[e]	Depression[f]
Methylphenidate[g]	Inhibit	160	40	22,000	Low	–	–	↑	?
					High	↑	↑	↓	?
D-Amphetamine[h]	Reverse	82	50	1840	Low	–	–	↑	?
					High	↑	↑	↓	?
Cocaine[i]	Inhibit	270	155	180	Low	↑	–	↑	?
					High	↑	↑	↓	?
Atomoxetine[j]	Inhibit	1600	1.9	750	Low	–	–	–	?
					High	↓	↑	–	?
Bupropion[k]	Inhibit	630	2300	15,600	Low	–	–	–	↓
					High	–	?	↓	?
Citalopram[l]	Inhibit	28,000	4000	1.3	Low	–	–	–	↓
					High	–	?	↓	?
Atomoxetine/ Bupropion[m]	Inhibit	+	++	–	Low	*	*	*	?
					High	?	?	?	?
MDMA[n]	Reverse	1572	462	238	Low	*	*	*	*
					High	*	*	*	*

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CHAPTER 2

Dopamine and norepinephrine transporter
inhibition for long-term fear memory enhancement

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Dopamine and norepinephrine transporter inhibition for long-term fear memory enhancement

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ABSTRACT

Psychostimulants are highly effective cognitive-enhancing therapeutics yet have a significant potential for abuse and addiction. While psychostimulants likely exert their rewarding and addictive properties through dopamine transporter (DAT) inhibition, the mechanisms of their procognitive effects are less certain. By one prevalent view, psychostimulants exert their procognitive effects exclusively through norepinephrine transporter (NET) inhibition, however increasing evidence suggests that DAT also plays a critical role in their cognitive-enhancing properties, including long-term memory enhancement. The present experiments test the hypothesis that combined strong NET and weak DAT inhibition will mimic the fear memory-enhancing but not the addiction-related effects of psychostimulants in mice. We examined the effects of the high affinity NET inhibitors atomoxetine or nisoxetine and the low affinity DAT inhibitor bupropion, either alone or in combination, on short- and long-term memory of Pavlovian fear conditioning. We also examined the addiction-related effects of combined strong NET and weak DAT inhibition using conditioned place preference and a locomotor activity test. While atomoxetine or nisoxetine alone enhanced short-term fear memory, the addition of bupropion was required to significantly enhance long-term fear memory. Additionally, combined atomoxetine and bupropion did not produce substantial motor stimulation or place preference. These findings suggest that combining strong NET and weak DAT inhibition could lead to the development of a highly effective cognitive enhancer that lacks the potential for addiction.

1. Introduction

Classical psychostimulants (*e.g.*, methylphenidate, amphetamine, and cocaine) all target the dopamine and norepinephrine transporters (DAT and NET) with high affinity—methylphenidate and cocaine are "reuptake inhibitors" and amphetamine is a "releaser" resulting in large increases in extracellular dopamine and norepinephrine levels [1,2]. The behavioral effects of psychostimulants are highly dose-dependent—low doses enhance cognition and rarely produce addiction, while high doses impair cognition and are closely associated with addiction [3]. Although amphetamine and methylphenidate have proven highly effective at enhancing cognition in patients with attention-deficit hyperactivity disorder (ADHD) and other disorders [4,5], these patients face a major public health deficit due to poor access to psychiatrists and other health providers [6–9], as well as complex and expensive procedures for obtaining refills [10]. Given that dose markedly dissociates the cognitive-enhancing and abuse-related effects of psychostimulants [3], it is likely possible to develop a drug that retains the therapeutic effects of psychostimulants but lacks abuse potential.

Our previous work explored if psychostimulant-induced memory enhancement is dependent on dose, and if efficacy for long-term memory (LTM) enhancement could be predicted based on DAT and/or NET affinity [3,11–16]. If LTM enhancement is due to exclusive action at one of these transporters, then a selective inhibitor of DAT or NET should also enhance LTM. However, given individually, bupropion (a low affinity DAT inhibitor) or atomoxetine (a high affinity NET inhibitor) did not enhance LTM [13] (see also Fig. 1), indicating that psychostimulant-induced LTM enhancement likely requires some combination of DAT and NET activity.

Although DAT inhibition appears to be required for LTM

Abbreviations: ADHD, attention-deficit hyperactivity disorder; ATX, atomoxetine; BUP, bupropion; CS, conditioned stimulus; DAT, dopamine transporter; LTM, long-term memory; NET, norepinephrine transporter; NIS, nisoxetine; PFC, prefrontal cortex; STM, short-term memory; US, unconditioned stimulus

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enhancement, increased extracellular dopamine levels are also responsible for the addictive potential of drugs, including psychostimulants [17–19]. However, drugs with weak activity at DAT (*i.e.*, low binding affinity, slow kinetics, and/or low doses) are not likely to produce addiction. For instance, the atypical antidepressant bupropion, a cathinone derivative, binds to DAT with low affinity, has slow kinetics, and has little abuse liability [1,20,21]. This suggests that weak DAT inhibition may be sufficient for LTM enhancement but insufficient for producing addiction-related behaviors.

While our previous work suggested that affinity for DAT and NET may be required for LTM enhancement and considered that it may be possible to develop a drug that retains the procognitive effects of psychostimulants but that lacks the potential for addiction [13], the present study aims to directly test these predictions. We hypothesized that combined strong NET and weak DAT inhibition will mimic the memory-enhancing but not the addiction-related effects of psychostimulants. Here, we use combinations of existing drugs—the high affinity NET inhibitors atomoxetine (ATX) or nisoxetine (NIS) and the low affinity DAT inhibitor bupropion (BUP). ATX is a non-stimulant ADHD medication that is non-controlled and lacks abuse potential but remains clinically inferior to psychostimulants [22–24]. NIS has a similar binding profile to ATX but has not been pursued clinically [1]. BUP is an atypical antidepressant that is occasionally used as a non-stimulant ADHD adjunct [21,25].

We examined the effects of these drugs alone and in combination on short-term memory (STM) and LTM using Pavlovian fear conditioning, a simple and efficient tool for modeling the effects of drugs on memory in rodents [13,26]. In Pavlovian fear conditioning, a discrete conditioned stimulus (CS) is paired with an aversive footshock unconditioned stimulus (US) in a novel context. After training, mice will exhibit freezing behavior to both the discrete CS as well as the context (*i.e.*, the conditioning chamber); both cued and contextual fear memory depend on the amygdala, whereas contextual fear memory further depends on the hippocampus [26–30]. When administered pre-training, we have found that clinically-relevant doses of several psychostimulants enhance short- and long-term fear memory [13–16]. In the present study, we found evidence that NET inhibition alone enhances short-term fear memory, but the addition of some DAT inhibition seems to be required to enhance long-term fear memory. We also examined the addiction-related effects of combined strong NET and weak DAT inhibition using conditioned place preference (a model of drug-seeking) and a locomotor activity test and found no substantial evidence of reward or motor stimulation.

2. Materials and methods

2.1. Subjects

480 hybrid C57BL6/Jx129T2/SvEmsJ (129B6) (Jackson Laboratory, West Sacramento, CA, USA) male (n = 255) and female (n = 225) mice were used. Separate cohorts of mice were used for the fear conditioning, locomotor activity, and conditioned place preference experiments. Mice were weaned at 3 weeks of age and group-housed (2–5 mice per same sex cage) with continuous access to food and water. The animal colony was maintained on a 14:10-h light/dark schedule and all testing occurred during the light phase of the cycle. Mice were at least 10 weeks old and handled for 3 days (1 min/day) prior to testing. All animal care and testing procedures were approved by the UCSD IACUC and compliant with the 8th NRC Guide for the Care and Use of Laboratory Animals.

2.2. Drugs

Atomoxetine HCl (Sigma-Aldrich, TCI America), Nisoxetine HCl (Abcam, Tocris Bioscience), and Bupropion HCl (Sigma-Aldrich, Spectrum Chemical, TCI America) were dissolved in 0.9% physiological saline, either alone or in combination (ATX + BUP, NIS + BUP). A range of doses were selected (0.1, 0.5, 1, and 10 mg/kg ATX; 0.1, 0.5, 1, 5, and 10 mg/kg NIS; 0.5, 2.5, 5, 10, and 20 mg/kg BUP; salt weights). Only clinically-relevant doses were given in combination, because previous experiments indicated that higher doses would produce deficits [13]. All injections were given intraperitoneally in a volume of 10 mL/kg. As further described, “on-drug” sessions were performed immediately or up to 30 min following drug injections (and necessarily includes all STM tests) and “off-drug” sessions were performed in a drug-naïve state.

2.3. Fear conditioning

The VideoFreeze system (Med-Associates Inc., St. Albans, VT, USA) and fear conditioning protocol were used as described previously [13,26,28,31,32]. Up to eight mice were trained/tested concurrently in individual conditioning chambers that contained stainless-steel rod floors, white acrylic sidewalls, and clear polycarbonate front walls. Training and context testing took place in the ‘training context’ in which the chambers were illuminated with moderate (80 lx) white light and were cleaned and scented with 7% isopropanol. Tone testing took place in the ‘alternate context’, as the chambers were transformed across multiple sensory dimensions to create a distinct context—a black plastic, triangular teepee was inserted into the chamber, white acrylic sheets were placed over the floors, only near-infrared light (980 nm) was used to create a dark environment, and the chambers were cleaned and scented with 5% vinegar. During all trials, the VideoFreeze system continuously scored locomotor activity (in arbitrary units [au], see [26] for a full description) and freezing behavior of each mouse.

425 mice were randomly assigned to drug dose groups as presented in Table 1. Groups were completely counterbalanced by sex and assigned chamber for training/testing.

2.3.1. Training

Mice were given an injection of drug or saline 15–30 min before being placed into one of eight identical chambers for training. Training began with a 3-min baseline period, followed by a single tone-shock pairing. The tone-shock consisted of a 30-s tone (2.8 kHz, 85 dBA) presented through a speaker in the chamber sidewall, which co-terminated with a 2-s scrambled footshock (0.75 mA, AC, RMS constant current) delivered through the rod floor. 1.5 min following the tone-shock paring, mice underwent a 5-min STM test. Locomotor activity

Table 1
Drug dose groups and sample sizes for fear conditioning experiments. 425 mice were randomly assigned to groups by dose of atomoxetine (ATX), nisoxetine (NIS), or bupropion (BUP), or dose combination of atomoxetine and bupropion (ATX + BUP) or nisoxetine and bupropion (NIS + BUP). [a]The NIS and NIS + BUP experiments were performed together and used the same saline control animals.

Drug	Dose (mg/kg)	*N*	Drug Combination	Dose (mg/kg)	*N*
ATX	0.0	20	ATX + BUP	0 + 0	43
	0.1	13		0.1 + 2.5	12
	0.5	13		0.5 + 2.5	12
	1.0	18		1 + 2.5	15
	10.0	19		0.1 + 5	22
NIS	0.0	16[a]		0.5 + 5	22
	1.0	8		1 + 5	19
	5.0	8		0.1 + 10	24
	10.0	8		0.5 + 10	21
BUP	0.0	14		1 + 10	16
	0.5	11	NIS + BUP	0 + 0	16[a]
	5.0	11		0.1 + 10	8
	10.0	10		0.5 + 10	8
	20.0	10		1 + 10	8
				5 + 10	8
				10 + 10	8

and freezing behavior were continuously scored to measure on-drug baseline locomotion, shock reactivity, and STM.

2.3.2. Context test

Seven to nine days after training, mice were returned to the training context, off-drug, for one 5-min context test. Freezing behavior was scored for all 5 min to measure contextual LTM.

2.3.3. Tone test

One to three days after context testing, mice were placed in the alternate context, off-drug, for one 5-min tone test. Tone testing consisted of a 2-min baseline period, followed by the presentation of 3, 30-s tones identical to the training tone (2.8 kHz, 85 dBA), each separated by 30 s. The difference in freezing behavior during the 3 tone presentations and the 2-min baseline period (tone minus baseline freezing) was used to measure tone LTM.

2.4. Locomotor activity

Eight mice were tested concurrently in individual chambers (one side of the two-compartment conditioned place preference chambers) (Med-Associates Inc.). Each chamber measured 21.6 × 43.2 × 30.5 cm, contained stainless steel rod flooring and polycarbonate walls (three white and one black), and was cleaned with glass cleaner between trials. Activity Monitor software (Med-Associates Inc.) used the interruption of infrared beams to identify mouse position and measure locomotor activity (ambulatory distance in cm).

Testing was conducted over 5 alternating days in a within-subjects design, such that 24 mice (not used in other experiments) were tested once at each of the five doses in a pseudorandom order: 0 + 0, 0.1 + 5, 0.5 + 5, 0.1 + 10, and 0.5 + 10 mg/kg ATX + BUP (all n's = 24). On each testing day, mice were given an injection and immediately placed in the testing chamber. Ambulatory distance was scored for a total of 60 min to measure acute drug effects on locomotor activity.

2.5. Conditioned place preference

Seven or eight mice were tested concurrently in individual chambers (Med-Associates Inc.) as described previously [13,31]. Each chamber (43.2 × 43.2 × 30.5 cm) consisted of two sides separated by a black wall with a removable insert (that was removed only for place preference testing). The two sides provided distinct tactile and visual cues, as they differed by flooring (stainless steel rods or wire-mesh) and walls (decorated white or undecorated clear polycarbonate). The chambers were counterbalanced by the combination of flooring/walls and were cleaned with glass cleaner between trials. Activity Monitor software (Med-Associates Inc.) used the interruption of infrared beams to identify mouse position and measure percent time spent on each side during testing.

31 mice were randomly assigned to drug dose groups: 0 + 0 (n = 11), 0.1 + 5 (n = 10), or 1 + 10 (n = 10) mg/kg ATX + BUP. Testing chamber and paired/unpaired side assignments were completely counterbalanced across groups.

2.5.1. Habituation

Mice were habituated to the testing chamber for two consecutive days prior to training. On each habituation day, mice were introduced to both sides for 30 min each, off-drug. The sequence of habituation to the paired/unpaired sides was counterbalanced across groups and day.

2.5.2. Training

The day following habituation, mice were trained for seven consecutive days. On each training day, mice were injected with saline and immediately placed into the unpaired side for 15 min, and then injected with drug and immediately placed into the paired side for 15 min.

2.5.3. Place preference test

24 h following training, mice were tested off-drug for place preference. The inserts that previously separated the two sides of the chambers were removed. Mice were placed into the center of the chamber (direction of entry was counterbalanced) and allowed access to both sides for 15 min. Time spent on each side was scored to evaluate place preference (percent time spent on the paired minus the unpaired side).

2.6. Statistical analyses

Univariate or multivariate analysis of variance (ANOVA) were used to identify overall group differences; these were followed by Fisher's Least Significant Difference (LSD) post-hoc tests against the saline control groups. Data from male and female mice were merged as we found no statistically significant sex differences that meaningfully influenced our findings (p values > 0.05).

3. Results

3.1. Effects of ATX, NIS, and BUP on fear learning and memory

The effects of ATX (0–10 mg/kg i.p.), NIS (0–10 mg/kg i.p.), and BUP (0–20 mg/kg i.p.) on fear learning and memory were examined alone[1] and in combination using Pavlovian fear conditioning. Mice were trained on-drug with a single tone-shock pairing, immediately tested for STM, and then tested off-drug at least one week later for contextual and tone LTM.

3.1.1. ATX alone

During the baseline period, a dose of 10 mg/kg ATX significantly reduced locomotor activity relative to saline controls ($p = 0.044$). All other doses had no effect on baseline locomotion (p values > 0.35). The shock elicited a large activity burst that did not significantly differ between groups ($F(4,78) = 0.883$, $p = 0.478$) (Fig. 1A). ATX dose-dependently modulated freezing during the STM test ($F(4,78) = 6.16$, $p < 0.001$). Doses of 0.5, 1, and 10 mg/kg ATX significantly enhanced STM relative to saline controls (p values < 0.04). A dose of 0.1 mg/kg ATX had no effect on STM ($p = 0.97$) (Fig. 1B). Freezing did not significantly differ between groups during the contextual ($F(4,78) = 0.59$, $p = 0.668$) (Fig. 1C) nor the tone ($F(4,78) = 0.94$, $p = 0.446$) LTM tests (Fig. 1D).

Low locomotor activity during training could be directly related to enhanced freezing, as seen in mice given 10 mg/kg ATX (*i.e.*, reduced baseline locomotion and enhanced STM freezing). However, such an effect could also reflect improved executive function, which could appear as both enhanced inhibition (*e.g.*, a 'calming' effect) and enhanced STM. Although one can never really completely separate these two views because STM tests are necessarily on-drug, we approached this problem by subtracting freezing behavior during baseline from that during the STM test. This eliminates the portion of post-shock freezing that may be due to the drug directly reducing activity and thereby enhancing freezing. Using this measure, a dose of 10 mg/kg ATX significantly enhanced STM relative to saline controls (data not shown; 0 mg/kg, 23.45 ± 4.67%; 10 mg/kg, 42.47 ± 3.62%; $p = 0.004$). Therefore, the significant reduction in baseline locomotion produced by 10 mg/kg ATX is not responsible for the significant enhancement in freezing during the STM test. This is typical of drug or lesion effects that produce small changes in locomotor activity – they are unlikely to affect freezing [29,30].

[1] Incomplete portions of this data (ATX alone and BUP alone) appear in the Supplemental Figures of Carmack et al. [13].

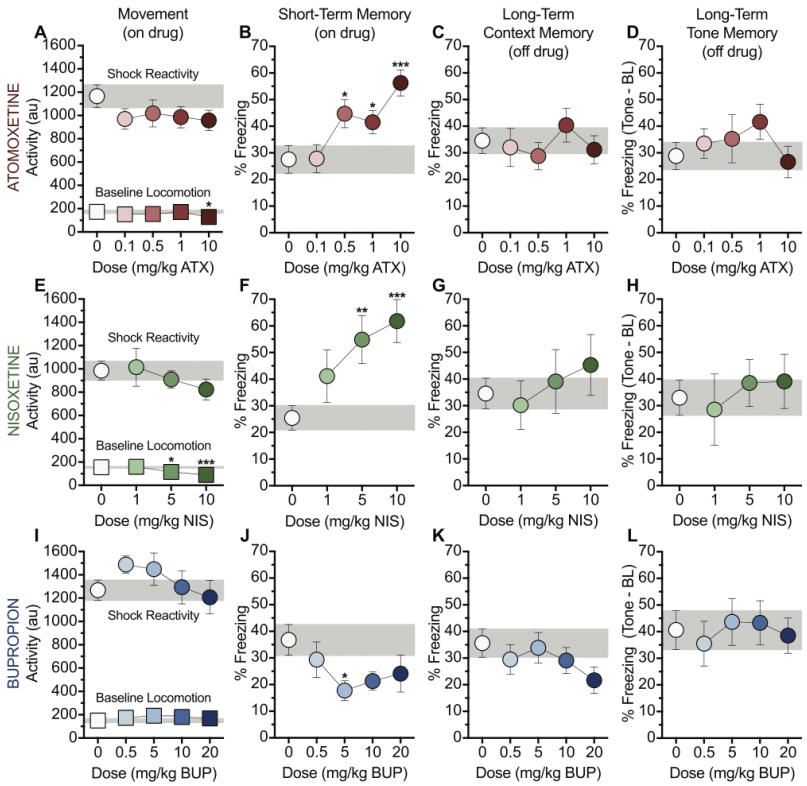

Fig. 1. The effects of atomoxetine (ATX; **a–d**), nisoxetine (NIS; **e–h**), and bupropion (BUP; **i–l**) on fear learning and memory. On-drug activity during the 3-min training baseline period and the 2-s footshock **(a, e and i)**, short-term memory as measured by percent freezing during the 5-min post-shock period **(b, f and j)**, and long-term context **(c, g and k)** and tone **(d, h and l)** memory as measured by percent freezing during off-drug testing, 1 week or more after training. **(a)** A dose of 10 mg/kg ATX significantly reduced baseline locomotion relative to saline controls. ATX had no effect on shock reactivity. **(b)** Doses of 0.5, 1, and 10 mg/kg ATX significantly enhanced short-term memory relative to saline controls. **(c and d)** ATX had no effect on long-term context or tone memory. **(e)** Doses of 5 and 10 mg/kg NIS significantly reduced baseline locomotion relative to saline controls. NIS had no effect on shock reactivity. **(f)** Doses of 5 and 10 mg/kg NIS significantly enhanced short-term memory relative to saline controls. **(g and h)** NIS had no effect on long-term context or tone memory. **(i)** BUP had no effect on baseline locomotion or shock reactivity. **(j)** A dose of 5 mg/kg BUP significantly impaired short-term memory relative to saline controls. **(k and l)** BUP had no effect on long-term context or tone memory. Each point represents the mean ± 1 standard error. The grey bar indicates standard error range for the comparison saline control group. Starred data points identify significant comparisons against the saline control group using Fisher's LSD (*P < 0.05, **P < 0.01, and ***P < 0.001). Incomplete portions of this data (ATX alone and BUP alone) appear in the Supplemental Figures of Carmack et al. [13].

3.1.2. NIS alone

NIS dose-dependently modulated locomotor activity during the baseline period ($F(3,36) = 7.06$, $p < 0.001$). Doses of 5 and 10 mg/kg NIS significantly reduced baseline locomotion relative to saline controls (p values < 0.02). A dose of 1 mg/kg NIS had no effect on baseline locomotion ($p = 0.793$). The shock elicited a large activity burst that did not significantly differ between groups ($F(3,36) = 0.60$, $p = 0.619$) (Fig. 1E). NIS dose-dependently modulated freezing during the STM test ($F(3,36) = 5.67$, $p = 0.003$). Doses of 5 and 10 mg/kg NIS significantly enhanced STM relative to saline controls (p values ≤ 0.005). A dose of 1 mg/kg NIS had no effect on STM ($p = 0.122$) (Fig. 1F). Freezing did not significantly differ between groups during the contextual ($F(3,36) = 0.45$, $p = 0.719$) (Fig. 1G) nor the tone ($F(3,36) = 0.24$, $p = 0.866$) LTM tests (Fig. 1H).

Similar to a dose of 10 mg/kg ATX, doses of 5 and 10 mg/kg NIS significantly reduced baseline locomotion and significantly enhanced freezing during the STM test. Again, we subtracted freezing behavior during baseline from that during the STM test and found that doses of 5 and 10 mg/kg NIS significantly enhanced STM relative to saline controls (data not shown; 0 mg/kg, 23.63 ± 4.57%; 5 mg/kg, 45.58 ± 6.90%; 10 mg/kg, 50.1 ± 8.3%; p values ≤ 0.026). Therefore, the significant reductions in baseline locomotion produced by 5 and 10 mg/kg NIS again are not responsible for the significant enhancements in freezing during the STM test.

3.1.3. BUP alone

Locomotor activity during the baseline period did not significantly differ between groups ($F(4,51) = 0.58$, $p = 0.679$). The shock elicited a large activity burst that also did not significantly differ between groups ($F(4,51) = 1.05$, $p = 0.389$) (Fig. 1I). During the STM test, a dose of 5 mg/kg BUP significantly reduced freezing relative to saline controls ($p = 0.015$). All other doses had no effect on STM (p values > 0.05) (Fig. 1J). Freezing did not significantly differ between groups during the contextual ($F(4,51) = 1.00$, $p = 0.416$) (Fig. 1K) nor the tone ($F(4,51) = 0.18$, $p = 0.949$) LTM tests (Fig. 1L).

3.1.4. Combined ATX and BUP

ATX + BUP dose-dependently modulated locomotor activity during the baseline period ($F(9,196) = 2.93$, $p = 0.003$). A dose of 0.5 + 2.5 mg/kg ATX + BUP significantly reduced baseline locomotion ($p = 0.021$) and a dose of 0.1 + 10 mg/kg ATX + BUP significantly enhanced baseline locomotion ($p = 0.024$) relative to saline controls. All other doses had no effect on baseline locomotion (p values > 0.06). The shock elicited a large activity burst that did not significantly differ between groups ($F(9,196) = 1.63$, $p = 0.11$). A dose of 0.1 + 2.5 mg/kg ATX + BUP did produce a statistically significant decrease in shock reactivity relative to saline controls ($p = 0.008$) (Fig. 2A). However, this was unlikely related to any effects seen in fear conditioning, as no memory effects were observed at this dose. ATX + BUP dose-dependently modulated freezing during the STM test ($F(9,196) = 3.48$, $p < 0.001$). Doses of 1 + 2.5, 0.5 + 5, and 1 + 5 mg/kg ATX + BUP significantly enhanced STM relative to saline controls (p values < 0.015). All other doses had no effect on STM (p values > 0.25) (Fig. 2B).

During the contextual LTM test, mice given 0.5 + 10 mg/kg ATX + BUP exhibited significantly enhanced freezing relative to saline controls ($p = 0.044$). Mice given 1 + 2.5 mg/kg ATX + BUP exhibited a trend towards significantly enhanced freezing relative to saline

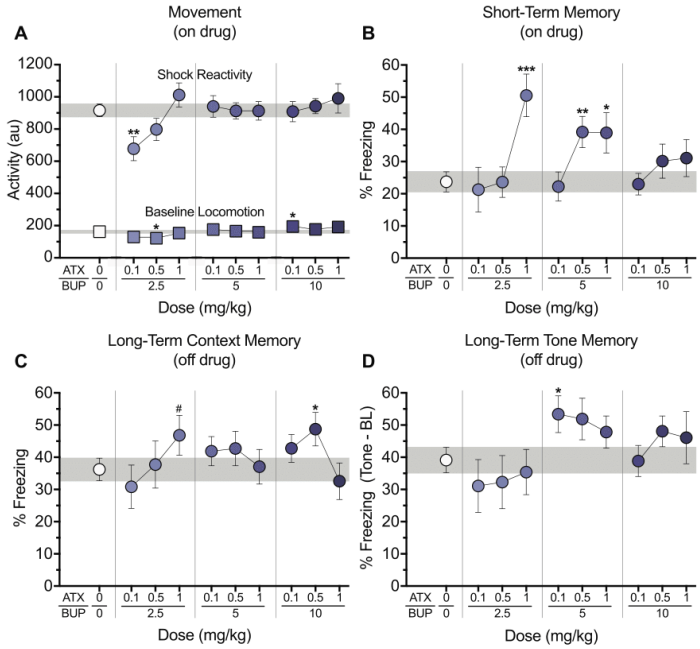

Fig. 2. The effects of combined atomoxetine (ATX) and bupropion (BUP) on fear learning and memory. **(a)** On-drug activity during the 3-min training baseline period and the 2-s footshock. A dose of 0.5 + 2.5 mg/kg ATX + BUP significantly reduced baseline locomotion relative to saline controls and a dose of 0.1 + 10 mg/kg ATX + BUP significantly enhanced baseline locomotion relative to saline controls. A dose of 0.1 + 2.5 mg/kg ATX + BUP significantly reduced shock reactivity relative to saline controls. **(b)** Short-term memory as measured by percent freezing during the 5-min post-shock period. Doses of 1 + 2.5, 0.5 + 5, and 1 + 5 mg/kg ATX + BUP significantly enhanced short-term memory relative to saline controls. **(c)** Long-term context memory as measured by percent freezing during off-drug context testing, 7–9 days after training. A pre-training dose of 0.5 + 10 mg/kg ATX + BUP significantly enhanced long-term context memory relative to saline controls. A pre-training dose of 1 + 2.5 mg/kg ATX + BUP significantly enhanced long-term context memory relative to saline controls during only the first minute of context testing. **(d)** Long-term tone memory as measured by percent freezing during off-drug tone testing (difference between tone presentations and tone baseline period), 1–3 days after context testing. A pre-training dose of 0.1 + 5 mg/kg ATX + BUP significantly enhanced long-term tone memory relative to saline controls. Hash-tagged data points identify significant comparisons against the saline control group during a certain portion of testing ($^{\#}P < 0.05$).

controls (p = 0.129), which was driven by a significant enhancement during the first minute of testing (data not shown; p = 0.017). During the tone LTM test, mice given 0.1 + 5 mg/kg ATX + BUP exhibited significantly enhanced freezing relative to saline controls (p = 0.041). All other doses had no effect on contextual (*p* values > 0.25) or tone (*p* values > 0.06) LTM (Figs. 2C and D).

3.1.5. Combined NIS and BUP

Locomotor activity during the baseline period did not significantly differ between groups ($F(5,50) = 1.25$, p = 0.302). The shock elicited a large activity burst that also did not significantly differ between groups ($F(5,50) = 0.84$, p = 0.526) (Fig. 3A). NIS + BUP dose-dependently modulated freezing during the STM test ($F(5,50) = 3.05$, p = 0.018). Doses of 0.5 + 10 and 5 + 10 mg/kg NIS + BUP significantly enhanced STM relative to saline controls (*p* values ≤ 0.045). All other doses had no effect on STM (*p* values > 0.1) (Fig. 3B).

During the contextual LTM test, mice given 0.1 + 10 and 0.5 + 10 mg/kg NIS + BUP exhibited a trend towards significantly enhanced freezing relative to saline controls (*p* values = 0.073 and 0.131), which were driven by significant enhancements during the fourth (data not shown, 0.1 + 10 mg/kg NIS + BUP, p = 0.021) or the second and third (data not shown, 0.5 + 10 mg/kg NIS + BUP, *p* values < 0.04) minutes of testing. During the tone LTM test, mice given 0.5 + 10 mg/kg NIS + BUP exhibited significantly enhanced freezing relative to saline controls (p = 0.01). All other doses had no effect on contextual (*p* values > 0.25) or tone (*p* values > 0.3) LTM (Fig. 3C and D).

3.2. Addictive potential of combined ATX and BUP

3.2.1. Locomotor activity

We selected a range of fear memory-enhancing dose combinations of ATX + BUP (0.1 + 5, 0.5 + 5, 0.1 + 10, and 0.5 + 10 mg/kg) and assessed their effects on locomotion over a 60-min period. There was no main effect of group on locomotor activity ($F(4,115) = 1.66$, p = 0.165). Doses of 0.1 + 10 and 0.5 + 10 mg/kg ATX + BUP significantly enhanced locomotor activity relative to saline during the first 10-min block (*p* values < 0.015) but not during any other blocks (*p* values > 0.2). Because increased locomotion was only observed during the first 10 min post-injection (before the peak of the drug), this effect may be a physical reaction to receiving a higher concentration of drug rather than an actual drug effect. All other doses of ATX + BUP had no effect on locomotion relative to saline during any time block (*p* values > 0.1) (Fig. 4A).

3.2.2. Conditioned place preference

We assessed the rewarding effects of ATX + BUP at two clinically-relevant dose combinations selected from the fear conditioning studies—a lower fear memory-enhancing dose (0.1 + 5 mg/kg) and the highest dose tested (1 + 10 mg/kg). Mice were trained for seven consecutive days to associate saline with one side and drug treatment with the other side of a two-compartment chamber. 24 h later, mice were returned off-drug with free access to both compartments. Place preference to the drug-paired side was scored as the difference in percent time spent on the paired side versus the unpaired side. None of the groups exhibited a significant preference for either side (one sample two-tailed *t*-test against hypothesized μ = 0, 0 + 0 mg/kg: $t(10) = 0.305$, p = 0.766, 0.1 + 5 mg/kg: $t(9) = 1.946$, p = 0.084, 1 + 10 mg/kg: $t(9) = 0.808$, p = 0.44). Place preference did not significantly differ between groups ($F(2,28) = 1.80$, p = 0.183). Mice given either ATX + BUP dose combination did not differ in place preference relative to saline controls (*p* values > 0.2) (Fig. 4B).

4. Discussion

We tested the effects of ATX, NIS, and BUP, alone and in combination, across a range of doses on Pavlovian fear conditioning. While ATX and NIS enhanced STM and BUP impaired STM, these drugs given alone failed to enhance LTM across a wide range of doses. However, BUP in combination with ATX or NIS produced enhancements in STM *and* LTM at certain dose combinations. On the locomotor activity and place preference tests, combined ATX and BUP did not produce

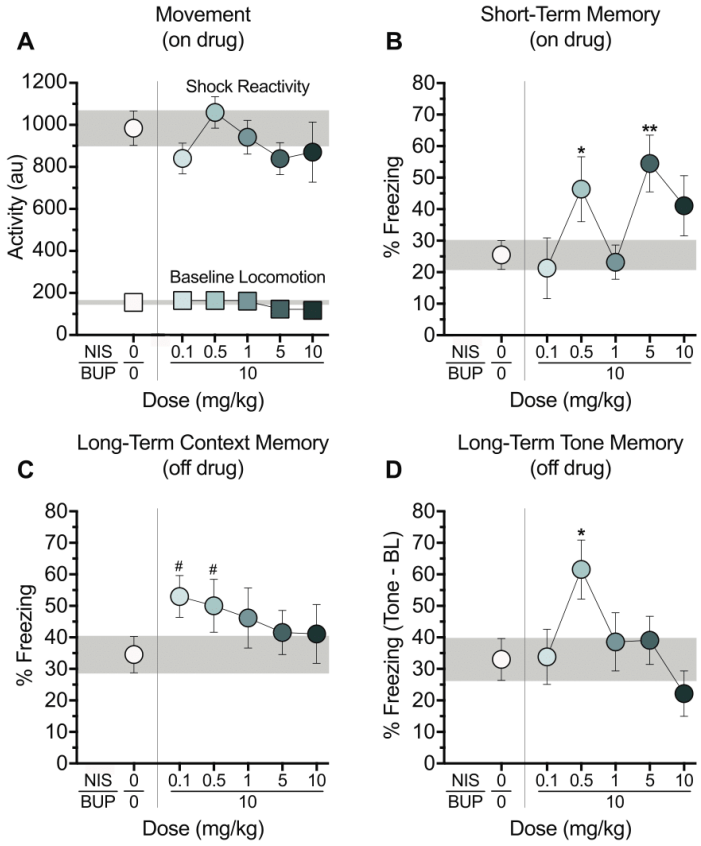

Fig. 3. The effects of combined nisoxetine (NIS) and bupropion (BUP) on fear learning and memory. **(a)** On-drug activity during the 3-min training baseline period and the 2-s foot-shock. NIS + BUP had no effect on baseline locomotion or shock reactivity. **(b)** Short-term memory as measured by percent freezing during the 5-min post-shock period. Doses of 0.5 + 10 and 5 + 10 mg/kg NIS + BUP significantly enhanced short-term memory relative to saline controls. **(c)** Long-term context memory as measured by percent freezing during off-drug context testing, 7–9 days after training. Pre-training doses of 0.1 + 10 and 0.5 + 10 mg/kg NIS + BUP significantly enhanced long-term context memory relative to saline controls during only the fourth minute (0.1 + 10 mg/kg) or the second and third minutes (0.5 + 10 mg/kg) of context testing. **(d)** Long-term tone memory as measured by percent freezing during off-drug tone testing (difference between tone presentations and tone baseline period), 1–3 days after context testing. A pre-training dose of 0.5 + 10 mg/kg NIS + BUP significantly enhanced long-term tone memory relative to saline controls.

substantial motor stimulation or reward. These findings indicate that NET inhibition alone is sufficient for short-term fear memory enhancement, but both DAT and NET inhibition seems to be needed for long-term fear memory enhancement. It also appears that *weak* DAT inhibition, when combined with strong NET inhibition, is sufficient for long-term fear memory enhancement but insufficient for producing addiction-related behaviors, at least in terms of motor stimulation or place preference.

In many previous experiments [14–16], LTM has been much more resistant than STM to enhancement or impairment by stimulant-like drugs (*e.g.*, modafinil, amphetamine, cocaine). Here, the STM and LTM tests differed in that STM was measured (unavoidably) on-drug and LTM was measured off-drug. Freezing behavior during the STM test could have been influenced by other drug effects, such as those on locomotor activity or fear. Only a few doses of ATX and NIS alone significantly reduced baseline locomotion and also enhanced freezing during the STM test. While reduced locomotor activity could reflect a 'calming' effect from improved executive function, we accounted for baseline drug effects on activity and found that these doses still enhanced STM (see Results section). It is unlikely that memory enhancements were confounded by drug-induced increases in fear or anxiety, as ATX, NIS, and BUP are typically *not* anxiogenic and both

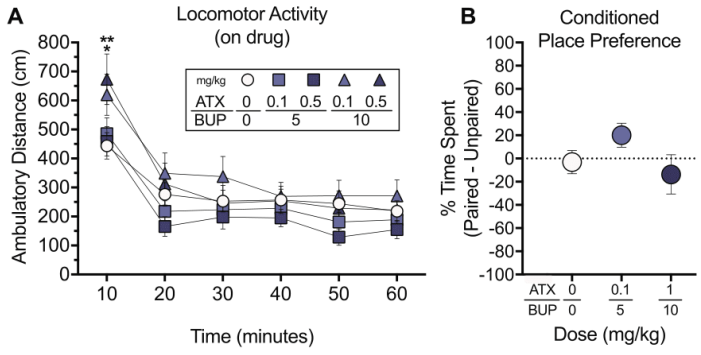

Fig. 4. The effects of combined atomoxetine (ATX) and bupropion (BUP) on addiction-related behaviors. **(a)** On-drug locomotor activity as measured by ambulatory distance during the 60 min (six 10-min blocks) immediately following drug administration. There was no main effect of dose on locomotor activity (total ambulatory distance in 60-min period). Doses of 0.1 + 10 and 0.5 + 10 mg/kg ATX + BUP significantly enhanced locomotor activity relative to saline during the first 10-min block only. **(b)** Conditioned place preference as measured by the difference in percent of time spent on the drug-paired side versus the unpaired side following seven days of training. None of the groups exhibited a significant preference for either side. Treatment with ATX + BUP had no significant effect on place preference relative to saline controls.

ATX and BUP are even prescribed for comorbid anxiety disorders [21,22,33,34]. It is also unlikely that memory enhancements were confounded by drug-induced increases in pain sensitivity, as we found no drug effects on nociception as measured by shock reactivity (except for the lowest dose of ATX + BUP, which had no effect on memory). If such confounds were present, we would expect nonspecific increases in freezing behavior across all tests; instead, we found that no doses significantly increased freezing across all three memory tests. Because the LTM tests were conducted in the absence of drug, we can conclude that certain dose combinations of DAT and NET inhibitors enhance fear memory acquisition and retention and the presence of drug is not required for retrieval.

Pavlovian fear conditioning is an efficient way to screen potential cognitive enhancers in rodents and is especially useful when testing many drugs at many doses [13,26]. Specifically, contextual fear memory is hippocampus-dependent and thus directly relevant to many conditions wherein memory is impaired [27,29,30]. Our previous work demonstrated that psychostimulants enhance both short- and long-term fear memory in mice at doses that are prescribed to treat ADHD and other cognitive disorders in humans [13–16], and these enhancements are also seen in other forms of learning and memory such as spatial memory [12,14]. Given this, we hypothesize that the drug combinations tested here may also be highly effective cognitive-enhancing therapeutics that target several forms of learning and memory.

LTM enhancement should be a critical therapeutic target of cognitive enhancers, as significant deficits in LTM are implicated in a wide range of disorders such as ADHD, dementia, Alzheimer's disease, schizophrenia, aphasia, and learning disabilities [35–40]. Despite this, clinical efficacy studies of cognitive enhancers often neglect LTM and focus primarily on attention, working memory, and response inhibition, conceivably because clinical assessment of these factors is far less laborious than long-term effects [41–45]. When left untreated, LTM deficits can lead to academic underachievement, poor job performance and retention, and limitations in major life activities [46]. LTM enhancement may be necessary to reverse deficits in academic and occupational achievement [44]. In particular, working and STM improvements are unlikely to improve school test performance unless LTM is also improved. We believe that an increased focus on LTM is crucial to develop novel, highly effective cognitive enhancers.

Existing theories suggest that psychostimulants and atomoxetine exert influence on "frontal" executive functions (*e.g.*, working memory, STM, attention, response inhibition) exclusively through NET inhibition in the prefrontal cortex (PFC) and all other procognitive effects, including LTM enhancement, are incidental to improvements in those functions [47–50]. It is believed that inhibiting NET in the PFC increases extracellular levels of both dopamine and norepinephrine, as there is a low density of DAT and a high density of NET in the PFC, and NET is non-selective in transporting either catecholamine [51–53]. In the present study, NET inhibition alone enhanced STM but did *not* enhance LTM unless combined with DAT inhibition. Thus, while increasing extracellular levels of dopamine and norepinephrine in the PFC may be responsible for enhancing STM and other executive functions, this mechanism is insufficient for enhancing LTM. We speculate that increasing extracellular dopamine levels in areas outside the PFC may also be necessary to enhance LTM. According to one view, the corelease of dopamine along with norepinephrine from the locus coeruleus to the dorsal hippocampus is key to successful learning and memory [54], which may explain our findings that the *combination* of DAT and NET affinity is necessary for LTM enhancement. Another possible mechanism by which the NET inhibitors enhanced STM may be increased brain-derived neurotrophic factor (BDNF) mRNA expression in the hippocampus, which atomoxetine has been shown to increase [55] and previous reports suggest is associated with improved STM [56–58].

There is much additional evidence implicating the critical role of DAT in learning and memory. DAT dysfunction is associated with age-related cognitive decline and several conditions wherein memory is impaired such as ADHD, dementia with Lewy bodies, Parkinson's disease, and chronic schizophrenia [59–62]. The 10-repeat VNTR allele of the dopamine transporter gene (DAT1) also correlates with ADHD as well as the combined inattentive/hyperactive-impulsive diagnostic subtype, higher levels of symptom severity, and an enhanced response to methylphenidate [63–65]. Taken together, some activity at DAT may be essential to treating learning and memory impairments.

We found that combinations of strong NET and weak DAT inhibitors mimic the short- and long-term fear memory-enhancing effects but lack the addiction-related effects of psychostimulants. Given that only certain dose combinations enhanced long-term fear memory, there is likely an ideal ratio of NET/DAT activity for maximal memory enhancement yet no addictive potential, and our future work will be aimed at exploring this. We propose that these drug combinations may be an effective alternative to psychostimulants in the treatment of cognitive dysfunction that may have decreased health risks and increased patient access.

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Declaration of Competing Interest

None.

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CHAPTER 3

Cognitive effects of MDMA in laboratory animals: a systematic review focusing on dose

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Cognitive Effects of MDMA in Laboratory Animals: A Systematic Review Focusing on Dose

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***Abstract*——±3,4-Methylenedioxymethamphetamine (MDMA) is a synthetic, psychoactive drug that is primarily used recreationally but also may have some therapeutic value. At low doses, MDMA produces feelings of relaxation, empathy, emotional closeness, and euphoria. Higher doses can produce unpleasant psychostimulant- and hallucinogen-like adverse effects and therefore are usually not taken intentionally. There is considerable evidence that MDMA produces neurotoxicity and cognitive deficits at high doses; however, these findings may not generalize to typical recreational or therapeutic use of low-dose MDMA. Here, we systematically review 25 years of research on the cognitive effects of MDMA in animals, with a critical focus on dose. We found no evidence that doses of less than 3 mg/kg MDMA—the dose range that users typically take—produce cognitive deficits in animals. Doses of 3 mg/kg or greater, which were administered most often and frequently ranged from 5 to 20 times greater than an average dose, also did not produce cognitive deficits in a slight majority of experiments. Overall, the preclinical evidence of MDMA-induced cognitive deficits is weak and, if anything, may be the result of unrealistically high dosing. While factors associated with recreational use such as polydrug use, adulterants, hyperthermia, and hyponatremia can increase the potential for neurotoxicity, the short-term, infrequent, therapeutic use of ultra low-dose MDMA is unlikely to pose significant cognitive risks. Future studies must examine any adverse cognitive effects of MDMA using clinically relevant doses to reliably assess its potential as a psychotherapeutic.**

I. Introduction

"Solely the dose determines that a thing is not a poison." – Paracelsus

±3,4-Methylenedioxymethamphetamine (MDMA, known as Ecstasy or Molly; Fig. 1) is a synthetic, psychoactive drug that is usually described as having mixed psychostimulant- and hallucinogen-like effects (i.e., effects like amphetamine and lysergic acid diethylamide) (Green et al., 2003). As with other phenethylamine and cathinone stimulant-psychedelics, MDMA primarily increases the neurotransmission of serotonin (5-HT) in the brain, specifically by reversing the 5-HT reuptake transporter (SERT) and causing the calcium-independent release of 5-HT (Rudnick and Wall, 1992; Wichems et al., 1995). MDMA also reverses the dopamine and norepinephrine transporters but to a lesser degree than SERT (Battaglia et al., 1988). These changes in brain chemistry produce desirable effects of relaxation, euphoria, arousal, and increased sociability as well as potential adverse effects such as nausea, headache, hallucinations, agitation, and palpitations. As dose is increased, MDMA produces more adverse effects and fewer desirable effects (Baylen and Rosenberg, 2006; Brunt et al., 2012), and therefore it is unlikely that MDMA is used intentionally at atypically high doses. As such, at the doses people typically take (i.e., 75–125 mg, see section II.A), MDMA primarily produces effects unlike classic psychostimulants or hallucinogens (Nichols, 1986). MDMA is usually described by its proponents as an "empathogen-entactogen"—a drug that increases empathy and closeness, both emotional and physical. It is these latter effects that are of significant therapeutic interest and are not shared with psychostimulants or hallucinogens. However, considerable evidence that MDMA is neurotoxic at high doses (see section III) has given considerable pause to this therapeutic interest.

Although MDMA is frequently described as the prototypical "designer drug," MDMA was synthesized and patented by Merck in 1912 as an unimportant precursor in a new chemical pathway (Freudenmann et al., 2006). The compound was shelved until Alexander Shulgin "rediscovered" MDMA in the 1970s. Shulgin produced the first reports on the psychoactive effects of MDMA and promoted its use as an adjunct to psychotherapy (Shulgin and Nichols, 1978). It was not until the early 1980s that MDMA began to be used recreationally, often at nightclubs, dance parties, and raves (Weir, 2000). The growing popularity of MDMA, in addition to new research findings on its adverse effects, led the U.S. Drug Enforcement Administration to classify MDMA as a Schedule I drug in 1985 for having "high abuse potential and no medical value" (Lawn, 1985, 1988; Shulgin, 1986). Despite its illegality, the recreational use of MDMA steadily increased through the 1990s with the rise of the underground rave scene (Schwartz and Miller, 1997) and plateaued in the early 2000s (Schulenberg et al., 2018). Any current increase in MDMA use may be related to the emergence of electronic dance music into mainstream culture (Fraser, 2012). Recently, scientific interest in the potential therapeutic value of MDMA has re-emerged as the result of findings that MDMA-assisted psychotherapy may be effective for treatment-resistant post-traumatic stress disorder (Bouso et al., 2008; Mithoefer et al., 2011, 2013, 2016; Oehen et al., 2013).

ABBREVIATIONS: 5-CSRT, 5-choice serial reaction time; CWM, Cincinnati water maze; DA, delayed alternation; DMS, delayed matching-to-sample; DNMS, delayed nonmatching-to-sample; FC, fear conditioning; 5-HT, serotonin; MDMA, ±3,4-methylenedioxymethamphetamine; MWM, Morris water maze; NOR, novel object recognition; NPR, novel place recognition; OST, odor span task; PA, passive avoidance; RAM, radial arm maze; SA, spontaneous alternation; SD, spatial discrimination; SERT, serotonin transporter; SR, social recognition.

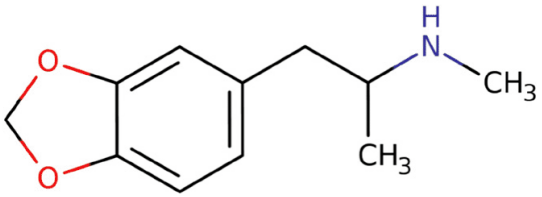

Fig. 1. Chemical structure of ±3,4-methylenedioxymethamphetamine (MDMA) (https://chem.nlm.nih.gov/chemidplus/name/mdma%20hcl, *Open Source*).

The United States "Monitoring the Future" national survey indicates that the lifetime prevalence of MDMA use among young adults (19–28 years of age) has remained relatively stable since 2000 (about 13%) and is significantly higher than in the 1990s (about 5%) (Schulenberg et al., 2018). Most MDMA users consume the drug relatively infrequently and only for a few years in their early twenties (Green et al., 2003; Kuypers et al., 2016). The 2016 U.S. National Survey on Drug Use and Health revealed that about one-third of lifetime MDMA users aged 18–25 years had used the drug in the past year, while less than 8% of lifetime MDMA users aged 26 years and older had used the drug within the past year (Center for Behavioral Health Statistics and Quality, 2016). Despite the low exposure, the long-term effects of MDMA use in young adulthood are a significant concern and one that is especially relevant to the current young adult population.

As both recreational and therapeutic interest in MDMA has increased over the past 40 years, so have concerns regarding the possible harmful effects of MDMA. There is evidence from both human and animal research that MDMA produces neurotoxicity and cognitive deficits. This evidence, however, is controversial and may have resulted from experiments with methodology that fail to generalize to typical MDMA users. The validity of MDMA toxicological findings rests particularly with respect to self-reported drug use and other confounding variables in human studies, the doses administered in animal studies, and the ability to generalize findings from animals to humans. Dose is a determinant of toxicity for virtually any substance, as even water and oxygen produce adverse effects and can lead to death at high doses. The question of critical importance then is: do the doses typical users actually take actually produce cognitive deficits and/or neurotoxicity? This question becomes even more acute when one considers that therapeutic dosing may be even lower than recreational dosing, meaning that MDMA could have therapeutic value at doses far below those for which any evidence of toxicity exists.

Others have extensively reviewed findings on the cognitive and neurotoxic effects of MDMA in humans as well as the neurotoxic effects of MDMA in animals (e.g., Baumann et al., 2007; Zakzanis et al., 2007; Mueller et al., 2016). In this paper, we summarize these review articles and discuss some potential methodological issues. Our aim is to provide the first ever full systematic review of findings on the cognitive effects of MDMA in animals. We review these studies with a critical focus on dose.

II. ±3,4-Methylenedioxymethamphetamine Dose

A. Human Use

MDMA is almost exclusively administered as a racemic mixture, although there is evidence that its two enantiomers have different pharmacological and behavioral effects (Fantegrossi, 2008; Pitts et al., 2018). MDMA is commonly sold as a tablet (i.e., "Ecstasy"; Fig. 2) or as crystalline powder (loose or in a capsule, i.e., "Molly"; Fig. 3) and is usually ingested orally; however, crushed tablets or crystalline powder can also be taken sublingually, buccally, or intranasally (Eisner, 1989). Because pure MDMA cannot be made into a pressed tablet by itself, Ecstasy tablets contain other substances, including excipients such as cellulose and often other active agents such as stimulants or other MDMA-like substances. "Molly" is often perceived by the purchasers to be pure MDMA, but is also frequently contaminated with other cheaper or more accessible substances (Palamar, 2017). Based on EcstasyData.org, an independent laboratory testing service for street MDMA, only 43.7% of the 4063 samples tested between 1996 and 2017 contained only MDMA. The remaining samples contained either MDMA with additional substance(s) (18%) or no MDMA (39%) (Fig. 4). Therefore, only 62% of street MDMA truly contained any MDMA, and 57% of street MDMA consisted partially or entirely of other substances (often a cocktail of substances). The most common substances mixed with or sold as MDMA included stimulants (55%; e.g., caffeine, methamphetamine, trifluoromethylphenylpiperazine, benzylpiperazine, pseudoephedrine), MDMA-like substances

Fig. 2. MDMA in the form of "Ecstasy" tablets (http://www.usdoj.gov/dea/programs/forensicsci/microgram/mg0103/mg0103.html, *Open Source*).

Fig. 3. MDMA crystalline powder in capsule form, commonly referred to as "Molly" (https://commons.wikimedia.org/w/index.php?curid=1884576, *Open Source*).

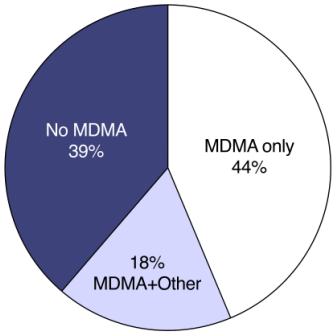

Fig. 4. Contents of 4063 samples of street MDMA tested by EcstasyData.org and other organizations between 1996 and 2017. Samples sold as MDMA contained either MDMA only, MDMA in combination with other substances, or no MDMA at all. Less than half of street MDMA samples contained MDMA only and more than half of street MDMA samples consisted partially or entirely of other substances (original figure; data redrawn with permission from https://www.ecstasydata.org/stats.php).

(20%; e.g., methylenedioxyamphetamine, methylenedioxyethylamphetamine, methylone), and/or dissociatives (11%; e.g., dextromethorphan, ketamine) (https://www.ecstasydata.org/stats.php).[1] It is important to note that the samples from EcstasyData.org are voluntarily submitted and are not a random sampling of available street MDMA. Nevertheless, given that the available data shows that more than half of street MDMA is adulterated and almost half of street MDMA does not contain any MDMA, MDMA users have most likely consumed these other psychoactive substance(s) in addition to and/or instead of MDMA.

MDMA users most commonly take doses of about 75–125 mg, or about 1 to 2 mg/kg, while doses higher than 200 mg are usually unintentional because they can produce unpleasant adverse effects, including hyperthermia and paranoia (https://erowid.org/chemicals/mdma/mdma_dose.shtml; Hayner and McKinney, 1986; Green et al., 2003; Gouzoulis-Mayfrank and Daumann, 2006; Morgan, 2000; Ricaurte et al., 2000). Consistent with this, Brunt et al. (2012) revealed that doses of 81–100 mg MDMA are associated with the highest probability of experiencing desirable subjective effects, while doses greater than 160 mg MDMA lead to more adverse than desirable effects. Analyses of street MDMA contents indicate that tablets usually contain doses in the range of those commonly used, yet there are some variations by batch and location. Older reports have suggested that MDMA tablets contain 70–120 mg on average (Parrott, 2004). Several more recent large-scale analyses in various countries indicated that MDMA tablets contain average doses of about 66–87 mg (close to 1 mg/kg) (Giraudon and Bello, 2007; Vogels et al., 2009; Brunt et al., 2012; Vidal Giné et al., 2016). Data from the 2016 European Drug Report indicated that tablets typically contain between 68 and 95 mg of MDMA (also close to 1 mg/kg) (EMCDDA, 2016).

MDMA users usually take one to two tablets per occasion and generally use MDMA once per week or less because of rapid tolerance to its desirable effects (Topp et al., 1999; Morgan, 2000; Winstock et al., 2001; Riley et al., 2001; Scholey et al., 2004; Gouzoulis-Mayfrank and Daumann, 2006; Parrott et al., 2006; ter Bogt et al., 2006). Only about 9%–17% of MDMA users take an average of three or four tablets per occasion, and about 3%–10% of MDMA users take an average of more than four tablets per occasion (Scholey et al., 2004; Parrott et al., 2006; ter Bogt et al., 2006). Because each tablet is expected to have a dose of about 1 mg/kg, a typical weekly dose of two tablets is about 2 mg/kg, but heavier users may be taking weekly doses of 3 mg/kg or more. In our review, we focus on understanding typical recreational MDMA users rather than atypical heavy users.

The therapeutic doses of MDMA used in current clinical trials are comparable to typical recreational doses yet are administered on only a few separate occasions. In the two completed phase 2 clinical trials testing MDMA-assisted psychotherapy for treatment-resistant post-traumatic stress disorder, patients were treated with a dose of 125 mg MDMA, plus a 62.5 mg supplemental dose in some cases, on two or three occasions (Mithoefer et al., 2011; Oehen et al., 2013). MDMA may potentially have therapeutic value at even lower doses, and we encourage investigators to explore those doses.

In summary, relatively low doses (<3 mg/kg) are used both therapeutically and recreationally. However, it is critical to differentiate between the therapeutic use of

[1]The values for this analysis were obtained from the Test Result Statistics: Summary Data on EcstasyData.org (Display as: Numbers; By date: Tested; Uncheck: EcstasyData Only). We included the laboratory testing results for all samples sold as MDMA between 1996 and 2017, which is listed as the total number of samples containing 1) MDMA Only, 2) MDMA + Something, or 3) No MDMA. Most of the samples (about three-fourths) were submitted from the United States. This analysis was conducted on 05/04/2017 and therefore includes all laboratory testing results up to that date.

pure MDMA in controlled medical settings and the recreational use of potentially impure MDMA in potentially high-risk settings. Our systematic review of preclinical MDMA research speaks to the use of pure MDMA in therapeutic settings or low-risk recreational settings.

B. Animal to Human Scaling

There has been significant controversy regarding whether the doses of MDMA administered to animals in preclinical studies accurately reflect those taken by human users. Given the average human weight of 70 kg, a typical MDMA dose of 75–125 mg is equivalent to about 1 to 2 mg/kg MDMA. Despite this, the majority of animal studies administer doses ranging from 10 to 20 mg/kg, which is equivalent to 700–1400 mg in a 70-kg human and is about 5–20 times larger than a typical MDMA dose.

Early MDMA researchers argued that the method of "interspecies scaling" (Mordenti and Chappell, 1989) should be used to translate MDMA doses across species (Ricaurte et al., 2000; McCann and Ricaurte, 2001). This method proposes that smaller animals require much larger doses than humans, using the equation $D_{human} = D_{animal} \times (W_{human}/W_{animal})^{0.7}$, where D is drug dose in milligrams, W is body weight in kilograms, and 0.7 is the "allometric constant" that accounts for differences in drug elimination. As a result, a dose of 98 mg in a 70 kg human (1.4 mg/kg) was equated to 7 mg/kg in rats and 5 mg/kg in monkeys. Most the studies reviewed here argued that doses of 10–20 mg/kg in rodents are suitable for modeling recreational use of MDMA, as they translate to a human dose of 140–280 mg under "allometric scaling." Allometric scaling results in animal doses that are exceedingly higher than those determined by a simple conversion of dose based on body weight, and the approach is not without controversy.

We have typically argued that one-to-one dosing should be used, unless further specific knowledge (for example, metabolic or actual exposure data) justifies some specific kind of alternative scaling (Shuman et al., 2009; Wood et al., 2014; Carmack et al., 2014). Furthermore, although doses vary somewhat in veterinary medicine, across a wide variety of indications, most drugs are given roughly on the same scale as human doses converted on a straight milligrams per kilogram basis. For example, fluoxetine dosing in dogs and cats is 1 to 2 mg/kg (https://www.reconcile.com/pdfs/prescribing-information.pdf), which is quite similar to human dosing (http://pi.lilly.com/us/prozac.pdf).

More recently, several researchers have argued that allometric scaling is not a valid approach for MDMA research. Specifically, this method does not take principles of pharmacokinetics/pharmacodynamics into account. Green et al. (2009, 2012a) explain that factors such as bioavailability, active metabolites, plasma protein binding differences, and patterns of systematic exposure are critical influences on drug effects, and these factors can vary markedly between species and methods. Humans almost always ingest MDMA orally, whereas animals are administered MDMA intraperitoneally or subcutaneously, which may lead to significant differences in bioavailability and/or metabolism (Green et al., 2009, 2012a). In humans, there is a nonlinear relationship between dose and plasma concentration such that a twofold increase in dose (from 1 to 2 mg/kg) results in a fourfold increase in plasma concentration, while the relationship between dose and plasma concentration in rats is approximately linear. As a result, the dose-plasma concentration curves of humans and rats are comparable at doses below 2.5 mg/kg but differ drastically at higher doses (Green et al., 2009, 2012a). Specifically, C_{max} (peak plasma concentration) of 1.6 mg/kg MDMA (orally) in humans and 2 mg/kg MDMA (intraperitoneally and subcutaneously) in rats is similar [humans (oral): 292 $\pm$ 76 ng/ml, rats (intraperitoneal): 210 $\pm$ 108 ng/ml, rats (subcutaneous): 196 $\pm$ 50 ng/ml). Time of drug peak, however, is much shorter in rats [0.14 $\pm$ 0.08 hours (intraperitoneal), 0.75 $\pm$ 0.29 hours (subcutaneous)] than in humans [2.4 $\pm$ 0.6 hours (oral)] (Kolbrich et al., 2008; Baumann et al., 2009). Thus, testing rats 10–45 minutes after parenteral doses of about 2 mg/kg is roughly equivalent to peak exposure in humans 2.4 hours after taking about one and a half oral tablets. The differences in time course are because MDMA is absorbed and metabolized much faster in rats than in humans and the proportion of metabolites formed differs strikingly between species (Green et al., 2009, 2012a). This is a major concern because the active metabolites of MDMA, rather than MDMA itself, appear to be responsible for long-term neurotoxicity. For instance, methylenedioxyamphetamine, an active and neurotoxic metabolite of MDMA, accounts for 23%–34% of MDMA metabolism in rats but only about 10% in humans (Green et al., 2012a). Nonetheless, MDMA is extensively metabolized in both animals and humans, a condition under which the allometric relationship does not hold true (Lin, 1998; Baumann et al., 2007).

For the reasons above, as well as others extensively discussed by Baumann et al. (2007) and Green et al. (2009, 2012a), allometric scaling in MDMA research is arguably flawed, and findings under this method should be interpreted with caution for using excessive dosing. Baumann et al. (2007) proposes the alternative method of "effect scaling" for extrapolating doses between species. Under this method, animal doses are determined based on the lowest dose of drug that produces a specific pharmacological response in animals and humans. Doses of about 1 to 2 mg/kg MDMA produce equivalent pharmacology effects in humans (orally) and rats (intraperitoneally, subcutaneously, or intravenously), including the in vivo release of serotonin and dopamine [humans (oral): 1.5 mg/kg, rats (intraperitoneal): 2.5 mg/kg, rats (subcutaneous): 1 mg/kg], secretion of prolactin and

glucocorticoids [humans (oral): 1.5 or 1.67 mg/kg, rats (intraperitoneal): 1–3 mg/kg], drug discrimination [humans (oral): 1.5 mg/kg, rats (intraperitoneal): 1.5 mg/kg], and drug reinforcement [humans (oral): 1 to 2 mg/kg, rats (intravenous): 1 mg/kg) (Baumann et al., 2007). Unlike Green et al.'s findings, Baumann et al. (2009) found that the pharmacokinetics of MDMA are not only nonlinear in monkeys and humans but also in rats. Regardless of this discrepancy, it is agreed that the pharmacokinetics of doses of about 1 to 2 mg/kg MDMA are similar across species. Because the pharmacologically relevant doses of MDMA are similar across species, there is not adequate scientific justification for using interspecies scaling to "adjust" MDMA doses (Baumann et al., 2007, 2009). This is especially true when considering toxicology, because most of the "adjustments" have been radical increases in dose, which tend to suggest a drug is more toxic than it actually is. Indeed, one might think this could impose a bias in "finding" toxic effects in drugs of abuse, in general.

Given that doses of about 1 to 2 mg/kg MDMA produce similar pharmacokinetic, pharmacological, and psychoactive effects across species and are analogous to the doses taken by human MDMA users, these low doses should be used in preclinical MDMA research in the absence of explicitly justified interspecies scaling. While low doses are unlikely to produce neurotoxicity, they may still have adverse cognitive effects (Green et al., 2012a,b). A central aim of this review is to determine if MDMA influences cognitive functioning at these doses.

III. Neurotoxicity in Animals and Humans

The long-term neurotoxic effects of MDMA have been studied extensively in animals and humans. Ricaurte et al. (2000), Green et al. (2003), and Lyles and Cadet (2003) were among the first to review the many findings on MDMA-induced neurotoxicity in animals. Research in rats and non-human primates demonstrated that MDMA produces significant reductions in biochemical markers of serotonergic activity that last for months to years. The most prominent reductions include decreased levels of 5-HT and 5-hydroxyindoleacetic acid (the major metabolite of 5-HT), decreased numbers of SERT, and decreased activity of tryptophan hydroxylase (the rate-limiting enzyme in 5-HT synthesis). Additional studies found through histologic methods (e.g., silver staining) that MDMA produces degeneration of 5-HT axons and terminals. These findings suggest that the long-lasting and selective serotonergic biomarker reductions produced by MDMA may reflect neurodegeneration. However, as with amphetamine neurotoxicity, there is no evidence of actual cell death.

These early studies used MDMA doses that are exceedingly large and not representative of those taken by typical users (as was done in early amphetamine neurotoxicity studies). Most rat strains (e.g., Lister Hooded, Sprague-Dawley, and Wistar) typically require several MDMA doses of 20 mg/kg or more to exhibit serotonergic deficits (Colado et al., 1993; Aguirre et al., 1998; Shankaran and Gudelsky, 1999; Green et al., 2003). Non-human primates show higher sensitivity to MDMA-induced serotonergic deficits, as doses of about 5 mg/kg will produce deficits that are more severe than those observed in rats (Ricaurte et al., 1988; Ricaurte and McCann, 1992; Green et al., 2003). Mice are far less sensitive than rats to MDMA-induced serotonergic deficits, as doses of up to 50 mg/kg produce only slight deficits (Stone et al., 1987; Logan et al., 1988; Green et al., 2003). Although there are differences between species/strains, MDMA-induced deficits in markers of serotonergic neurons require fairly high and often sustained dosing (Green et al., 2003).

In a more recent review, Baumann et al. (2007) analyzed findings on MDMA-induced neurotoxicity in rats with respect to dose. Several studies have demonstrated that behaviorally relevant doses of MDMA (i.e., 1 to 2 mg/kg; see section II.B) do not produce reductions in biochemical markers of 5-HT neurons. The doses of MDMA that do produce serotonergic deficits (i.e., 10–20 mg/kg) are five or more times greater than behaviorally relevant doses of MDMA. Even so, these high doses are not reliably associated with 5-HT neuron degeneration. Rather, even more extreme doses of MDMA were used in the histology studies that found neurotoxic damage. For instance, massive cumulative doses of 100–600 mg/kg (i.e., up to 42,000 mg or 600 MDMA tablets in humans) were given to rats that exhibited increased silver-positive staining in degenerating 5-HT neurons. Thus, MDMA-induced reductions in biochemical markers of 5-HT neurons do not necessarily reflect neurotoxic damage (see Baumann et al. for additional supporting evidence). There is insufficient evidence that the MDMA doses typically used by humans result in serotonergic neurotoxicity in animal models.

Nevertheless, evidence of possible MDMA-induced neurotoxicity in animals has raised concern for neurotoxicity in human MDMA users. Reneman et al. (2006) and Cowan (2007) provided reviews on some of the latest neuroimaging studies in human MDMA users. While there has been much debate regarding the methods used in early human studies on MDMA-induced neurotoxicity, modern neuroimaging techniques such as positron and single photon emission tomography provide updated findings on the effects of MDMA in the human brain. The most consistent finding is that MDMA users exhibit a reduction in SERT density that appears to be associated with the degree of MDMA exposure, while findings on other serotonergic deficits are largely inconsistent. It remains unclear whether the SERT reductions in MDMA users are a direct reflection of serotonergic neurodegeneration.

A concern regarding the above findings is that most studies investigated samples of heavy MDMA users, with a mean lifetime consumption ranging from

173 to 880 MDMA tablets. Only about 13%–18% of MDMA users report having taken MDMA on more than 100 occasions (Scholey et al., 2004; Parrott et al., 2006), and while this research may be indicative of neurotoxicity in these heavy users, it is not indicative of the effects of MDMA in the typical user and is highly unlikely to reflect patients treated therapeutically only a few times with MDMA. Mueller et al. (2016) addressed this issue with a systematic review of neuroimaging studies in moderate MDMA users (those with lifetime use of <50 occasions or <100 tablets). The 19 studies that met inclusion criteria provided little, if any, evidence for brain alterations in moderate MDMA users.

The animal and human data together suggest that heavy use of MDMA may produce neurotoxicity, but typical (i.e., low to moderate) MDMA use may have no effect on brain structure and function. Human MDMA research, however, may have issues with experimental design, confounding variables, and methodological techniques (explained further in section IV and by Gouzoulis-Mayfrank and Daumann, 2006). In this review, we explore the functional consequences of MDMA use, specifically the effects on cognition, as potential indicators of MDMA-induced neurotoxicity.

IV. Cognitive Effects in Humans

Numerous review articles have evaluated findings on cognitive functioning in MDMA users. Recent meta-analyses and systematic reviews suggest that MDMA users, when compared with drug-naïve or polydrug controls, are impaired in several cognitive domains including decision-making (Betzler et al., 2017), attention (Verbaten, 2003; Zakzanis et al., 2007), executive functioning (Zakzanis et al., 2007; Murphy et al., 2009; Roberts et al., 2016), verbal and visuospatial working memory, short-term memory, and long-term memory (Verbaten, 2003; Laws and Kokkalis, 2007; Zakzanis et al., 2007; Murphy et al., 2009, 2012; Nulsen et al., 2010). Others, however, have found that MDMA users and controls show no differences in executive switching (Murphy et al., 2009), executive inhibition (Roberts et al., 2016), visual short- and long- term memory (Laws and Kokkalis, 2007), and verbal long-term memory (Kuypers et al., 2016). Like the neurotoxicity studies, many of these reviews include data from heavy MDMA users only (Verbaten, 2003; Laws and Kokkalis, 2007; Nulsen et al., 2010; Roberts et al., 2016). Some deficits have been attributed to polydrug/cannabis use rather than MDMA use specifically, such as those in decision-making, visual short-term memory, and verbal long-term memory (Verbaten, 2003; Nulsen et al., 2010; Betzler et al., 2017), although there is some evidence of the contrary regarding verbal memory (Laws and Kokkalis, 2007). Overall, the most consistent findings are that heavy MDMA users exhibit long-term deficits in attention, executive updating, verbal and visuospatial working memory, and verbal short-term memory; findings regarding other cognitive domains are fairly inconsistent.

Research on the cognitive effects of MDMA in humans face a multitude of potential methodological issues. Dose-related, double-blind, placebo-controlled paradigms are the strongest in human psychopharmacology research, but there is a lack of such prospective studies in MDMA research due to the ethical and legal barriers of administering MDMA to human volunteers (Verbaten, 2003). As a result, retrospective cross-sectional designs dominate in this field, in which a group of self-reported MDMA users are compared with a control group. In contrast to prospective designs, retrospective designs decrease the ability to control potential confounds. A potential confounding variable in the studies reviewed above is that MDMA users are typically polydrug users, either knowingly or due to the impurity of street MDMA. While some studies controlled for self-reported polydrug use, the contents of impure street MDMA is typically unbeknownst to all and therefore cannot be controlled for. Self-reported drug use also introduces uncertainty in drug use patterns, including doses, number of exposures, and duration of abstinence. Another potential issue with retrospective designs is that the observed effects could be due to pre-existing differences, such as intelligence, cognitive, psychologic, neurochemical, genetic, or personality differences in the selected control group. It is conceivable that individuals with cognitive deficits may be more likely to use MDMA, and therefore the cognitive deficits observed in MDMA users could have been a cause of MDMA use rather than a consequence, although the direction of causality has been sparsely explored and is still a matter of debate (Curran, 2000; Roberts et al., 2016; Betzler et al., 2017).

To summarize, there has been consistent evidence of some cognitive deficits in heavy MDMA users (specifically in attention, executive updating, working memory, and verbal short-term memory), but we cannot be certain that these deficits are exclusively due to MDMA use rather than the use of other drugs, pre-existing conditions, and/or other confounding variables (Curran, 2000; also discussed further in the reviews/meta-analyses cited above). It is likely that MDMA poses considerable risk at high doses, as does high-dose amphetamine. However, as with amphetamine, low-dose MDMA may have great clinical potential and should not be barred based on unfounded concerns about behavioral or neural toxicity.

V. Cognitive Effects in Animals—Systematic Review

Because of the methodological issues in human MDMA research, animal models may be ideal for studying the cognitive effects of MDMA, specifically with respect to the therapeutic use of pure MDMA. Here we provide the first systematic review of findings on the cognitive effects

of MDMA in animal models. A total of 90 experiments (from 68 articles) provide such findings using a variety of tasks. We divided research findings by task into five major cognitive domains: 1) attention, 2) working memory, 3) spatial learning and memory, 4) nonspatial learning and memory, and 5) fear-motivated learning and memory. Findings on both the on-drug (acute) and post-drug (long-lasting) effects of MDMA are included. In some cases, on-drug impairments are associated with task performance impairments (e.g., impaired movement or altered state while intoxicated) rather than actual cognitive impairment. All findings are reviewed with respect to methodology, with a specific emphasis on the doses of MDMA administered. Again, we stress that most of these studies used doses that are exceedingly higher than low, behaviorally relevant doses of 1 to 2 mg/kg MDMA. The ability to generalize high-dose (≥3 mg/kg MDMA) findings to typical MDMA use, and specifically therapeutic use, is limited.

A. Attention

1. 5-Choice Serial Reaction Time. The 5-choice serial reaction time (5-CSRT) task (Robbins, 2002) is commonly used to assess attention and impulsivity in non-human primates. As to the studies reviewed here, the task is conducted in an operant chamber that contains a monitor and a single response lever. On each trial, five circles connected by lines are presented on the monitor, and the trial begins when the animal presses and holds down the response lever. After a variable delay period of 0.75–2.5 seconds, a yellow circle is quickly presented on one of the five circles for 20, 100, or 1000 milliseconds. The animal must touch the circle that contained the yellow circle within 2 seconds for reinforcer delivery. The release latency (time to release the lever) and movement time (time to move from lever to target) are used to measure attentional performance, with longer release latencies/movement times representing poorer attention.

Taffe et al. (2001, 2002) investigated the effects of MDMA on the 5-CSRT task, and these studies are listed in Table 1. Taffe et al. (2001) trained adult male rhesus monkeys on the task for 4 weeks prior to drug treatment. Monkeys were then given two daily injections of 10 mg/kg i.m. MDMA at a 12-hour interval for 4 consecutive days. Testing continued during the treatment week (3–5 hours after the first injection of each day, so testing occurred after the peak drug effect) and also for the following 21 weeks. During all three testing periods (pretreatment weeks, treatment week, and posttreatment weeks), MDMA-treated monkeys and saline controls did not significantly differ in release latency or movement time. However, the release latency of MDMA-treated monkeys was significantly longer during the treatment week than during the pretreatment weeks. Taffe et al. (2002) tested the same group of rhesus monkeys 13 months later, and again MDMA-treated monkeys and saline controls did not significantly differ in release latency or movement time. Together, these findings indicate that treatment with repeated doses of 10 mg/kg MDMA may produce slight attentional deficits during the treatment period but have no residual effects on attention for more than 1 year later.

B. Working Memory

1. Delayed (Non)matching-to-Sample. The delayed matching-to-sample (DMS) and delayed nonmatching-to-sample (DNMS) tasks (see Dudchenko, 2004) are widely used to study working memory in many species, including rodents, birds, and non-human primates. These tasks assess recognition memory for a visual stimulus and can be conducted using stimuli such as retractable levers, color illuminated keys, or visual stimuli displayed on a press-plate or touchscreen. Each trial has three main phases: sample presentation, delay, and choice. During the sample presentation, a single visual stimulus is presented to the animal (i.e., right or left lever, red or green key, a geometric shape on the press-plate or touchscreen). After the animal makes an observing response (i.e., a press or nose-poke) to the sample stimulus, the stimulus is removed for a delay period of a specified duration. The delay period is followed by the choice phase, when two or three visual stimuli are presented to the animal, only one of which is identical to the sample stimulus. The animal must respond (i.e., a press or nose-poke) to the sample stimulus in the DMS task or the novel stimulus in the DNMS task for accuracy, food reinforcer delivery, and initiation of the next trial.

Sessions are typically conducted daily, and a range of delay periods are tested, with each animal performing multiple trials at each delay duration. Accuracy is

TABLE 1
Studies examining the effects of MDMA on attention

Article	Task[a]	Subjects[b]	Doses/Frequency[c]	Timeline[d]	Effects[e]
Taffe et al., 2001	5-CSRT	Monkeys (R), Adult, Male	10 mg/kg (i.m.) × 2/day, 4 days	Training: Predrug Testing: On-Drug, Postdrug	↓ (On-Drug), ⌀ (Postdrug)
Taffe et al., 2002	5-CSRT	Monkeys (R), Adult, Male	10 mg/kg (i.m.) × 2/day, 4 days	Training: Predrug Testing: Postdrug	⌀

[a]Studies used the 5-choice serial reaction time (5-CSRT) task.
[b]Species (strain), age, and sex of subjects. Strains include rhesus (R) monkeys.
[c]Dose, route, and frequency of MDMA administration. Treatment days/weeks are consecutive unless noted as "spaced."
[d]When training and testing occurred in relation to drug treatment. Pre- and post-drug training/testing were always conducted off-drug.
[e]Effects of drug treatment on attention: ⌀ No Effect, ↓ Impairment, ↑ Enhancement.

determined at each delay by average percent correct (i.e., percent of trials that a correct response was made). Under normal working memory function, accuracy will decrease as the duration of the delay period increases. A working memory deficit is usually indicated by normal accuracy under no delay or ultra-short delays but a significant decrease in accuracy relative to normal at longer delays. A significant decrease in accuracy relative to normal across all delays [i.e., no/ultra-short delay(s) and long delays] does not represent impaired working memory, but rather a performance impairment.

Table 2 includes the seven studies that tested the effects of MDMA on the DMS (5 studies) and DNMS (2 studies) tasks. All of these studies trained animals on the task to a criterion level before beginning on-drug testing, and some of the studies continued testing after the on-drug trials.

Harper et al. (2005) and Harper (2011) trained and tested adult male Sprague-Dawley rats on a DMS task using retractable levers and delays of 0.1, 3, 9, and 18 seconds. Harper et al. (2005) gave rats 0, 0.3, 1, 2, or 3 mg/kg i.p. MDMA in a within-subjects design 10 minutes before on-drug test sessions. Relative to saline, doses of 0.3 and 1 mg/kg MDMA had no effect on accuracy, and doses of 2 and 3 mg/kg MDMA significantly decreased accuracy across all delays. Harper (2011) gave rats 0 or 3 mg/kg i.p. MDMA in a within-subjects design 5 min before on-drug test sessions. A dose of 3 mg/kg MDMA significantly decreased accuracy across all delays relative to saline. Together, these results indicate that while doses of 2 and 3 mg/kg MDMA impair performance on the task, doses of 0.3, 1, 2, and 3 mg/kg MDMA have no effect on working memory.

Frederick et al. (1995a) trained and tested adult male rhesus monkeys on a DMS task using a press-plate apparatus and delays of 2, 4, 8, 16, 32, and 48 seconds. Monkeys were given 0, 0.1, 0.3, or 1 mg/kg i.m. MDMA in a within-subjects design 30 minutes before on-drug test sessions. MDMA had no effect on overall accuracy relative to saline at any of the doses tested. In continuation of this study, Frederick et al. (1995b) tested the same rhesus monkeys on the same task under different MDMA treatments. Monkeys were given two daily injections of i.m. MDMA at an 8-hour interval for 14 consecutive days. The dose of MDMA was increased every 2 weeks, such that doses of 0, 0.1, 0.3, 1, 3, 5.6, 10, and 20 mg/kg MDMA were each given for 14 consecutive days in sequential order. On-drug testing took place 30 minutes after the first injection of each day, and MDMA had no effect on overall accuracy relative to saline at any dose. Five months later, monkeys were given 0, 0.3, 1, 1.75, 3, or 5.6 mg/kg i.m. MDMA in a within-subjects design 30 min before daily on-drug test sessions. Doses of 0.3, 1, 1.75, and 3 mg/kg MDMA had no effect on overall accuracy relative to saline, and the effect of 5.6 mg/kg MDMA could not be determined due to performance failure. In all, these studies indicate that doses of 0.1, 0.3, 1, 1.75, 3, 5.6, 10, and 20 mg/kg MDMA may have no effect on working memory.

LeSage et al. (1993) trained and tested adult White Carneau pigeons on a DMS task using color illuminated keys and delays of 0, 3, and 6 seconds. Pigeons were given 0, 0.32, 1, 1.7, 3.2, 4.2, or 5.6 mg/kg i.m. MDMA in a within-subjects design 10 minutes before on-drug test sessions. Relative to saline, doses of 0.32, 1, and 1.7 mg/kg MDMA had no effect on accuracy across all delays, doses of 3.2 and 4.2 mg/kg MDMA significantly decreased accuracy across all delays, and a dose of 5.6 mg/kg MDMA completely suppressed responding. Ten days later, pigeons were given i.m. MDMA at doses of 0 mg/kg for 2 days (baseline), followed by 3.2 mg/kg for 20 days, 4.2 mg/kg for 1 day (challenge dose), 3.2 mg/kg for 5 days, and 5.6 mg/kg for 1 day (challenge dose) (all consecutive days). The final dose of 3.2 mg/kg MDMA and the challenge doses of 4.2 and 5.6 mg/kg MDMA had no effect on accuracy relative to saline (baseline) across all delays. These findings suggest that doses of 3.2, 4.2, and 5.6 mg/kg MDMA initially impair performance on the DMS task but these impairments diminish after treatment with repeated doses of MDMA. Nevertheless, doses of 0.32, 1, 1.7, 3.2, 4.2, and 5.6 mg/kg MDMA appear to have no effect on working memory.

Taffe et al. (2001) trained adult male rhesus monkeys on a DNMS task using touchscreen stimuli. Monkeys were first tested under delays of 0, 16, 32, and 64 seconds for 4 weeks. The following week, monkeys were given two daily injections of 10 mg/kg i.m. MDMA at a 12-hour interval for 4 consecutive days. Testing continued during the MDMA treatment week (3–5 hours after the first injection of each day, so testing occurred after the peak drug effect) and for the 21 weeks following treatment. During all three testing periods (pretreatment weeks, treatment week, and posttreatment weeks), MDMA-treated monkeys and saline controls did not significantly differ in accuracy across all four delays. The accuracy of MDMA-treated monkeys was significantly reduced during the treatment week compared with the pretreatment weeks at delays of 0 and 64 seconds, but this effect can be attributed to performance deficits rather than working memory deficits as the reductions were seen at both no delay and a long delay. In all, these findings indicate that treatment with repeated doses of 10 mg/kg MDMA may have no effect on working memory during treatment and for at least 5 months later.

Marston et al. (1999) trained and tested adult male Lister Hooded rats on a DNMS task using retractable levers and delays of 0.3, 1, 3, 5.6, 10, 17.6, and 30 seconds. Rats were given two daily injections of i.p. MDMA at a 10-hour interval for 3 consecutive days at doses of 10 mg/kg MDMA on day 1, 15 mg/kg MDMA on day 2, and 20 mg/kg MDMA on day 3. Testing continued during MDMA treatment (45–130 min after the first injection of each day) and for the 3–16 days following treatment. MDMA suppressed responding during the

TABLE 2
Studies examining the effects of MDMA on working memory

Article	Task[a]	Subjects[b]	Doses/Frequency[c]	Timeline[d]	Effects[e]
Harper et al., 2005	DMS	Rats (SD), Adult, Male	0.3, 1, 2, or 3 mg/kg (i.p.)*	Training: Predrug / Testing: On-Drug	⌀
Harper, 2011	DMS	Rats (SD), Adult, Male	3 mg/kg (i.p.)*	Training: Predrug / Testing: On-Drug	⌀
Frederick et al., 1995a	DMS	Monkeys (R), Adult, Male	0.1, 0.3, or 1 mg/kg (i.m.)*	Training: Predrug / Testing: On-Drug	⌀
Frederick et al., 1995b	DMS	Monkeys (R), Adult, Male	0.1 → 0.3 → 1 → 3 → 5.6 → 10 → 20 (14 days each) mg/kg (i.m.) × 2/day, 14 wk AND 0.3, 1, 1.75 or 3 mg/kg (i.m.)*	Training: Predrug / Testing: On-Drug	⌀
LeSage et al., 1993	DMS	Pigeons (WC), Adult	0.32, 1, 1.7, 3.2, or 4.2 mg/kg (i.m.)* AND 3.2 (20 days) → 4.2 (1 day) → 3.2 (5 days) → 5.6 (1 day) mg/kg (i.m.) × 1/day, 27 days	Training: Predrug / Testing: On-Drug	⌀
Taffe et al., 2001	DNMS	Monkeys (R), Adult, Male	10 mg/kg (i.m.) × 2/day, 4 days	Training: Predrug / Testing: On-Drug, Postdrug	⌀
Marston et al., 1999	DNMS	Rats (LH), Adult, Male	10 → 15 → 20 (1 day each) mg/kg (i.p.) × 2/day, 3 days	Training: Predrug / Testing: Postdrug	↓
Hawkey et al., 2014	OST	Rats (SD), Adult, Male	0.3, 1, 1.8, or 3 mg/kg (i.p.)* OR 10 mg/kg (i.p.) × 2/day, 4 days	Training: Predrug / Testing: On-Drug (Single), Postdrug (Repeated)	⌀
Costa et al., 2014	SA	Mice (B6), Adol.→Adult, Male	10 mg/kg (i.p.) × 2/day, 2 days/wk (spaced), 9 wk	Testing: Off-Drug[1], Postdrug	⌀
Edut et al., 2011	SA	Mice (ICR), Adult, Male	10 mg/kg (i.p.) × 1	Testing: Postdrug	⌀
Cassel et al., 2005	SA	Rats (LE), Adult, Male	10 mg/kg (i.p.) × 1/day, 4 days	Testing: Postdrug	⌀
Kolyaduke and Hughes, 2013	SA	Rats (PVG/c), Adol.→ Adult, Male/Female	10 mg/kg (i.p.) × 1/day, 10 days	Testing: Postdrug	⌀
Ricaurte et al., 1993	DA	Rats (LE), Adult, Male	20 mg/kg (s.c.) × 2/day, 4 days/wk, 2 wk (spaced)	Training/Testing: Postdrug	⌀
Young et al., 2005	DA	Rats (W), Adult, Male	1.25, 2.25, or 5 mg/kg (i.p.)*	Training: Predrug / Testing: On-Drug	⌀ (1.25 and 2.25 mg/kg) ↓ (5 mg/kg)
Viñals et al., 2012	DA	Mice (B6), Adult, Male	3 or 30 mg/kg (i.p.) × 2/day, 4 days	Training: Predrug / Testing: Postdrug	⌀
Hernandez-Rabaza et al., 2010	RAM	Rats (LE), Adol., Male	10 mg/kg (i.p.) × 2	Training: Postdrug	⌀
Ros-Simó et al., 2013	RAM	Mice (CD1), Adol., Male	20 mg/kg (i.p.) × 2	Training: Predrug, Off-Drug[2], Postdrug	⌀
Kay et al., 2010	RAM	Rats (SD), Adult, Male	0.75, 3, or 4 mg/kg (i.p.)*	Training: Predrug, On-Drug	⌀ (0.75 and 3 mg/kg) ↓ (4 mg/kg)
Kay et al., 2011	RAM	Rats (SD), Adult, Male	10 mg/kg (i.p.) × 4 AND 4 mg/kg (i.p.)*	Training: Postdrug (10 mg/kg) and On-Drug (4 mg/kg)	⌀
Harper et al., 2013	RAM	Rats (SD), Adult, Male	10 mg/kg (i.p.) × 4 AND/OR 4 mg/kg (i.p.) × 1/wk, 6 wk	Training: Postdrug (10 mg/kg) and On-Drug (4 mg/kg)	⌀
Braida et al., 2002	RAM	Rats (W), Adult, Male	1, 2, or 3 mg/kg (i.p.) × 1/day, 3 days	Training: Predrug, On-Drug	⌀ (1 and 2 mg/kg) ↓ (3 mg/kg)
Robinson et al., 1993	MWM	Rats (SD), Adult, Male	10 mg/kg (i.p.) × 2/day, 4 days	Training: Postdrug	⌀
Galizio et al., 2014	MWM	Rats (SD), Adult, Male	0.3, 1, 1.7, 3, or 5.6 mg/kg (i.p.)*	Training: Predrug / Testing: On-Drug	⌀

[a]Studies used the delayed matching-to-sample (DMS), delayed nonmatching-to-sample (DNMS), odor span task (OST), spontaneous alternation (SA), delayed alternation (DA), radial arm maze (RAM), and Morris water maze (MWM) tasks.

[b]Species (strain), age, and sex of subjects. Strains include rhesus (R) monkeys; White Carneau (WC) pigeons; Lister Hooded (LH), Long Evans (LE), PVG/c hooded, Sprague-Dawley (SD), and Wistar (W) rats; and C57BL/6 (B6), CD1, and ICR mice.

[c]Dose, route, and frequency of MDMA administration. Treatment days/weeks are consecutive unless noted as "spaced."

[d]When training and testing occurred in relation to drug treatment. Pre- and post-drug training/testing were always conducted off-drug.

[e]Effects of drug treatment on nonspatial working memory [DMS/DNMS, OST, DA (only Viñals et al., 2012)] and spatial working memory [SA/DA (except Viñals et al., 2012), RAM, MWM]: ⌀ No Effect, ↓ Impairment, ↑ Enhancement.

[1]Off-drug training/testing took place on day(s) without drug administration.

[2]Off-drug training/testing took place immediately before drug administration on (last) day of treatment.

*Within-subjects design (all animals were tested multiple times at each dose including saline).

treatment days, so the results on accuracy were not reported. During the posttreatment days, the accuracy of MDMA-treated rats was significantly reduced relative to saline controls at the longer delays of 17.6 and 30 seconds (but not the shorter delays) during the last few days of testing. These results suggest that treatment with increasing doses of 10–20 mg/kg MDMA impairs working memory for up to about 2 weeks later.

a. Odor span task. Hawkey et al. (2014) conducted a variation of the DNMS task, the odor span task (OST), and this study is included in Table 2. In the OST, the stimuli are plastic cups that contain sand and a food reinforcer with different scented lids. On the first trial, a single olfactory stimulus is presented. On the second trial, the familiar olfactory stimulus is presented with a novel olfactory stimulus. On each subsequent trial, an additional olfactory stimulus is added so that the number of familiar olfactory stimuli increases with each trial, but there is always only one novel olfactory stimulus. Beyond the fifth trial, the number of stimuli does not increase, but the familiar and novel scents are still changed between trials. Simple discrimination trials are also interspersed between OST trials, which test for simple task performance but not working memory functioning. On each simple discrimination trial, the same five olfactory stimuli are presented and the single stimulus that is reinforced remains constant for all trials (while responses to the other four stimuli are never reinforced). In this study, each test session consisted of 24 OST trials and 6 simple discrimination trials. OST percent correct, simple discrimination percent correct, span (number of trials completed before an error), and longest run (longest series of correct responses) were scored for each session.

Hawkey et al. (2015) trained adult male Sprague-Dawley rats on the OST and simple discrimination tasks to a criterion level prior to testing. Rats were given 0, 0.3, 1, 1.8, or 3 mg/kg i.p. MDMA in a within-subjects design 15 minutes before on-drug test sessions. Doses of 0.3, 1, and 1.8 mg/kg MDMA had no effect on OST percent correct, simple discrimination percent correct, span, or longest run relative to saline. A dose of 3 mg/kg MDMA significantly decreased span and longest run relative to saline but had no effect on OST percent correct or simple discrimination percent correct. The reductions in span and longest run were due to a significant increase in response omissions on both simple discrimination and OST trials, rather than being due to working memory deficits. Another group of rats was given two daily injections of 10 mg/kg i.p. MDMA for 4 consecutive days and then tested off-drug 3 days later for a total of 10 sessions. MDMA-treated rats and saline controls did not significantly differ in OST percent correct, simple discrimination percent correct, span, or longest run. In all, these findings demonstrate that doses of 0.3, 1, 1.8, and 3 mg/kg MDMA have no on-drug effect on working memory (although 3 mg/kg MDMA did produce performance deficits), and pretreatment with repeated doses of 10 mg/kg MDMA also have no subsequent effect on working memory.

2. Spontaneous and Delayed Alternation. The spontaneous alternation (SA) and delayed alternation (DA) tasks (see Dudchenko, 2004; Hughes, 2004) are used to assess spatial working memory in rodents, typically on a T- or Y-maze. The main difference between these two tasks is that SA responses are driven by the natural tendency for rodents to explore novel environments, and DA responses are driven by food reinforcement. In both tasks, the goal of the animal is to investigate a new arm of the T- or Y-maze rather than one that they recently visited.

There are two main versions of the SA task, continuous SA and two-trial SA. The continuous SA task is completed in one trial, during which the animal is allowed to freely explore all three arms of the maze for the entire duration (usually several minutes). Number of alternations, defined as consecutive entries into all three arms without repeated entries, is scored for each animal and converted to percent alternation (ratio of actual to possible alternations given number of arm entries). The two-trial SA task consists of a forced trial and a test trial. On the forced trial, the animal is placed at the end of the "start" arm and is only allowed to enter one other arm (the "familiar" arm), as the third arm (the "novel" arm) is blocked by a door. Normally, a delay period is placed after the forced trial and before the test trial. On the test trial, the animal is returned to the end of the "start" arm and allowed to enter either the "familiar" arm or the "novel" arm (all three arms are open). A correct response or alternation is defined as an entry into the "novel" arm on the test trial.

The DA task is quite similar to the two-trial SA task. Each session usually consists of one forced trial followed by several choice trials. The forced trial is conducted in the same manner as the SA task, except a food reinforcer is placed at the end of the "familiar" arm. On the first choice trial, the food reinforcer is placed at the end of the "novel" arm, and for all subsequent choice trials, the food reinforcer is placed at the end of the arm that was not entered on the previous trial. Only one entry is permitted per trial, and a correct response or alternation is defined as a reinforced response, an entry into the arm that was not entered on the previous trial.

Table 2 includes the seven studies that explored the effects of MDMA on the SA (four studies) and DA (three studies) tasks. Of these studies, only Costa et al. (2014) conducted the continuous SA task. In this study, male C57BL/6 mice were given two daily injections of 10 mg/kg i.p. MDMA at a 4- to 6-hour interval on the 2nd and 5th days of each week for 9 weeks, which began in adolescence and extended into adulthood. Mice were tested on a Y-maze, off-drug, on the 7th day of drug treatment weeks 1, 4, and 9 and postdrug treatment weeks 2 and 3. The percent alternations of MDMA-treated mice and saline controls did not significantly differ at any time

point, suggesting that treatment with repeated doses of 10 mg/kg MDMA has no effect on spatial working memory for up to 3 weeks after treatment.

Edut et al. (2011) and Cassel et al. (2005) tested rodents on the two-trial SA task at least 1 week after MDMA treatment. Edut et al. gave adult male ICR mice a single injection of 10 mg/kg i.p. MDMA and tested them 7 and 30 days later on a Y-maze. Mice were permitted to enter multiple arms during a 5-minute forced trial and a 2-minute choice trial, which were separated by a 2-minute delay period. The preference index [(time at "novel" arm – time at "familiar" arm)/(time at "novel" arm + time at "familiar" arm)] of MDMA-treated mice and saline controls did not significantly differ at both 7 and 30 days later. Cassel et al. gave adult male Long Evans rats a daily injection of 10 mg/kg i.p. MDMA for 4 consecutive days. Four days later, rats began testing on a T-maze, and were tested once daily for 4 days and then twice on a 5th day. Rats were permitted to enter only one arm during each trial, which were separated by a 30-second delay period. The overall percent alternation of MDMA-treated rats and saline controls did not significantly differ. These two studies suggest that pretreatment with a dose of 10 mg/kg MDMA, whether administered once or repeatedly, has no subsequent effect on spatial working memory.

Kolyaduke and Hughes (2013) performed a variation of the two-trial SA task in which all three arms of a Y-maze were open during both trials (now referred to as the acquisition and retention trials). During the acquisition trial, one arm contained a black insert and one arm contained a white insert, and during the retention trial, both arms contained a black insert (the changed arm = the novel arm). Multiple choices were allowed during the 6-minute acquisition trial and the 3-minute retention trial, and there was no delay period between the two trials. Male and female PVG/c hooded rats were given a daily injection of 10 mg/kg i.p. MDMA for 10 consecutive days during early adolescence (postnatal days 35–45) or late adolescence (postnatal days 45–55). Rats were tested as adults on two separate days at least 35 days after MDMA treatment (after postnatal day 90). Both early and late adolescence MDMA-treated rats did not significantly differ from saline controls in percent novel entries and percent time spent in the novel arm, indicating that pretreatment with repeated doses of 10 mg/kg MDMA may have no subsequent effect on spatial working memory.

Ricaurte et al. (1993) gave adult male Long Evans rats two daily injections of 20 mg/kg s.c. MDMA at an 8-hour interval for 4 consecutive days, and this treatment was repeated again about 1 week later. About 1 month later, rats began training for a DA task on a T-maze. Seven weeks after MDMA treatment, rats began daily test sessions consisting of one forced trial followed by 10 choice trials under a constant delay of 5 seconds. The percent correct of MDMA-treated rats and saline controls increased at a similar rate over the 20 test sessions, and there were no significant differences between groups. After 5 weeks of testing under a constant delay, variable delays of 5, 30, 60, 120, and 180 seconds were introduced, and testing continued for an additional 3 weeks. The percent correct of MDMA-treated rats and saline controls decreased at a similar rate as the duration of the delay period increased, and again there were no significant differences between groups. Findings from this study indicate that pretreatment with repeated doses of 20 mg/kg MDMA may have no subsequent effect on spatial working memory.

Young et al. (2005) performed a two-part task on a double Y-maze: the first part was a spatial discrimination (SD) task (described in section V.C.3.a) and the second part was a DA task (summarized here). Young adult male Wistar rats were trained to criterion on the task prior to being introduced to delays of 0, 15, or 60 second and then on-drug testing. Rats were injected with 0, 1.25, 2.25, or 5 mg/kg i.p. MDMA in a within-subjects design 20 minutes before on-drug test sessions. Each test session consisted of one forced trial followed by 24 choice trials with randomly allocated delays. Overall, the percent correct of all rats significantly decreased as the duration of the delay increased. Relative to saline, doses of 1.25 and 2.25 mg/kg MDMA had no effect on percent correct at any delay, a dose of 2.25 mg/kg MDMA produced a small increase in percent correct under a 60-second delay, and a dose of 5 mg/kg MDMA significantly decreased percent correct at all delays. Typically, this deficit would be attributed to a performance impairment, but since 5 mg/kg MDMA had no effect on accuracy in the SD component, which required the same performance abilities (see section V.C.3.a), this may be due to a working memory impairment. In all, these findings suggest that a dose of 1.25 or 2.25 mg/kg MDMA has no effect on spatial working memory, but a dose of 5 mg/kg MDMA may produce spatial working memory deficits.

Viñals et al. (2012) performed an operant/nonspatial version of the DA task in which adult male C57BL/6 mice were trained to alternate nose-poking between two nose-poking holes. Mice were trained to a criterion level on the task, and then given two daily injections of 3 or 30 mg/kg i.p. MDMA at a 4-hour interval for 4 consecutive days. Mice were tested off-drug for 7 days after MDMA treatment and introduced to delays of 2, 4, 6, or 8 seconds in a random order. Mice given 3 mg/kg MDMA injections and saline controls did not significantly differ in percent correct over all 7 days of testing. The percent correct of mice given 30 mg/kg MDMA injections was significantly higher than saline controls on the 1st day of testing but did not significantly differ from saline controls for the remaining 6 days. The increased accuracy on the 1st day of testing may be because of a slowed reaction time rather than working memory enhancements, as mice given 30 mg/kg MDMA injections also

demonstrated a significantly longer response latency compared with saline controls. In all, these results suggest that pretreatment with repeated doses of 3 or 30 mg/kg MDMA has no subsequent effect on nonspatial working memory.

3. Radial Arm Maze. The radial arm maze (RAM) (see Dudchenko, 2004; Quillfeldt, 2016) is a useful tool to study spatial working and reference memory in rodents. The goal of this task is to learn and remember the location of food pellets using spatial cues. Performance can be separated by type of memory (working vs. reference). The effects of MDMA on working memory in the RAM task will be discussed here, while the effects on reference memory will be reviewed in section V.C.2.

The RAM consists of a central hub that provides access to eight radiating arms. All eight arms are equal in length, and a food well is attached to the end of each arm. The entire maze is typically elevated above the floor in a room with many distal spatial cues at fixed locations. Prior to training, each animal is randomly assigned a set of four baited arms and four nonbaited arms, which remains fixed for the remainder of the experiment. Training is usually conducted daily (or sometimes spaced by 1 to 2 days), with all animals completing several trials per day (2–6 trials/day for the studies reviewed). Before each trial, food pellets are placed in the food wells of the four baited arms assigned to that animal. The trial then begins by placing the animal in the central hub facing arm number one. The animal is typically allowed to enter four arms per trial before being removed from the maze. The number of working memory errors, defined as entries into a baited arm that has already been visited in that same trial, is scored for each trial. A single entry into each baited arm reflects accurate spatial working memory of the food pellet locations.

Table 2 includes the six studies that used the RAM to evaluate the effects of MDMA on spatial working memory. Five of the studies used the general methods outlined above, while Braida et al. (2002) used an alternative procedure that is described below.

Hernandez-Rabaza et al. (2010) and Ros-Simó et al. (2013) treated adolescent male rodents with two injections of MDMA on a single day. Hernandez-Rabaza et al. gave Long Evans rats two injections of 10 mg/kg i.p. MDMA at a 6-hour interval, 12 days prior to training. MDMA-treated rats and saline controls exhibited a decrease in working memory errors over the 5 days of training, and the number of working memory errors did not significantly differ between groups. Ros-Simó et al. began training CD1 mice prior to any MDMA administration. Each animal was assigned only three baited arms, and animals were not limited to a certain number of arm entries within each trial. Mice were trained for a total of 12 consecutive days and were given two injections of 20 mg/kg i.p. MDMA on the 12th training day, one immediately after training and another 2 hours later. Three days later, mice were subject to an additional training session, during which the number of working memory errors produced by MDMA-treated mice and saline controls did not significantly differ. The findings of these two studies suggest that pretreatment with two doses of 10 or 20 mg/kg MDMA has no later effect on spatial working memory.

Figure 5 presents the findings of Kay et al. (2010), which exemplify dose-dependent effects of MDMA on working memory. Kay et al. trained adult male Sprague-Dawley rats off-drug until all rats reached a criterion of at least 75% correct arm entries for 7 days. After reaching criterion, rats began on-drug training. Rats were given 0, 0.75, 3, or 4 mg/kg i.p. MDMA in a within-subjects design 15 minutes before each day of training. Relative to saline, a dose of 0.75 mg/kg MDMA had no effect on the mean overall percent correct, but doses of 3 and 4 mg/kg MDMA significantly decreased the mean overall percent correct (Fig. 5A). The deficits produced by 3 mg/kg MDMA were not due to working memory impairments, as this dose did not significantly impact the percent of working memory errors (number of errors/number of errors possible per day). A dose of 4 mg/kg MDMA did significantly increase the percent of working memory errors relative to saline; however the percent of working memory errors was still significantly smaller than the percent of reference

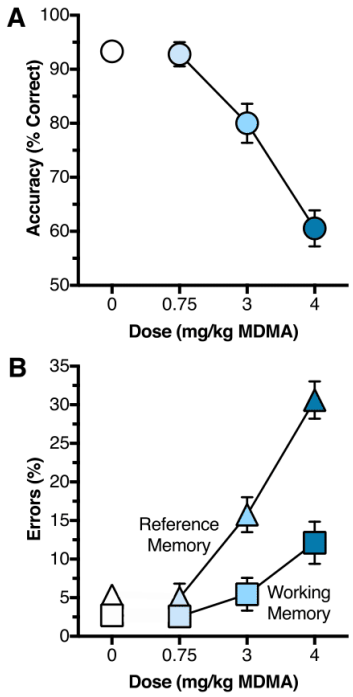

Fig. 5. Dose-dependent effects of MDMA on the radial arm maze task. 0.75 mg/kg MDMA had no effect on overall accuracy (A) or errors (B), while 3 and 4 mg/kg MDMA impaired overall accuracy (A) and increased working (4 mg/kg only) and reference memory errors (B). Data redrawn with permission from Figs. 1 and 3 in Kay et al. (2010).

memory errors (Fig. 5B). These findings indicate that doses of 0.75 and 3 mg/kg MDMA have no effect on spatial working memory, but a larger dose of 4 mg/kg MDMA slightly impairs spatial working memory.

Kay et al. (2011) and Harper et al. (2013) conducted similar experiments in which adult male Sprague-Dawley rats were treated with MDMA before and/or during training. Kay et al. gave rats four injections of 10 mg/kg i.p. MDMA at 2-hour intervals, 2 days prior to training. Rats were trained off-drug until all animals reached training criterion (28 days later). Over the 24 off-drug training sessions, the average percent correct of MDMA-treated rats increased at a slower rate than saline controls. Two days after off-drug training, on-drug training began and continued for a total of 12 days. Rats were given 0 or 4 mg/kg i.p. MDMA in a within-subjects design 20 minutes before each on-drug training session. MDMA significantly decreased the average percent correct relative to saline, but this impairment was significantly smaller in rats treated with MDMA prior to off-drug training relative to saline controls. Nonetheless, the impairments observed during off- and on-drug training were not due to working memory deficits, as working memory error percentage was not significantly affected by any MDMA treatment.

Harper et al. also gave rats four injections of 10 mg/kg i.p. MDMA at 2-hour intervals. Training began after MDMA treatment and lasted for 47 days. Most training sessions took place off-drug, except some rats were given 4 mg/kg i.p. MDMA before training sessions on days 8, 15, 22, 28, 34, and 41. The average percent correct of rats pretreated with MDMA prior to all training sessions was significantly lower than saline controls on both off-drug and on-drug training days. MDMA also significantly reduced average percent correct during on-drug training days relative to saline. Again, none of these impairments were due to working memory deficits, as working memory error percentage was not significantly affected by any MDMA treatment. The findings of Kay et al. and Harper et al. suggest that pretreatment with four doses of 10 mg/kg MDMA has no subsequent effect on spatial working memory, and a dose of 4 mg/kg MDMA also has no on-drug effect on spatial working memory.

Braida et al. (2002) conducted an alternative working memory task on the RAM. In this version, all eight arms of the maze are baited, and the animal's goal is to enter all eight arms only one time during each trial. The number of errors are scored for each trial, which is synonymous with working memory errors on the typical RAM task (i.e., re-entries into a baited arm). Here, the task was conducted both without a delay and with a 2-hour delay between the fourth and fifth arm entry. Adult male Wistar rats were trained on the task to a criterion level, and then began on-drug training for 3 consecutive days. Rats were given a single injection of 1, 2, or 3 mg/kg i.p. MDMA 20 minutes before each on-drug training session. Without a delay, MDMA had no effect on the total number of errors relative to saline. With a 2-hour delay, doses of 1 and 2 mg/kg MDMA had no effect on the total number of errors during the pre-delay period (first 4 choices) and post-delay period (last 4 choices) relative to saline. A dose of 3 mg/kg MDMA also had no effect on the total number of errors during the pre-delay period, but significantly increased the total number of errors during the post-delay period relative to saline. These results suggest that doses of 1 and 2 mg/kg MDMA have no effect on spatial working memory, but a dose of 3 mg/kg MDMA impairs spatial working memory.

4. Other Working Memory Tasks.

a. Morris water maze. The standard Morris water maze (MWM) task (Morris, 1984) typically assesses spatial learning and spatial reference memory (see section V.C.1); however the task procedures can be manipulated to measure spatial working memory (see Vorhees and Williams, 2006). On the standard MWM task, the hidden platform remains in the same location throughout acquisition training, and therefore long-term memory is required to navigate to the platform. On the working memory version of the MWM task, the location of the hidden platform is changed each day, and therefore long-term memory of the platform location is not required and rather the task demands working memory functioning. The two studies that examined the effects of MDMA on the working memory MWM task are included in Table 2. The methods for these studies are briefly discussed here, but see section V.C.1 for a full description of the MWM apparatus/methods.

Robinson et al. (1993) conducted a spatial navigation task on the MWM that consisted of three parts: an initial learning set, a retention test (reviewed in section V.C.1.a), and a second learning set. The learning sets assessed working memory and were each 3 consecutive days in total. On each day, the platform location was chosen randomly, which remained constant for that day only. Eight trials were performed per day, with two trials from each of the four starting locations. Adult male Sprague-Dawley rats were given two daily injections of 10 mg/kg i.p. MDMA at a 12-hour interval for 4 consecutive days. The initial learning set began 2 days after MDMA treatment, and the second learning set began 8 days after MDMA treatment. On the initial learning set, the escape latency of MDMA-treated rats was significantly higher than saline controls on the first few trials of each day, but both groups demonstrated a significant decrease in escape latency across trials and showed no significant differences by the last few trials. On the second learning set, both MDMA-treated rats and saline controls demonstrated a significant decrease in escape latency across trials, and there were no significant differences between groups. These results suggest that pretreatment with repeated doses of 10 mg/kg MDMA has no subsequent effect on spatial working memory.

Galizio et al. (2014) performed a repeated acquisition/performance procedure on the MWM. The acquisition component assessed working memory, as the platform location changed each day, while the performance component (see section V.C.1.a) assessed reference memory, as the platform location remained fixed over all days. Each day consisted of 12 trials that alternated between acquisition and performance trials. Adult male Sprague-Dawley rats were trained on the task prior to on-drug testing. Rats were given of 0, 0.3, 1.0, 1.7, 3.0, or 5.6 mg/kg i.p. MDMA in a within-subjects design 15 minutes before each on-drug test session. On the acquisition component, doses of 0.3, 1.0, and 1.7 mg/kg MDMA had no effect on escape latency, while doses of 3.0 and 5.6 mg/kg MDMA significantly increased escape latency relative to saline. Doses of 3.0 and 5.6 mg/kg MDMA also produced significant increases in latency on the performance component (see section V.C.1.a), and therefore these deficits can be attributed to performance impairments rather than working memory impairments. In all, these results reveal that doses of 0.3, 1.0, 1.7, 3.0, and 5.6 mg/kg may have no effect on spatial working memory.

C. Spatial Learning and Memory

1. Morris Water Maze. The Morris water maze (MWM) (Morris, 1984) is one of the most widely used tasks for studying spatial learning and memory in rodents. The objective of this task is to learn to navigate to a hidden platform in a large circular pool of water using spatial cues. The pool is arbitrarily divided into four quadrants and is in a room with many distal visual cues at fixed locations (e.g., furniture, wall art, etc.). There are two main stages of the task: acquisition training and the probe test, which assess spatial learning and spatial reference memory, respectively.

Acquisition training takes place over a few consecutive days (3–5 days for the studies reviewed), with all animals completing several trials per day (3–8 trials/day for the studies reviewed). On each trial, an animal is placed into the water facing the wall of the pool and is expected to swim and escape onto the hidden platform. The hidden platform remains in the same location throughout acquisition training, but the starting location of the animal is varied between trials. As a result, spatial memory of the distal visual cues is required to identify the location of the hidden platform. The escape latency (i.e., time taken to reach the platform), and often the path length (i.e., distance swam to reach the platform), is recorded for all trials. A significant decrease in escape latency/path length over days of acquisition training suggests spatial learning of the platform location.

The probe test takes place after the last acquisition training session, either the same day or the following day. The procedure is similar to acquisition training, except the hidden platform is removed from the pool and each animal performs only one trial. The total time spent swimming in each quadrant of the pool, or sometimes the average distance from the platform location, is recorded. A significantly greater amount of time spent swimming in the target quadrant (i.e., quadrant where the hidden platform used to be located) relative to the other three quadrants indicates spatial reference memory of the platform location.

Table 3 presents the 14 studies that report the effects of MDMA on the standard MWM task. Most of these studies completed both acquisition training and the probe test. Some studies do not report the change in escape latency/path length over days of acquisition training, and a few other studies do not report findings on the probe test. In these particular studies, effects on spatial learning or spatial reference memory (respectively) cannot be properly assessed. All 14 studies used high doses of 5–20 mg/kg MDMA, and none used lower, typical doses of less than 3 mg/kg MDMA.

Taghizadeh et al. (2016) were the only group to conduct on-drug acquisition training. Adult male Wistar rats were given 5, 10, or 15 mg/kg i.p. MDMA 30 minutes before the first trial of acquisition training on all 4 days. During the probe test on the following day (off-drug), MDMA-treated rats spent significantly less time in the target quadrant than saline controls. These results suggest that doses of 5, 10, and 15 mg/kg MDMA impair spatial reference memory when acquisition occurs on-drug.

The remaining studies explored the effects of administering MDMA one or more days prior to acquisition training. Mirzaei et al. (2013) gave adult male Wistar rats a single injection of 10 mg/kg i.p. MDMA, 3 days prior to acquisition training. The escape latency and path length of MDMA-treated rats were significantly higher than that of saline controls on the 1st day of training, but these values decreased significantly over the 2nd and 3rd day to a level comparable to that of the saline controls. This suggests that pretreatment with a dose of 10 mg/kg MDMA has no subsequent effect on spatial learning.

Sprague et al. (2003), Cohen et al. (2005), Able et al. (2006), Skelton et al. (2008), and Cunningham et al. (2009) all gave adult male Sprague-Dawley rats multiple injections of MDMA on a single day prior to acquisition training. Sprague et al. gave rats two injections of 20 mg/kg s.c. MDMA at a 12-hour interval, 1 week prior to acquisition training. The escape latency and path length of MDMA-treated rats decreased significantly over 3 days of acquisition training, and there were no significant differences between MDMA-treated rats and saline controls during acquisition. During the probe test directly after the last acquisition session, MDMA-treated rats spent significantly less time in the target quadrant than saline controls, yet significantly more time in the target quadrant than two of the other three quadrants. Similarly, Cunningham

TABLE 3
Studies examining the effects of MDMA on spatial learning and memory

Article	Task[a]	Subjects[b]	Doses/Frequency[c]	Timeline[d]	Effects[e]
Taghizadeh et al., 2016	MWM	Rats (W), Adult, Male	5, 10, or 15 mg/kg (i.p.) × 1/day, 4 days	Acquisition: On-Drug / Probe: Postdrug	Probe: ↓
Mirzaei et al., 2013	MWM	Rats (W), Adult, Male	10 mg/kg (i.p.) × 1 OR × 2/day, 7 days	Acquisition: Postdrug	Acquisition: ⌀
Sprague et al., 2003	MWM	Rats (SD), Adult, Male	20 mg/kg (s.c.) × 2	Acquisition/Probe: Postdrug	Acquisition: ⌀ / Probe: ↓
Cunningham et al., 2009	MWM	Rats (SD), Adult, Male	7.5 mg/kg (i.p.) × 4	Acquisition/Probe: Postdrug	Acquisition: ⌀ / Probe: ↓
Able et al., 2006	MWM	Rats (SD), Adult, Male	15 mg/kg (s.c.) × 4	Acquisition/Probe: Postdrug	Acquisition: ⌀ / Probe: ↓
Cohen et al., 2005	MWM	Rats (SD), Adult, Male	15 mg/kg (s.c.) × 4	Acquisition/Probe: Postdrug	Probe: ⌀
Skelton et al., 2008	MWM	Rats (SD), Adult, Male	15 mg/kg (s.c.) × 4 OR × 4/day, 1 day/wk, 5 wk	Acquisition/Probe: Postdrug	Acquisition/Probe: ⌀
Camarasa et al., 2008	MWM	Rats (LE), Adult, Male	15 mg/kg (s.c.) × 2/day, 4 days	Acquisition/Probe: Postdrug	Acquisition/Probe: ↓
Abad et al., 2014	MWM	Rats (SD), Adol., Male	20 mg/kg (s.c.) × 2/day, 4 days	Acquisition/Probe: Postdrug	Acquisition: ↑ / Probe: ⌀
Busceti et al., 2008	MWM	Mice (B6), Adult, Male	5 or 15 mg/kg (i.p.) × 2/day, 6 days	Acquisition/Probe: Postdrug	Acquisition: ↓ / Probe: ↓ (7 days later; 40 days later - 15 mg/kg), ⌀ (40 days later - 5 mg/kg)
Kermanian et al., 2012	MWM	Rats (SD), Adult, Male	10 or 20 mg/kg (i.p.) × 1/day, 7 days	Acquisition: Postdrug	Acquisition: ↓
Soleimani Asl et al., 2015	MWM	Rats (SD), Adult, Male/Female	5 mg/kg (i.p.) × 2/day, 7 days	Acquisition/Probe: Postdrug	Probe: ↓
Soleimani Asl et al., 2011	MWM	Rats (SD), Adult, Male	5, 10, or 20 mg/kg (i.p.) × 2/day, 7 days	Acquisition/Probe: Postdrug	Probe: ⌀
Soleimani Asl et al., 2013	MWM	Rats (SD), Adult, Male	5, 10, or 20 mg/kg (i.p.) × 2/day, 7 days	Acquisition/Probe: Postdrug	Probe: ↓
Robinson et al., 1993	MWM Var.	Rats (SD), Adult, Male	10 mg/kg (i.p.) × 2/day, 4 days	Training/Probe: Postdrug	⌀
Galizio et al., 2014	MWM Var.	Rats (SD), Adult, Male	0.3, 1, 1.7, 3, or 5.6 mg/kg (i.p.)*	Training: Predrug / Acquisition: On-Drug	⌀ (0.3, 1, and 1.7 mg/kg) ↓ (3 and 5.6 mg/kg)
Compton et al., 2011	MMW Var.	Rats (LE), Adol.→Adult, Male	10 mg/kg (i.p.) × 1/day, 6 days (spaced)	Training/Acquisition: Postdrug	↓
Edut et al., 2011	MWM Var.	Mice (ICR), Adult, Male	10 mg/kg (i.p.) × 1	Acquisition: Postdrug	⌀
Hernandez-Rabaza et al., 2010	RAM	Rats (LE), Adol., Male	10 mg/kg (i.p.) × 2	Training: Postdrug	⌀
Ros-Simó et al., 2013	RAM	Mice (CD1), Adol., Male	20 mg/kg (i.p.) × 2	Training: Predrug, Off-Drug[2], Postdrug	↓
Kay et al., 2010	RAM	Rats (SD), Adult, Male	0.75, 3, or 4 mg/kg (i.p.)*	Training: Predrug, On-Drug	⌀ (0.75 mg/kg), ↓ (3 and 4 mg/kg)
Kay et al., 2011	RAM	Rats (SD), Adult, Male	10 mg/kg (i.p.) × 4 AND 4 mg/kg (i.p.)*	Training: Postdrug (10 mg/kg) and On-Drug (4 mg/kg)	↓
Harper et al., 2013	RAM	Rats (SD), Adult, Male	10 mg/kg (i.p.) × 4 AND/OR 4 mg/kg (i.p.) × 1/wk, 6 wk	Training: Postdrug (10 mg/kg) and On-Drug (4 mg/kg)	↓
Young et al., 2005	SD	Rats (W), Adult, Male	1.25, 2.25, or 5 mg/kg (i.p.)*	Training: Predrug / Testing: On-Drug	⌀

[a]Studies used the Morris water maze (MWM), radial arm maze (RAM), and spatial discrimination (SD) tasks.
[b]Species (strain), age, and sex of subjects. Strains include Long Evans (LE), Sprague-Dawley (SD), and Wistar (W) rats; and C57BL/6 (B6), CD1, and ICR mice.
[c]Dose, route, and frequency of MDMA administration. Treatment days/weeks are consecutive unless noted as "spaced."
[d]When training and testing occurred in relation to drug treatment. Pre- and post-drug training/testing were always conducted off-drug.
[e]Effects of drug treatment on spatial learning (MWM-Acquisition) and spatial reference memory (MWM-Probe, RAM, SD): ⌀ No Effect, ↓ Impairment, ↑ Enhancement.
[2]Off-drug training/testing took place immediately before drug administration on (last) day of treatment.
*Within-subjects design (all animals were tested multiple times at each dose including saline).

et al. gave rats four injections of 7.5 mg/kg i.p. MDMA at 2-hour intervals, 24 days prior to acquisition training. There were no significant differences between MDMA-treated rats and saline controls in the decrease in escape latency and path length over 5 days of acquisition training. During the probe test directly after the last acquisition session, MDMA-treated rats spent the same amount of time in all four quadrants, unlike the saline controls that spent significantly more time in the target quadrant than the other three quadrants. Able et al. gave rats four injections of 15 mg/kg s.c. MDMA at 2-hour intervals, 12 days prior to acquisition training. Again, there were no significant differences between MDMA-treated rats and saline controls during acquisition training, as both groups exhibited similar decreases in escape latency over all 5 days. During the probe test on the following day, the average distance from the platform location of MDMA-treated rats was significantly greater than that of saline controls. Cohen et al. also gave rats four injections of 15 mg/kg s.c. MDMA at 2-hour intervals, but at least 2 weeks prior to 5 days of acquisition training. On the probe test the day after the last acquisition session, there were no significant differences between MDMA-treated rats and saline controls in percent time spent in the target quadrant. Skelton et al. also gave rats four injections of 15 mg/kg s.c. MDMA at 2-hour intervals, 14 days prior to acquisition training. The latency of MDMA-treated rats and saline controls decreased in a similar manner over 5 days of acquisition training. During the probe test on the following day, the average distance from the platform location of MDMA-treated rats and saline controls did not significantly differ.

The findings from the five studies above suggest that pretreatment with two doses of 20 mg/kg MDMA or four doses of 7.5 or 15 mg/kg MDMA has no later effect on spatial learning. The results from the probe test of these studies suggest that pretreatment with two doses of 20 mg/kg MDMA or four doses of 7.5 mg/kg MDMA subsequently impairs spatial reference memory, while pretreatment with four doses of 15 mg/kg MDMA has no later effect on spatial reference memory (apart from Able et al.'s findings that this dose produces spatial reference memory impairments).

The remainder of the studies investigated the consequences of administering multiple daily injections of MDMA on multiple days prior to acquisition training. Camarasa et al. (2008) and Abad et al. (2014) treated rats with two daily injections of MDMA for 4 consecutive days. Camarasa et al. gave adult male Long Evans rats two daily injections of 15 mg/kg s.c. MDMA at a 7-hour interval for 4 consecutive days, 9 days prior to acquisition training. Unlike saline controls, the escape latency of MDMA-treated rats did not significantly decrease over 4 days of acquisition training. On the probe test the following day, MDMA-treated rats also did not spend significantly more time in the target quadrant than that predicted by random (1/4th of the total time). Abad et al. gave adolescent male Sprague-Dawley rats two daily injections of 20 mg/kg s.c. MDMA for 4 consecutive days, 1 week prior to acquisition training. The escape latency of MDMA-treated rats decreased at a faster rate than saline controls over 4 days of acquisition training. MDMA-treated rats and saline controls spent significantly more time in the target quadrant than the opposite quadrant on the probe test the next day. These two studies have opposing findings. The results of Camarasa et al. suggest that pretreatment with repeated doses of 15 mg/kg MDMA later impairs both spatial learning and spatial reference memory, while the results of Abad et al. suggest that pretreatment with repeated doses of 20 mg/kg MDMA later enhances spatial learning and has no effect on spatial reference memory. The use of different rat strains or ages (Camarasa et al. tested adult Long Evan rats and Abad et al. tested adolescent Sprague-Dawley rats) may account for this discrepancy in findings.

Busceti et al. (2008) used a similar MDMA regimen as Camarasa et al. and Abad et al., but instead gave adult male C57BL/6 mice two daily injections of 5 or 15 mg/kg i.p. MDMA at a 2-hour interval for 6 consecutive days. Acquisition training began 7 or 40 days after MDMA treatment, and at both time points, the escape latency of MDMA-treated mice did not significantly decrease over the 4 days. On the probe test, mice given 5 mg/kg MDMA injections spent significantly less percent time than saline controls in the target quadrant when tested 7 days later but not when tested 40 days later. Mice given 15 mg/kg MDMA injections spent significantly less percent time than saline controls in the target quadrant when tested 7 or 40 days later. These results suggest that pretreatment with repeated doses of 5 or 15 mg/kg MDMA subsequently results in spatial learning and spatial reference memory deficits, but spatial reference memory may return to normal by 40 days after treatment with repeated doses of 5 mg/kg MDMA only.

The next group of studies treated adult rats with MDMA for 7 consecutive days. Kermanian et al. (2012) gave adult male Sprague-Dawley rats a daily injection of 10 or 20 mg/kg i.p. MDMA for 1 week. Acquisition training began 1 week later, and unlike saline controls, the escape latency of MDMA-treated rats did not significantly decrease over the 4 days. Soleimani Asl et al. (2015) gave male and female adult Sprague-Dawley rats two daily injections of 5 mg/kg i.p. MDMA for 1 week. On the following probe test, MDMA-treated rats spent significantly less percent time in the target quadrant than saline controls. Soleimani Asl et al. (2011) and Soleimani Asl et al. (2013) gave adult male Sprague-Dawley rats two daily injections of 5, 10, or 20 mg/kg i.p. MDMA at an 8-hour interval for 1 week. Soleimani Asl et al. (2011) began 3 days of acquisition training 1 week after MDMA treatment. The probe test

took place the day after acquisition training, during which MDMA-treated rats and saline controls spent the same percent time in the target quadrant. Soleimani Asl et al. (2013) began 3 days of acquisition training the day after MDMA treatment. The probe test also took place the day after acquisition training, but MDMA-treated rats spent significantly less percent time in the target quadrant than saline controls. In addition to testing the effects of a single dose of MDMA (described above), Mirzaei et al. (2013) gave another group of adult male Wistar rats two daily injections of 10 mg/kg i.p. MDMA for 1 week and began acquisition training the following day. The escape latency and path length of MDMA-treated rats were significantly higher than that of saline controls on the 1st day of training, but these values decreased significantly over the 2nd and 3rd day to a level comparable to that of saline controls.

The findings from the above studies are mixed. The results of Kermanian et al. suggest that pretreatment with repeated doses of 10 or 20 mg/kg MDMA leads to spatial learning deficits, while the results of Mirzaei et al. suggest that pretreatment with repeated doses of 10 mg/kg MDMA has no effect on spatial learning. Likewise, the results of Soleimani Asl et al. (2013, 2015) suggest that pretreatment with repeated doses of 5, 10, or 20 mg/kg MDMA leads to spatial reference memory deficits. The results of Soleimani Asl et al. (2011), however, suggest that pretreatment with repeated doses of 5, 10, or 20 mg/kg MDMA has no effect on spatial reference memory. These differences in findings could be due to the timing of training/testing relative to MDMA treatment. Soleimani Asl et al. (2013) and Mirzaei et al. began acquisition training 1 day after MDMA treatment and found learning and memory deficits, while Soleimani Asl et al. (2011) and Kermanian et al. (2012) began acquisition training 1 week after MDMA treatment and found no effects. The differences between the findings of Kermanian et al. and Mirzaei et al. could also be due to the use of different rat strains (Sprague-Dawley vs. Wistar). In all, it appears that pretreatment with MDMA for 7 consecutive days may produce spatial learning and memory deficits within the week after treatment, but not after 1 week.

Skelton et al. (2008), in addition to studying the effects of multiple MDMA injections on a single day (above), gave another group of animals the same treatment weekly. Adult male Sprague-Dawley rats were given four daily injections of 15 mg/kg s.c. MDMA at 2-hour intervals once weekly for 5 weeks. Acquisition training began 14 days later, during which the latency of MDMA-treated rats and saline controls decreased in a similar manner over all 5 days. During the probe test on the following day, the average distance from the platform location of MDMA-treated rats and saline controls did not significantly differ. These results suggest that pretreatment with repeated doses of 15 mg/kg MDMA has no subsequent effect on spatial learning or spatial reference memory.

a. Morris water maze variations. Four studies included in Table 3 used variations of the water maze to assess the effects of MDMA on spatial learning and memory. Robinson et al. (1993) conducted a spatial navigation task that is similar to the standard MWM task. Adult male Sprague-Dawley rats were given two daily injections of 10 mg/kg i.p. MDMA at a 12-hour interval for 4 consecutive days, 2 days prior to training. The location of the hidden platform changed each day of training, and therefore this phase measured working memory rather than spatial learning (see section V.B.4.a). Training lasted for 3 days, and on the 4th day the hidden platform was left in the same location as the previous day for a retention test of spatial reference memory of the platform location (similar to the probe test in the standard MWM task). The escape latencies of MDMA-treated rats and saline controls showed no significant differences over all four trials of the retention test, which suggests that pretreatment with repeated doses of 10 mg/kg MDMA has no subsequent effect on spatial reference memory.

Figure 6 portrays the findings of Galizio et al. (2014), which exemplify dose-dependent effects of MDMA on spatial learning and memory. Galizio et al. conducted a repeated acquisition/performance procedure on the MWM. Adult male Sprague-Dawley rats were trained on the acquisition and performance components of the task prior to any MDMA administration. Each training day consisted of 12 trials that alternated between acquisition and performance trials. The acquisition component (see section V.B.4.a) assessed working memory, as the platform location changed each training day but remained fixed for all trials on a particular day. The performance component corresponded to acquisition training on the standard MWM, as the platform location remained fixed over all days of training. Once rats reached criterion on training, the same procedure was repeated on-drug. Unlike the other MWM studies, MDMA was tested across a wide range of doses. Rats were given 0, 0.3, 1.0, 1.7, 3.0, or 5.6 mg/kg i.p. MDMA in a within-subjects design 15 minutes before each on-drug session. On the performance component, doses of 0.3, 1.0, and 1.7 mg/kg MDMA had no effect on escape latency while doses of 3.0 and 5.6 mg/kg MDMA significantly increased escape latency relative to saline. These results suggest that doses of 0.3, 1.0, and 1.7 mg/kg MDMA have no effect on spatial learning but doses of 3.0 and 5.6 mg/kg MDMA impair spatial learning.

Compton et al. (2011) used a constant-start training and novel-start testing procedure on the MWM. Adolescent male Long Evans rats were given a daily injection of 10 mg/kg i.p. MDMA for 6 alternating days. Rats were trained and tested as adults about 3 months later. The experiment started with constant-start

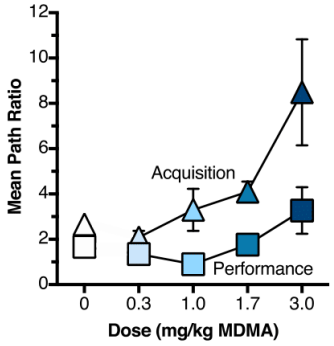

Fig. 6. Dose-dependent effects of MDMA on a variation of the Morris water maze task. 0.3, 1.0, 1.7 mg/kg MDMA had no effect on working memory (acquisition) or spatial learning (performance), while 3 mg/kg MDMA impaired spatial learning (performance). Data redrawn with permission from Fig. 4 (middle) in Galizio et al. (2014).

training, during which the starting location of each rat and the platform location were invariable. Novel-start testing began after rats reached training criterion, which is executed in the same manner as acquisition training on the standard MWM (variable starting locations and a fixed platform location). Rats were tested for 3 days, with each day consisting of six trials, trials one, two, four, and five were constant-start trials and trials three and six were novel-start trials. The escape latency of MDMA-treated rats on novel-start trials were significantly greater than that of saline controls, suggesting that pretreatment with repeated doses of 10 mg/kg MDMA during adolescence will impair spatial learning as adults.

Edut et al. (2011) tested the effects of MDMA treatment on the dry maze test, a variation of the MWM that does not require swimming. The dry maze consists of a circular arena with 20 tiny wells arranged in a circular manner. The goal of the task is to learn the location of the single well that is filled with water. Adult male ICR mice were first trained to drink from all 20 wells, and then introduced to a procedure identical to acquisition training on the standard MWM to learn the water well location. Mice were given a single injection of 10 mg/kg i.p. MDMA and tested 7 and 30 days later. Seven days after MDMA treatment, the latency to reach the water well of MDMA-treated mice and saline controls decreased in a similar manner over all 7 days of testing. Thirty days after MDMA treatment, the latency of MDMA-treated mice was significantly higher than saline controls on days 4 and 6 of acquisition, but both groups showed significant decreases in latency over all 7 days and by the last day of acquisition there were no significant differences. These results suggest that pretreatment with a dose of 10 mg/kg MDMA has no subsequent effect on spatial learning.

2. Radial Arm Maze. As described in section V.B.3, the radial arm maze (RAM) (see Dudchenko, 2004; Quillfeldt, 2016) is a useful tool to study spatial working and reference memory in rodents. Here, we review the five studies that examined the effects of MDMA on spatial reference memory using the RAM task, which are outlined in Table 3. The methods for these studies are as previously explained; however, now the outcome variable of interest is the number of reference memory errors per trial. Reference memory errors are defined as entries into a nonbaited arm. Entries into only baited arms reflect accurate spatial reference memory of the food pellet locations.

Hernandez-Rabaza et al. (2010) gave adolescent male Long Evans rats two injections of 10 mg/kg i.p. MDMA at a 6-hour interval, 12 days prior to training. MDMA-treated rats and saline controls exhibited a similar decrease in reference memory errors over 5 days of training. The total reference memory errors during all 5 days also did not significantly differ between groups. Similarly, Ros-Simó et al. (2013) gave adolescent male CD1 mice two injections of 20 mg/kg i.p. MDMA, but on the 12th and last day of training (using the alternative methods described in section V.B.3). One injection was given immediately after training and the second was given 2 hours later. Three days later, mice were subject to an additional training session, during which MDMA-treated mice produced significantly more reference memory errors than saline controls. Although these two studies administered similar MDMA treatments, the findings of Hernandez-Rabaza et al. suggest that pretreatment with a two doses of 10 mg/kg MDMA has no later effect on spatial reference memory, while the findings of Ros-Simó et al. suggest that treatment with two doses of 20 mg/kg MDMA impairs consolidation of spatial reference memory.

Kay et al. (2010) gave adult male Sprague-Dawley rats (that were pretrained on the task) 0, 0.75, 3, or 4 mg/kg i.p. MDMA in a within-subjects design 15 minutes before each day of training. As summarized previously, a dose of 0.75 mg/kg MDMA had no effect on the mean overall percent correct, but doses of 3 and 4 mg/kg MDMA significantly decreased the mean overall percent correct relative to saline. The deficits produced by doses of 3 and 4 mg/kg MDMA are primarily attributed to reference memory impairments, as both doses significantly increased the percent of reference memory errors relative to saline, and the percent of reference memory errors were significantly higher than the percent of working memory errors. As illustrated in Fig. 5, these findings indicate that a dose of 0.75 mg/kg MDMA has no effect on spatial reference memory but doses of 3 and 4 mg/kg MDMA impair spatial reference memory.

Kay et al. (2011) and Harper et al. (2013) gave adult male Sprague-Dawley rats MDMA before and/or during training. Kay et al. gave rats four injections of 10 mg/kg i.p. MDMA at 2-hour intervals, 2 days prior to off-drug training. After 28 days of off-drug training, on-drug training began and continued for a total of 12 days. Rats were given 0 or 4 mg/kg i.p. MDMA in a within-subjects

design 20 minutes before each on-drug training session. The effects of MDMA on average percent correct that were previously reported in section V.B.3 can be attributed to reference memory deficits, as the effects on average percent correct and reference memory error percentage follow the same pattern. During off-drug training, the reference memory error percentage of MDMA-treated rats decreased at a slower rate than saline controls. During on-drug training, MDMA significantly decreased the reference memory error percentage relative to saline (this impairment was also significantly smaller in rats treated with MDMA prior to off-drug training vs. saline controls).

Harper et al. (2013) gave rats four injections of 10 mg/kg i.p. MDMA at 2-hour intervals and began training after MDMA treatment. Training lasted for 47 days, with a mix of on-drug sessions (days 8, 15, 22, 28, 34, and 41) and off-drug sessions (all other days). Before the on-drug training sessions, some rats were given 4 mg/kg i.p. MDMA. Again, the effects of MDMA on average percent correct that were previously reported in section V.B.3 can be attributed to reference memory deficits, as the effects on average percent correct and reference memory error percentage follow the same pattern. The reference memory error percentage of rats pretreated with MDMA prior to all training sessions was significantly higher than saline controls on both off-drug and on-drug training days. MDMA also significantly increased reference memory error percentage during on-drug training days relative to saline. The findings of Kay et al. and Harper et al. suggest that pretreatment with four doses of 10 mg/kg MDMA subsequently impairs spatial reference memory, and a dose of 4 mg/kg MDMA also impairs spatial reference memory when on-drug.

3. Other Spatial Tasks.

a. Spatial discrimination. Young et al. (2005) used a double Y-maze for a two-part task, spatial discrimination (SD) task, which assesses spatial reference memory and is outlined in Table 3, and a delayed alternation task, which assesses working memory and is described in section V.B.2. The double Y-maze consists of four end arms connected to a central stem (2 arms on each side of stem). Every arm is virtually identical from inside the maze, but the entire maze is in a room with many distal visual cues. On every trial, the animal is placed on the end of one of the arms on the left side of the maze, and the goal is to navigate to a food reward that is on one of the arms on the right side of the maze. The first part of the task is the SD task, as the animal is faced with the decision to turn left or right—one way leading to the adjacent arm and the other leading to the central stem and ultimately the food reward. Both options appear identical to the animal because there is a door placed in the central stem before the arms on the right side. The task therefore requires spatial reference memory of the location of the central stem relative to the distal visual cues.

For this study, young adult male Wistar mice were trained on the task above prior to any MDMA treatment. After reaching training criterion, mice were introduced to intertrial delays of 15 and 60 seconds and then tested on-drug with the same procedure. Mice were given 0, 1.25, 2.25, or 5 mg/kg i.p. MDMA in a within-subjects design 20 minute before each test session. MDMA had no effect on percent correct choices at any delay relative to saline. These findings demonstrate that doses of 1.25, 2.25, and 5 mg/kg MDMA do not influence spatial reference memory retrieval.

D. Nonspatial Learning and Memory

1. Novel Object Recognition. The novel object recognition (NOR) task (Ennaceur and Delacour, 1988) is a relatively simple test of nonspatial memory. This method is based on the natural tendency for rodents to explore a novel object more than a familiar object. Animals are first habituated to the testing environment, a box or circular arena that is typically under dim lighting, on 1 or more days prior to testing. Testing consists of two trials, a training trial and a test trial, separated by a delay ranging from 1 minute to 24 hours. This task can measure short-term memory or long-term memory, depending on the duration of the delay. Short-term memory does not require protein synthesis but long-term memory does require protein synthesis, and the transition from protein synthesis-independent to protein synthesis-dependent long-term potentiation begins about 2 hours after memory acquisition (Frey and Morris, 1997; Lu et al., 2008). Therefore, we can consider that delays of less than 2 hours measure short-term recognition memory, and delays of 2 hours or more measure long-term recognition memory.

During the training trial, the animal is presented with two identical objects (“A”), and the total time spent exploring the two objects is measured. During the test trial (following the delay), the animal is presented with one familiar object (“A”) and one novel object (“B”), and the time spent exploring each object is measured. Object exploration is defined as touching, sniffing, or directing the nose and vibrissae toward the object at a distance of less than 1 to 2 cm. Significantly more exploration of the novel object B than of the familiar object A in the test trial is an indicator of object recognition memory. A “discrimination index” or “discrimination ratio” is usually calculated to capture this data. The “discrimination index” is the difference in exploration times of the novel object B and the familiar object A, divided by the total exploration time of the two objects in the test trial. The discrimination ratio is the exploration time of the novel object B divided by the total exploration time of the two objects in the test trial. A higher discrimination index or discrimination ratio reflects greater memory retention of the familiar object.

Table 4 outlines the 22 studies that investigated the effects of MDMA on the NOR task. Similar to the MWM

studies, most of the NOR studies used high doses of 3–20 mg/kg MDMA, and none used lower, typical doses of less than 3 mg/kg MDMA. For most of these studies, the rodents were pretreated with MDMA and then trained and tested on the task at a later time point. Two of the 22 studies (Ros-Simó et al., 2013; Shortall et al., 2013), however, administered MDMA on the same day as the training and/or test trials, and these studies will be discussed first.

Instead of conducting only one training trial, Ros-Simó et al. (2013) trained adolescent male CD1 mice daily for 3 days. Mice were given two injections of 20 mg/kg i.p. MDMA, one directly after the third training trial and another 2 hours later. The test trial took place 72 hours later. The discrimination index of MDMA-treated mice was significantly less than that of saline controls. These results suggest that two doses of 20 mg/kg MDMA administered after memory acquisition leads to impairments in long-term recognition memory. Shortall et al. (2013) gave young adult male Lister Hooded rats a daily injection of 10 mg/kg i.p. MDMA for 2 consecutive days. The training trial began 30 minutes after the drug injection on the 2nd day, which was followed by a 2-hour delay and then the test trial. Rats treated with MDMA did not explore the novel object more than the familiar object, and the discrimination ratio of MDMA-treated rats was significantly less than that of saline controls. This suggests that a dose of 10 mg/kg MDMA impairs long-term recognition memory when administered on the day before and the day of memory acquisition/retrieval. The findings of Ros-Simó et al. and Shortall et al. together suggest that MDMA impairs long-term recognition memory when on-drug during the memory consolidation phase.

Nawata et al. (2010) and Edut et al. (2011) gave adult male CD1 and ICR (respectively) mice a single injection of 10 mg/kg i.p. MDMA. Nawata et al. ran the training and test trials 1 or 7 days after MDMA treatment, with a 3-hour delay between trials. The discrimination indexes of MDMA-treated mice and saline controls were comparable at both 1 and 7 days posttreatment. Edut et al. ran the training and test trials 7 or 30 days after MDMA treatment, with a 24-hour delay between trials. The discrimination indexes of MDMA-treated mice and saline controls were comparable at 7 days posttreatment, but MDMA-treated mice showed significant reductions relative to saline controls at 30 days posttreatment. Together, these findings suggest that pretreatment with a dose of 10 mg/kg MDMA has no effect on long-term recognition memory up to 1 week after treatment, but deficits may arise 30 days after treatment.

The following group of studies treated rats with multiple injections of MDMA on a single day prior to testing. Figure 7 depicts the findings of Rodsiri et al. (2011), which exemplify dose-dependent effects of MDMA on nonspatial learning and memory. Rodsiri et al. gave adult male Lister Hooded rats three injections of 3 or 6 mg/kg i.p. MDMA at 2-hour intervals. Rats were tested 2 weeks later, with a 2-hour delay between trials. Rats given 3 mg/kg MDMA injections and saline controls did not exhibit differences in discrimination ratios, but the discrimination ratio of rats given 6 mg/kg MDMA injections was significantly less than that of saline controls (Fig. 7B). These findings indicate that there may be dose-dependent effects of MDMA on long-term recognition memory, as pretreatment with three doses of MDMA had no later effect at 3 mg/kg MDMA but led to memory impairments at 6 mg/kg.

In a similar study, Piper et al. (2008) gave young adult male Sprague-Dawley rats four injections of 10 mg/kg s.c. MDMA at 1-hour intervals. Rats were tested at 15–17 and 17–19 days after MDMA treatment with shorter 15- and 60-minute delays, respectively. The discrimination ratios of MDMA-treated rats and saline controls did not significantly differ during either test. Cohen et al. (2005), Able et al. (2006), and Skelton et al. (2008) all gave adult male Sprague-Dawley rats four injections of 15 mg/kg s.c. MDMA at 2-hour intervals. Able et al. tested their rats 30 days after MDMA treatment and Cohen et al. and Skelton et al. tested their rats at least 5 weeks after MDMA treatment. All three studies used a 1-hour delay period and found that during the test trial MDMA-treated rats and saline controls explored the novel object more than the familiar object, and both groups explored the novel object for a similar amount of time. The findings from the above studies suggest that pretreatment with four doses of 10 or 15 mg/kg MDMA has no subsequent effect on short-term recognition memory.

The remaining studies treated animals with MDMA over several days prior to testing. Morley et al. (2001) and McGregor et al. (2003) treated adult male Wistar rats with MDMA for 2 consecutive days. Morley et al. gave rats one or four (at 1-hour intervals) daily injections of 5 mg/kg i.p. MDMA for 2 consecutive days. Rats were trained and tested 14 weeks later with a 15-minute delay and again 1 more week later with a 60-minute delay. The discrimination ratio of rats given one daily injection did not significantly differ from that of saline controls at either delay. The discrimination ratio of rats given four daily injections did not significantly differ from that of saline controls at the 60-minute delay, but was significantly less than saline controls at the 15-minute delay. McGregor et al. also gave rats four daily injections of 5 mg/kg i.p. MDMA at 1-hour intervals for 2 consecutive days. Approximately 10–12 weeks later, rats were tested with a 1-hour delay between trials. Two “preliminary” days of testing were conducted followed by a third identical day of testing that provided the reported data. The discrimination ratio of MDMA-treated rats was significantly less than that of saline controls. In all, the findings from these two studies suggest that treatment with one dose of 5 mg/kg

TABLE 4
Studies examining the effects of MDMA on nonspatial learning and memory

Article	Task[a]	Subjects[b]	Doses/Frequency[c]	Timeline[d]	Effects[e]
Ros-Simó et al., 2013	NOR	Mice (CD1), Adol., Male	20 mg/kg (i.p.) × 2	Training: Predrug, Off-Drug[2] / Testing: Postdrug *[72 h delay]*	↓
Shortall et al., 2013	NOR	Rats (LH), Adult, Male	10 mg/kg (i.p.) × 1/day, 2 days	Training/Testing: On-Drug *[2 h delay]*	↓
Nawata et al., 2010	NOR	Mice (CD1), Adult, Male	10 mg/kg (i.p.) × 1 OR × 1/day, 7 days	Training/Testing: Postdrug *[3 h delay]*	⌀ (Single), ↓ (Repeated)
Edut et al., 2011	NOR	Mice (ICR), Adult, Male	10 mg/kg (i.p.) × 1	Training/Testing: Postdrug *[24 h delay]*	⌀ (7 days later), ↓ (30 days later)
Rodsiri et al., 2011	NOR	Rats (LH), Adult, Male	3 or 6 mg/kg (i.p.) × 3	Training/Testing: Postdrug *[2 h delay]*	⌀ (3 mg/kg), ↓ (6 mg/kg)
Piper et al., 2008	NOR	Rats (SD), Adult, Male	10 mg/kg (s.c.) × 4	Training/Testing: Postdrug *[15 min and 60 min delays]*	⌀
Able et al., 2006	NOR	Rats (SD), Adult, Male	15 mg/kg (s.c.) × 4	Training/Testing: Postdrug *[1 h delay]*	⌀
Cohen et al., 2005	NOR	Rats (SD), Adult, Male	15 mg/kg (s.c.) × 4	Training/Testing: Postdrug *[1 h delay]*	⌀
Skelton et al., 2008	NOR	Rats (SD), Adult, Male	15 mg/kg (s.c.) × 4 OR × 4/day, 1 day/wk, 5 wk	Training/Testing: Postdrug *[1 h delay]*	⌀
Morley et al., 2001	NOR	Rats (W), Adult, Male	5 mg/kg (i.p.) × 1/day, 2 days OR × 4/day, 2 days	Training/Testing: Postdrug *[15 min and 60 min delays]*	⌀ (Single; Repeated - 60 min delay) ↓ (Repeated - 15 min delay)
McGregor et al., 2003	NOR	Rats (W), Adult, Male	5 mg/kg (i.p.) × 4/day, 2 days	Training/Testing: Postdrug *[1 h delay]*	↓
Abad et al., 2014	NOR	Rats (SD), Adol., Male	20 mg/kg (s.c.) × 2/day, 4 days	Training/Testing: Postdrug *[1 h delay]*	⌀
van Nieuwenhuijzen et al., 2010	NOR	Rats (W), Adult, Male	5 mg/kg (i.p.) × 1/day, 10 days	Training/Testing: Postdrug *[1 h delay]*	↓
Kolyaduke and Hughes, 2013	NOR	Rats (PVG/c), Adol.→Adult, Male/Female	10 mg/kg (i.p.) × 1/day, 10 days	Training/Testing: Postdrug *[15 min delay]*	⌀
García-Pardo et al., 2017	NOR	Mice (OF1), Adol., Male	10 mg/kg (i.p.) × 1/day, 4 days (spaced)	Training/Testing: Postdrug *[1 min delay]*	⌀
Llorente-Berzal et al., 2013	NOR	Rats (W), Adol.→Adult, Male/Female	10 mg/kg (s.c.) × 2/day, 4 days (spaced)	Training/Testing: Postdrug *[4 h delay]*	⌀
Piper and Meyer, 2004	NOR	Rats (SD), Adol., Male	10 mg/kg (s.c.) × 2/day, 6 days (spaced)	Training/Testing: Postdrug *[15 min delay]*	↓
Piper et al., 2005	NOR	Rats (SD), Adol., Male	5 mg/kg (s.c.) × 4/day, 6 days (spaced)	Training/Testing: Postdrug *[15 min and 30 min delays]*	⌀
Clemens et al., 2007	NOR	Rats (W), Adult, Female	8 mg/kg (i.p.) × 1/wk, 16 wk	Training/Testing: Postdrug *[20 min delay]*	⌀
Costa et al., 2014	NOR	Mice (B6), Adol.→Adult, Male	10 mg/kg (i.p.) × 2/day, 2 days/wk (spaced), 9 wk	Training/Testing: Off-Drug[1], Postdrug *[1 h delay]*	⌀ (Off-Drug), ↓ (Postdrug)
Schulz et al., 2013	NOR	Rats (W), Adol./Adult, Male	7.5 mg/kg (s.c.) × 1/day (10 days) and 2/day (5 days), 15 days (spaced/randomized)	Training/Testing: Postdrug *[25 min delay]*	↓
Abad et al., 2016	NOR	Mice (B6), Adol.→Adult, Male	5 (2 wk) → 7.5 (3 wk) → 10 (3 wk) mg/kg (s.c.) × 3/day, 1 day/wk, 8 wk	Training/Testing: Postdrug *[1 h and 24 h delays]*	⌀ (1 h delay), ↓ (24 h delay)
Skelton et al., 2008	NPR	Rats (SD), Adult, Male	15 mg/kg (s.c.) × 4 OR × 4/day, 1 day/wk, 5 wk	Training/Testing: Postdrug *[1 h delay]*	⌀
Pompei et al., 2002	SR	Rats (SD), Adult, Male	1, 5, or 10 mg/kg (i.p.) × 1/day, 8 days	Training: Off-Drug[2] / Testing: On-Drug *[2 h delay]*	↑ (1 and 5 mg/kg), ⌀ (10 mg/kg)
Able et al., 2006	CWM	Rats (SD), Adult, Male	15 mg/kg (s.c.) × 4	Testing: Postdrug	↓
Skelton et al., 2008	CWM	Rats (SD), Adult, Male	15 mg/kg (s.c.) × 4 OR × 4/day, 1 day/wk, 5 wk	Testing: Postdrug	⌀
Vorhees et al., 2011	CWM	Rats (SD), Adult, Male	15 mg/kg (s.c.) × 4	Testing: Postdrug	⌀

[a]Studies used the novel object recognition (NOR), novel place recognition (NPR), social recognition (SR), and Cincinnati water maze (CWM) tasks.
[b]Species (strain), age, and sex of subjects. Strains include Lister Hooded (LH), PVG/c hooded, Sprague-Dawley (SD), and Wistar (W) rats; and C57BL/6 (B6), CD1, ICR, and OF1 mice.
[c]Dose, route, and frequency of MDMA administration. Treatment days/weeks are consecutive unless noted as "spaced."
[d]When training and testing occurred in relation to drug treatment, and duration of *delay* period between training and testing for recognition tasks. Pre- and post-drug training/testing were always conducted off-drug.
[e]Effects of drug treatment on nonspatial learning (CWM) and recognition memory (NOR, NPR, SR): ⌀ No Effect, ↓ Impairment, ↑ Enhancement.
[1]Off-drug training/testing took place on day(s) without drug administration.
[2]Off-drug training/testing took place immediately before drug administration on (last) day of treatment.

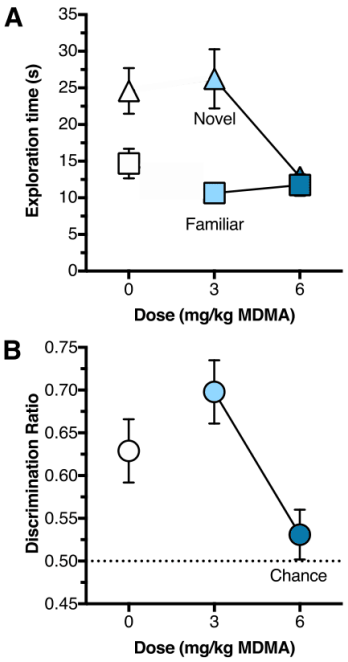

Fig. 7. Dose-dependent effects of MDMA on novel object recognition. Pretreatment with three doses of 3 mg/kg MDMA had no effect on exploration time of the novel and familiar objects during the test trial (A) or the discrimination ratio [novel/(novel+familiar)] (B), while pretreatment with three doses of 6 mg/kg MDMA significantly decreased exploration time of the novel object (A) and the discrimination ratio (B). Data redrawn with permission from Fig. 3, A and C in Rodsiri et al. (2011).

MDMA for 2 consecutive days has no subsequent effect on short-term recognition memory, while treatment with four doses of 5 mg/kg MDMA for 2 consecutive days may impair short-term recognition memory (with the exception of Morley et al.'s findings at the 60-minute delay).

Abad et al. (2014) gave adolescent male Sprague-Dawley rats two daily injections of 20 mg/kg s.c. MDMA for 4 consecutive days. Rats were tested 1 week later with a 1-hour delay between trials. MDMA-treated rats and saline controls explored the novel object for significantly more percent of the total exploration time than the familiar object. These findings indicate that pretreatment with repeated doses of 20 mg/kg MDMA may have no subsequent effect on short-term recognition memory.

In addition to testing a single dose of MDMA, Nawata et al. (2010) gave another group of adult male CD1 mice a daily injection of 10 mg/kg i.p. MDMA for 1 week. These mice were also tested 1 or 7 days later with a 3-hour delay between trials. The discrimination index of MDMA-treated mice was significantly less than that of saline controls at 1 and 7 days posttreatment; however this value was significantly above chance at 1 day posttreatment. Although this study found that pretreatment with a single dose of 10 mg/kg MDMA had no effect on long-term recognition memory (see above), these additional findings suggest that pretreatment with repeated doses of 10 mg/kg MDMA may lead to long-term recognition memory deficits, with more pronounced deficits 1 week after treatment versus 1 day.

van Nieuwenhuijzen et al. (2010) and Kolyaduke and Hughes (2013) treated rats with a daily injection of MDMA for 10 consecutive days. van Nieuwenhuijzen et al. treated adult male Wistar rats with a daily dose of 5 mg/kg i.p. MDMA. Rats were tested 6 weeks after MDMA treatment with a 1-hour delay between trials. The discrimination ratio of MDMA-treated rats was significantly less than that of saline controls. Kolyaduke and Hughes treated male and female adolescent PVG/c hooded rats with a higher daily dose of 10 mg/kg i.p. MDMA during early adolescence (postnatal days 35–45) or late adolescence (postnatal days 45–55). Both groups were tested as adults at no less than 90 days old (around 5–8 weeks postdrug) with a short 15-minute delay between trials. The exploratory behavior of MDMA-treated rats and saline controls during the test trial led to similar discrimination indexes. The findings from these two studies demonstrate that pretreatment with repeated doses of MDMA may lead to short-term recognition memory impairments at doses of 5 mg/kg but surprisingly may have no effect at doses of 10 mg/kg. This unexpected outcome may be because van Nieuwenhuijzen et al. and Kolyaduke and Hughes tested rats from different strains, ages, and sexes.

García-Pardo et al. (2017) gave adolescent male OF1 mice four injections of 10 mg/kg i.p. MDMA over 2 weeks, one on each of postnatal days 55, 57, 60, and 62. Testing took place on postnatal day 64, 2 days after MDMA treatment, with an ultra-short 1-minute delay between trials. The discrimination indexes of MDMA-treated mice and saline controls did not significantly differ, suggesting that pretreatment with four doses of 10 mg/kg MDMA may have no later effect on short-term recognition memory.

The following group of studies treated adolescent rats with MDMA every 5 days (for a specific number of total days), with multiple injections given on each treatment day. Llorente-Berzal et al. (2013) gave male and female Wistar rats two injections of 10 mg/kg s.c. MDMA at a 4-hour interval every 5 days from postnatal day 30–45. Rats were tested 1 month later as adults on postnatal day 75 with a 4-hour delay between trials. There were no significant differences between the discrimination indexes of MDMA-treated rats and saline controls. These findings demonstrate that pretreatment with repeated doses of 10 mg/kg has no subsequent effect on long-term recognition memory. Piper and Meyer (2004) gave male Sprague-Dawley rats two injections of 10 mg/kg s.c. MDMA at a 4-hour interval every 5 days from postnatal day 35 to 60. Rats were tested 1 week later with a 15-minute delay between trials. The

discrimination ratio of MDMA-treated rats was significantly less than that of saline controls. Piper et al. (2005) gave male Sprague-Dawley rats four injections of 5 mg/kg s.c. MDMA at 1-hour intervals every 5 days from postnatal day 35 to 60. Rats were tested 1 week later (postnatal day 67) with a 15-minute delay and again 1 to 2 days later (postnatal day 68 or 69) with a 30-minute delay. There were no significant differences between the discrimination ratios of MDMA-treated rats and saline controls under either delay condition. The findings from these two studies are mixed; the results of Piper et al. suggest that pretreatment with repeated doses of 5 mg/kg MDMA has no subsequent effect on short-term recognition memory, while the findings of Piper and Meyer suggest that pretreatment with repeated doses of 10 mg/kg produces short-term recognition memory deficits. Although the same cumulative daily doses were given, the difference in number and dose of injections (two daily injections of 10 mg/kg vs. four daily injections of 5 mg/kg) could account for this discrepancy in findings.

The next group of studies treated animals with MDMA over several weeks. Clemens et al. (2007) gave adult female Wistar rats a single injection of 8 mg/kg i.p. MDMA once weekly for 16 weeks. Two days of testing were performed 8 weeks after MDMA treatment (with 1 day between the 2 days), and a 20-minute delay was used for both tests. The discrimination ratio of MDMA-treated rats and saline controls did not significantly differ during either test. In addition to testing the effects of multiple MDMA injections on a single day (see above), Skelton et al. (2008) gave another group of adult male Sprague-Dawley rats the same treatment of four injections of 15 mg/kg s.c. MDMA at 2-hour intervals once weekly for 5 weeks. Again, rats were tested 5 weeks after the MDMA treatment with a 1-hour delay period. MDMA-treated rats and saline controls explored the novel object more than the familiar object, and there were no significant differences between groups. Costa et al. (2014) gave male C57BL/6 mice two injections of 10 mg/kg i.p. MDMA at a 4- to 6-hour interval on the 2nd and 5th days of each week for 9 weeks (which started in adolescence and continued into adulthood). Mice completed a total of 5 days of testing—on the 6th day of drug treatment weeks 1, 4, and 9 and posttreatment weeks 2 and 3. A 1-hour delay was used for all five tests. The discrimination ratio of MDMA-treated mice and saline controls did not significantly differ during drug treatment weeks 1, 4, or 9 but was significantly reduced in MDMA-treated mice compared with saline controls during posttreatment weeks 2 and 3. The findings of Clemens et al. and Skelton et al. suggest that pretreatment with repeated doses of 8 or 15 mg/kg MDMA has no subsequent effect on short-term recognition memory. On the other hand, the findings of Costa et al., suggest that pretreatment with repeated doses of 10 mg/kg MDMA has no effect on short-term recognition memory 1 day posttreatment but produces impairments by 2 weeks posttreatment. This discrepancy may be because Costa et al. used mice as subjects rather than rats, or possibly because the mice were tested repeatedly throughout drug treatment.

Schulz et al. (2013) treated adolescent and adult male Wistar rats with a varying number of s.c. MDMA injections over 25 days. A single injection of 7.5 mg/kg MDMA was given on 10 of the 25 days, two injections of 7.5 mg/kg MDMA were given at a 4-hour interval on 5 of the 25 days, and no drug was given on 10 of the 25 days (treatment schedule was randomized). All rats were tested 10 days after the 25-day treatment period, and the adolescent rats were tested again as adults 6 weeks after the first test. Unlike the other NOR studies reviewed here, only one object was presented during the training trial, but the remainder of the methods were as described above. A 25-minute delay was placed between the training and test trials. The adult saline controls explored the familiar object significantly less in the test trial than the same object in the training trial, and significantly less than the novel object in the test trial. The adolescent saline controls, however, explored all three objects for a comparable amount of time during the first test. Because the adolescent saline controls did not exhibit normal recognition memory, the effects of MDMA cannot be accurately determined. During the second test, the adolescent saline controls explored the familiar object significantly less in the test trial than the same object in the training trial (but not significantly less than the novel object in the test trial). The adult and adolescent MDMA-treated rats explored all three objects for comparable amounts of time during all tests. These results suggest that pretreatment with repeated doses of 7.5 mg/kg MDMA during adulthood impairs short-term recognition memory, and the same treatment during adolescence may produce some deficits as in adults (but the effects during adolescence cannot be determined).

Abad et al. (2016) gave adolescent male C57BL/6 mice three injections of s.c. MDMA at 1-hour intervals once weekly for 8 weeks—at doses of 5 mg/kg MDMA for the first 2 weeks, 7.5 mg/kg MDMA for the next 3 weeks, and 10 mg/kg MDMA for the last 3 weeks. Mice were tested as adults, 1 week and 3 months after MDMA treatment with 1- and 24-hour delays. The discrimination indexes of MDMA-treated mice and saline controls did not significantly differ with a 1-hour delay but was significantly reduced in MDMA-treated mice compared with saline controls with a 24-hour delay at both 1 week and 3 months posttreatment. These findings suggest that pretreatment with repeated doses of MDMA (increasing from 5 to 10 mg/kg) has no subsequent effect on short-term recognition memory but may lead to long-term recognition memory deficits.

a. Novel object recognition variations. Pompei et al. (2002) and Skelton et al. (2008) tested the effects of MDMA on a novel place recognition (NPR) test and a

social recognition (SR) test, respectively, and these studies are outlined in Table 4. The procedures of these tests are similar to the NOR test, but the NPR test assesses recognition memory of an object's orientation and the SR test assesses recognition memory of another animal. In the NPR test, the two objects presented in the training trial are identical to those presented in the test trial, but in the test trial one object is placed 90° clockwise compared with its location in the training trial. The exploration time of each object is recorded during both trials, and recognition memory is revealed by significantly less exploration of the non-rotated object compared with the rotated object in the test trial or either object in the training trial. In the SR test, a juvenile rat is introduced into the cage of an adult male rat (the test subject) in the training trial, and the same juvenile rat is reintroduced into the cage of the adult in the test trial. The time that the adult rat spends exploring the juvenile rat (i.e., nosing, sniffing, grooming, pawing, or close following) is recorded during both trials. A decrease in exploration time from the training trial to the test trial reflects recognition memory of the juvenile rat.

Skelton et al. (2008) gave adult male Sprague-Dawley rats four injections of 15 mg/kg s.c. MDMA at 2-hour intervals on a single day or once weekly for 5 weeks. Testing took place 40 days after MDMA treatment with a 1-hour delay between trials. MDMA-treated rats did not significantly differ from saline controls on any measure of object exploration. This suggests that pretreatment with repeated doses of 15 mg/kg MDMA has no later effect on short-term recognition memory.

Pompei et al. (2002) gave adult male Sprague-Dawley rats a daily injection of 1, 5, or 10 mg/kg i.p. MDMA for 8 consecutive days. The SR test took place on the 8th day of MDMA treatment. Rats were given their final MDMA injection immediately after the training trial and tested after a 120-minute delay. All groups explored the juvenile rat less in the test trial than in the training trial. This decrease in exploration time was significantly enhanced in rats given 1 or 5 mg/kg MDMA injections compared with saline controls and did not significantly differ between rats given 10 mg/kg MDMA injections and saline controls. These findings reveal that pretreatment with repeated doses MDMA may enhance short-term recognition memory at doses of 1 or 5 mg/kg MDMA (although the authors' conclusions are inconsistent with their graphical data) and may have no effect on short-term recognition memory at a dose of 10 mg/kg MDMA when memory consolidation and retrieval occur on-drug.

2. Other Nonspatial Tasks.

a. Cincinnati water maze. The Cincinnati water maze (CWM) task (Vorhees, 1987) is a nonspatial variation of the MWM task. The CWM is a 9-unit multiple T-maze that is filled with water. Animals are required to swim through the maze to escape onto a hidden platform. The maze is configured so that the path to the goal runs along only the long arms of each T. Testing is performed under red light or complete darkness to limit or eliminate the use of distal visual cues, and therefore animals must rely on egocentric cues to navigate to the hidden platform. Typically, each animal completes two trials per day for several days. The starting location of the animal and the platform location remain constant over all trials and days. The escape latency (i.e., time taken to reach the hidden platform) and number of errors (i.e., entries into one of the short arms of a T) are recorded during all trials. A decrease in escape latency/number of errors over the days of testing reflects nonspatial learning of the platform location.

Three studies assessed the effects of MDMA on the CWM task, which are listed in Table 4. Prior to testing, Able et al. (2006), Skelton et al. (2008), and Vorhees et al. (2011) all gave adult male Sprague-Dawley rats four injections of 15 mg/kg s.c. MDMA at 2-hour intervals on a single day, and Skelton et al. gave another group of rats this same treatment once weekly for 5 weeks. Able et al. began testing 4 days after MDMA treatment and tested rats for a total of 6 days. The rate at which the number of errors and escape latency of MDMA-treated rats decreased over the 6 testing days was slower than that of saline controls. Specifically, MDMA-treated rats made significantly more errors than saline controls on days 4 and 5, and a trend toward significantly more errors on day 6. Skelton et al. began testing 1 week after MDMA treatment and tested rats for a total of 6 days. While the average number of errors and the average escape latency of MDMA-treated rats (both single day and weekly) were significantly higher than saline controls, these measures decreased at a similar rate over the 6 testing days in all three groups. Both groups of MDMA-treated rats therefore exhibited performance impairments but not learning impairments. Vorhees et al. began testing 2 weeks after MDMA treatment and tested rats for a total of 21 days. The number of errors and escape latency of MDMA-treated rats and saline controls decreased at a similar rate over the 21 testing days, and the overall average number of errors and average escape latency also did not significantly differ between groups. The findings from these three studies reveal pretreatment with repeated doses of 15 mg/kg MDMA has no effect on nonspatial learning when tested 1 week or more after treatment but produces nonspatial learning impairments when testing begins less than 1 week after treatment.

E. Fear-Motivated Learning and Memory

1. Passive Avoidance. The passive avoidance (PA) task is a fear-motivated task that is used to evaluate learning and memory in rodents. A common version of this task is the step-through PA task (Jarvik and Kopp, 1967), which takes place in a two-compartment chamber consisting of one bright (e.g., illuminated, white

walls) compartment and one dark (e.g., nonilluminated, black walls) compartment connected by a guillotine door. The task requires animals to inhibit their natural tendency to prefer dark areas/avoid bright areas to avoid an aversive stimulus. Each animal is first habituated to both compartments of the chamber as well as crossing through the guillotine door prior to training. Training is usually completed in a single trial, which begins by placing the animal in the bright compartment with the guillotine door closed. After a brief period, the guillotine door is opened, and once the animal enters the dark component, the guillotine door is closed, and the animal receives an inescapable foot shock. Testing typically takes place 24 hours after training, during which the animal is returned to the bright compartment, and again the guillotine door is opened after a brief period. If the animal remembers that entering the dark compartment lead to a foot shock during training, then the animal will inhibit its natural tendency to enter the dark compartment.

The step-through latency (i.e., time taken to enter the dark compartment once the guillotine door is opened) is measured during both the training and test trials, and the cutoff time/maximum latency recorded is usually 300 seconds. A significant increase in step-through latency from training to testing reflects normal memory retention, whereas the lack of this increase reflects memory deficits. A significantly lower step-through latency relative to normal during testing is also an indicator of memory deficits. The type of memory measured here involves both explicit memory (i.e., association with the context) and implicit memory (i.e., operant conditioning to the shock).

Table 5 lists the 12 studies that explored the effects of MDMA on the PA task. Eleven of these studies conducted the step-through PA task, whereas only one study (McNamara et al., 1995) performed another version, the step-down PA task (methods described below). The animals from most of these studies were treated with MDMA 1 or more days prior training, 30 minutes before training, and/or immediately after training. All of the studies evaluated long-term memory as delays of 24 hours or more were placed between the training and test trials.

Moyano et al. (2004, 2005) and Barrionuevo et al. (2000) all tested the effects of on-drug training. Adult male Wistar rats were given a single injection of MDMA 30 minutes before training and then tested 24 hours later. Moyano et al. (2004, 2005) found that rats injected with 10 mg/kg i.p. MDMA before training exhibited a significantly lower step-through latency than saline controls during testing, and Barrionuevo et al. (2000) found the same results with a dose of 20 mg/kg i.p. MDMA. These findings suggest that doses of 10 and 20 mg/kg MDMA produce long-term memory deficits when memory acquisition occurs on-drug.

Shariati et al. (2014) and Budzynska et al. (2017) explored the effects of administering the drug immediately after training. Figure 8 exhibits the findings of Budzynska et al., which exemplify the dose-dependent effects of MDMA on fear-motivated learning and memory. Budzynska et al. gave adult male Swiss Webster mice a single injection of 1, 2.5, 5, or 10 mg/kg i.p. MDMA immediately after training and tested the mice 24 hours later. The step-through latency of mice treated with 1 or 10 mg/kg MDMA did not significantly differ from that of saline controls, while mice treated with 2.5 or 5 mg/kg MDMA showed a significantly higher step-through latency than saline controls. Shariati et al. tested two groups of adult male Wistar rats—one group received a single injection of 10 mg/kg i.p. MDMA following two training trials, and another group received a daily injection of 10 mg/kg i.p. MDMA on 2 consecutive days per week for 3 weeks, with the last injection administered immediately following training. All rats were tested 24 hours after MDMA treatment, and both groups of MDMA-treated rats demonstrated a significantly shorter step-through latency than saline controls. Together, the above results indicate that administering MDMA immediately after memory acquisition has no effect on long-term memory retention at a dose of 1 mg/kg but enhances long-term memory retention at doses of 2.5 or 5 mg/kg. The findings regarding a dose of 10 mg/kg MDMA are mixed, as Budzynska et al. found that this dose has no effect on long-term memory retention, while Shariati et al. found that single or repeated administration of this dose impairs long-term memory retention. This discrepancy could be due to the use of different species (mice vs. rats) or the number of training trials (one vs. two).

Jahanshahi et al. (2013) also treated young adult male Wistar rats with MDMA between training and testing, but the MDMA treatment began 24 hours after two training trials and lasted for 4 weeks. Rats were given three injections of 2.5, 5, or 10 mg/kg i.p) MDMA at 3-hour intervals once weekly for 4 weeks. Testing took place following drug treatment, and all three groups of MDMA-treated rats exhibited a significantly longer step-through latency than saline controls. These results reveal that treatment with doses of 2.5, 5, and 10 mg/kg MDMA after acquisition may enhance long-term memory retention.

The next group of studies treated animals with a specific MDMA regimen prior to training and testing. Timár et al. (2003) and Murnane et al. (2012) gave animals four injections of MDMA at 2-hour intervals. Timár et al. gave adolescent male Wistar rats doses of 10 mg/kg s.c. MDMA and tested them 3 days and 4 weeks after MDMA treatment, with 48 hours separating training and testing. During both tests, MDMA-treated rats and saline controls did not significantly differ in step-through latency. Murnane et al. gave adolescent male Swiss Webster mice doses of 10 or 20 mg/kg i.p. MDMA. Rats were trained 2 days after MDMA treatment and tested 2 days after training. Again, MDMA-treated rats

TABLE 5
Studies examining the effects of MDMA on fear-motivated learning and memory

Article	Task[a]	Subjects[b]	Doses/Frequency[c]	Timeline[d]	Effects[e]
Moyano et al., 2004	PA	Rats (W), Adult, Male	10 mg/kg (i.p.) × 1	Training: On-Drug Testing: Postdrug	↓
Moyano et al., 2005	PA	Rats (W), Adult, Male	10 mg/kg (i.p.) × 1 AND/OR × 2/day, 4 days	Training: On-Drug (Single), Postdrug (Repeated) Testing: Postdrug	↓ (Single) ⌀ (Repeated)
Barrionuevo et al., 2000	PA	Rats (W), Adult, Male	20 mg/kg (i.p.) × 1	Training: On-Drug Testing: Postdrug	↓
Budzynska et al., 2017	PA	Mice (SW), Adult, Male	1, 2.5, 5, or 10 mg/kg (i.p.) × 1	Training: Off-Drug[2] Testing: Postdrug	↑ (2.5 and 5 mg/kg) ⌀ (1 and 10 mg/kg)
Shariati et al., 2014	PA	Rats (W), Adult, Male	10 mg/kg (i.p.) × 1 OR × 1/day, 2 day/wk, 3 wk	Training: Off-Drug[2] Testing: Postdrug	↓
Jahanshahi et al., 2013	PA	Rats (W), Adult, Male	2.5, 5, or 10 mg/kg (i.p.) × 3/day, 1 day/wk, 4 wk	Training: Predrug Testing: Postdrug	↑
Timár et al., 2003	PA	Rats (W), Adol., Male	10 mg/kg (s.c.) × 4	Training/Testing: Postdrug	⌀
Murnane et al., 2012	PA	Mice (SW), Adol., Male	10 or 20 mg/kg (i.p.) × 4	Training/Testing: Postdrug	⌀
García-Pardo et al., 2015	PA	Mice (OF1), Adol., Male	10 mg/kg (i.p.) × 1/day, 4 days (spaced)	Training/Testing: Postdrug	↓
García-Pardo et al., 2017	PA	Mice (OF1), Adol., Male	10 mg/kg (i.p.) × 1/day, 4 days (spaced)	Training/Testing: Postdrug	↓
Rodríguez-Arias et al., 2011	PA	Mice (OF1), Adol., Male	10 or 20 mg/kg (i.p.) × 2/day, 2 days/wk, 2 wk	Training/Testing: Postdrug	⌀
McNamara et al., 1995	PA	Rats (SD), Adult, Male	5, 10, or 20 mg/kg (i.p.) × 2/day, 4 days	Testing: Postdrug	⌀
Shortall et al., 2013	FC	Rats (LH), Adult, Male	10 mg/kg (i.p.) × 1/day, 3 days (spaced)	Training: Off-Drug[2] Testing: Postdrug	⌀
Johansson et al., 2015	FC	Mice (ICR), Adult, Male	20 mg/kg (i.p.) × 2	Training/Testing: Postdrug	↓

[a]Studies used the passive avoidance (PA) and contextual fear conditioning (FC) tasks.
[b]Species (strain), age, and sex of subjects. Strains include Lister Hooded (LH), Sprague-Dawley (SD), and Wistar (W) rats; and ICR, OF1, and Swiss Webster (SW) mice.
[c]Dose, route, and frequency of MDMA administration. Treatment days/weeks are consecutive unless noted as "spaced."
[d]When training and testing occurred in relation to drug treatment. Pre- and post-drug training/testing were always conducted off-drug.
[e]Effects of drug treatment on learning and memory: ⌀ No Effect, ↓ Impairment, ↑ Enhancement.
[2]Off-drug training/testing took place immediately before drug administration on (last) day of treatment.

and saline controls did not significantly differ in step-through latency. In all, these studies reveal that pretreatment with four doses of 10 or 20 mg/kg MDMA has no subsequent effect on long-term memory retention.

In addition to testing the effects of on-drug training (see above), Moyano et al. (2005) gave another group of adult male Wistar rats two daily injections of 10 mg/kg i.p. MDMA for 4 consecutive days. Rats were trained 1 week after MDMA treatment and tested 24 hours after training. The step-through latency of MDMA-treated rats and saline controls did not significantly differ. Moyano et al. tested an additional group, which received both of the previously described treatments, two daily injections of 10 mg/kg i.p. MDMA for 4 consecutive days, 1 week prior to training, and a single injection of 10 mg/kg i.p. MDMA, 30 minutes before training. This group of MDMA-treated rats exhibited a significantly slower step-through latency than saline controls. Together these findings suggest that pretreatment with repeated doses of 10 mg/kg MDMA has no subsequent effect on long-term memory retention, while a single dose of 10 mg/kg MDMA impairs long-term memory retention when memory acquisition occurs on-drug.

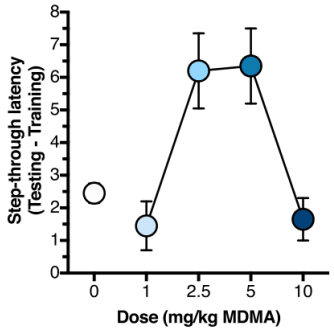

Fig. 8. Dose-dependent effects of MDMA on passive avoidance. Posttraining doses of 2.5 and 5 mg/kg MDMA enhanced long-term memory, while posttraining doses of 1 and 10 mg/kg MDMA had no effect on long-term memory. Data redrawn with permission from Fig. 5 in Budzynska et al. (2017).

García-Pardo et al. (2015, 2017) gave adolescent male OF1 mice a single injection of 10 mg/kg i.p. MDMA on four alternating days (2 to 3 days between each injection). García-Pardo et al. (2015) trained their mice 4 days after MDMA treatment and García-Pardo et al. (2017) trained their mice 5 days after MDMA treatment, and all mice were tested at 24 hours and 1 week after training. Both studies had identical findings—the step-through latency of MDMA-treated mice did not change significantly from training to testing (24 hours and 1 week later), and the step-through latency of MDMA-treated mice was significantly shorter than that of saline controls at 1 week after training (but not at 24 hours). These findings suggest that pretreatment with four doses of 10 mg/kg MDMA leads to long-term memory impairments that are more significant at 1 week versus 24 hours after acquisition.

Rodríguez-Arias et al. (2011) gave adolescent male OF1 mice two daily injections of 10 or 20 mg/kg i.p. MDMA at a 4-hour interval on 2 consecutive days per

week for 2 weeks. Mice were trained 22 days after MDMA treatment and tested 24 hours later. The step-through latency of MDMA-treated mice and saline controls did not significantly differ, revealing that pretreatment with repeated doses of 10 or 20 mg/kg MDMA may have no subsequent effect on long-term memory.

Unique to the other studies reviewed here, McNamara et al. (1995) tested adult male Sprague-Dawley rats on the step-down version of the PA task. Rats were given two daily injections of 5, 10, or 20 mg/kg i.p. MDMA at a 12-hour interval for 4 consecutive days and tested 6 days later. On each trial, a rat was placed on a triangular platform that was mounted above a grid floor. When the rat stepped off the platform, it received a foot shock. Repeated trials were conducted until the rat remained on the platform for at least 2 minutes. MDMA-treated rats and saline controls did not significantly differ in the number of trials it took for them to reach this threshold, suggesting that pretreatment with repeated doses of 5, 10, or 20 mg/kg MDMA has no effect on memory function.

2. Contextual Fear Conditioning. The contextual fear conditioning (FC) paradigm (Fanselow, 1986; Anagnostaras et al., 1999, 2010, 2015) is an efficient model to measure hippocampal-dependent learning and memory in rodents. In contextual FC, an animal learns to associate an aversive stimulus (typically a foot shock) with a specific context. As a result, the initially neutral context elicits a fear response in the animal. In rodents, this fear response arises as freezing behavior, which is a measure of contextual fear memory. Thus a significant decrease in freezing is indicative of memory deficits. Two studies explored the effects of MDMA on contextual FC and are outlined in Table 5. These studies used two different variations of the typical FC procedure, which are described below.

Shortall et al. (2013) conducted a "conditioned emotional response" task, which is a variation of the contextual FC task. The task took place in a two-compartment box that consisted of a dark side and a light side separated by a computer-operated door. For training, each animal was placed on the light side of the box, and after 30 seconds the door was opened. When the animal entered the dark side of the box, the door was closed, and the animal was subject to two light/tone and foot-shock pairings (a 5-second light and tone cue that coterminated with a 1-second foot shock) with a 1-minute interval between pairings. For testing, each animal was returned to the dark side of the box, and freezing was measured for 5-minute without any light/tone or foot shock presentation. In this study, young adult male Lister Hooded rats were given a single injection of 10 mg/kg i.p. MDMA on experiment days 1, 2, and 8. Rats were trained on experiment day 8 immediately prior to MDMA treatment and tested 24 hours later. MDMA-treated rats and saline controls did not significantly differ in freezing time during the test. These results suggest that treatment with repeated doses of 10 mg/kg MDMA prior to and following memory acquisition has no effect on long-term context memory.

Johansson et al. (2015) performed another variation of the contextual FC task, contextual fear discrimination. This task took place in two contexts, Context A and Context B, which differed by a variety of sensory modalities (different floor/walls, noise, illumination, and scent). Training took place in Context A, and each animal completed one training trial per day for 3 days. For each trial, the animal was introduced to Context A, and after a 3-minute baseline period they received a 2-second foot shock and then remained in the context for an additional 15 seconds. Testing began 3 days later, and each animal was exposed to both Context A and Context B on all 12 days of testing (random order of exposure with a 1.5- to 2-hour interval between each exposure). The trials in Context A were identical to training (3-minute baseline + 2-second foot shock + 15-second postshock period), and the trials in Context B were 3 minutes in duration with no foot shock. In this study, adult male ICR mice were given two injections of 20 mg/kg i.p. MDMA at a 2-hour interval, 4 days prior to training. The freezing behavior of saline controls increased significantly in Context A and decreased significantly in Context B over the 12 days of testing and overall was significantly greater in Context A than in Context B on the last 8 days of testing. Conversely, the freezing behavior of MDMA-treated rats remained constant over the 12 days of testing and did not significantly differ between Context A and Context B. These results suggest that pretreatment with two doses of 20 mg/kg MDMA leads to later deficits in learning to discriminate between two contexts.

VI. Analysis of Findings

This review includes a total of 90 experiments on the cognitive effects of MDMA in animals. Clearly, findings are mixed on whether MDMA impairs, enhances, or has no effect on cognition. Figure 9 depicts the breakdown of findings from all experiments reviewed here. Of the 90 total experiments, MDMA produced cognitive enhancements in one experiment, mixed parameter-dependent cognitive enhancements/no effects in three experiments, no cognitive effects in 46 experiments, mixed parameter-dependent impairments/no effects in 17 experiments, and cognitive impairments in 23 experiments.[2] MDMA produced cognitive impairments in

[2]All experiments in the current review were categorized by whether MDMA treatment produced: 1) impairments, 2) a mix of impairments and no effects, 3) no effects, 4) a mix of no effects and enhancements, 5) enhancements. The "mixed" categories (2 and 4) include experiments with findings that are inconsistent across different treatment and/or task parameters (e.g., MDMA dose, frequency of drug administration, experimental timeline, etc.). See Tables 1–5 ("Effects" columns) for examples of experiments with mixed findings.

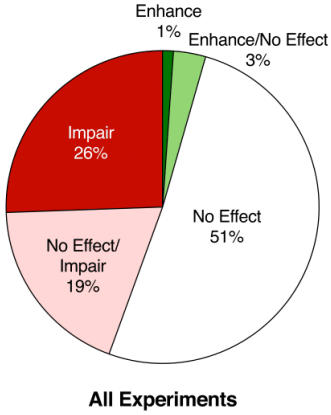

Fig. 9. Breakdown of findings from all 90 experiments. Most experiments (46 out of 90) found no effects of MDMA on cognition.

only 40 of the 90 experiments (44.4%), and in 17 of these experiments only certain parameters led to impairments. Thus MDMA did not influence cognition in the majority of these experiments, even when dose is ignored. MDMA did not produce any cognitive impairments in 50 of the 90 experiments (55.6%), and an additional 17 experiments showed negligible effects under certain parameters. Therefore, some negligible effects were found in 74.4% of all experiments. To better understand these findings, we further analyze the factors that may modulate the cognitive effects of MDMA.

A. Findings by Cognitive Domain

We first analyze the findings within each major section —attention (2 studies), working memory (23 studies), spatial learning and memory (24 studies), nonspatial learning and memory (27 studies), and fear-motivated learning and memory (14 studies). Figure 10 illustrates the breakdown of findings by cognitive domain.

The effects of MDMA on attention were examined in two studies on the 5-CSRT task (Table 1). Taffe et al. (2001) found that MDMA produced attention deficits on-drug but no effects postdrug, and Taffe et al. (2002) found that MDMA produced no effects postdrug. Therefore, it appears that MDMA produces attention deficits when on-drug but not following drug treatment. However, there are not enough studies to reach a definitive conclusion of these findings.

The effects of MDMA on working memory were examined in 23 studies using the DMS/DNMS, OST, SA/DA, RAM, or MWM tasks (Table 2). Of these 23 studies, 19 found no effects on working memory, three found no effects at doses of 1.25–3 mg/kg and working memory impairments at doses of 3–5 mg/kg, and one found working memory impairments only (Fig. 10A). Thus the majority of these studies found that MDMA treatment does not alter working memory. While Braida et al. (2002), Young et al. (2005), and Kay et al. (2010) found that doses of 3–5 mg/kg impair spatial working memory while on-drug, most of the studies with similar testing parameters found no effects on spatial working memory. In Wistar rats, the on-drug effects appear to be dose-dependent, as doses of 1–2.25 mg/kg had no effects but doses of 3–5 mg/kg impaired spatial working memory (Braida et al., 2002; Young et al., 2005). Marston et al. (1999) found that treatment with doses of 10–20 mg/kg leads to postdrug working memory impairments, but several other studies concluded that similar treatments lead to no postdrug effects. In all, it appears that MDMA generally has no on-drug or postdrug impact on working memory.

The effects of MDMA on spatial learning and memory were explored in 24 studies using the MWM, RAM, and SD tasks (Table 3). Of these 24 studies, one found spatial learning enhancements and no effect on spatial reference memory, eight found no effects on spatial learning and memory, six found a mix of no effects and spatial learning and memory impairments (impairments found with doses of 3–5.6 mg/kg but not 0.3–1.7 mg/kg, spatial reference memory but not spatial learning, or later postdrug testing), and nine found spatial learning and memory impairments only (Fig. 10B). Here, the slight majority of studies found impairments, but the true effect of MDMA on spatial learning and memory remains unclear. The effects of on-drug training and/or testing appear to be dose-dependent yet differ by strain. In Wistar rats, doses of 1.25–5 mg/kg had no effects (Young et al., 2005) and doses of 5–15 produced impairments (Taghizadeh et al., 2016). In Sprague-Dawley rats, doses of 0.3–1.7 mg/kg had no effects (Kay et al., 2010; Galizio et al., 2014) and doses of 3–5.6 mg/kg produced impairments (Kay et al., 2010, 2011; Harper et al., 2013; Galizio et al., 2014). The postdrug findings remain mixed, as there is evidence that highly similar/identical experimental designs produced dissimilar effects. In the MWM studies, spatial reference memory during the probe test appears to be more sensitive to impairment than spatial learning during acquisition. Overall, these findings reveal that the effects of MDMA on spatial learning and memory while on-drug may be dose-dependent but the postdrug effects are still unclear.

The effects of MDMA on nonspatial learning and memory were explored in 27 studies using the NOR, NPR, SR, and CWM tasks (Table 4). Of these 27 studies, one found nonspatial learning and memory enhancements at doses of 1 and 5 mg/kg and no effects at a dose of 10 mg/kg, 13 found no effects, 6 found a mix of no effects and nonspatial learning and memory impairments (impairments found with a dose of 6 mg/kg but not 3 mg/kg, more drug administrations, longer delay periods, or later postdrug testing), and 7 found nonspatial learning and memory impairments only

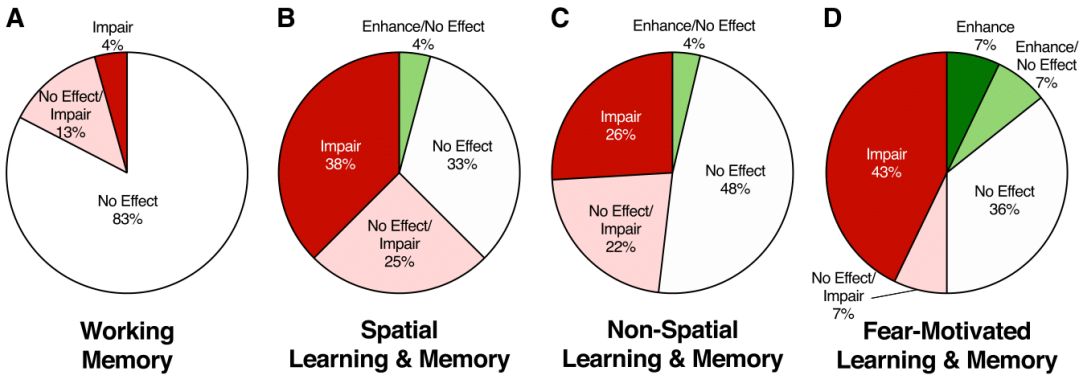

Fig. 10. Breakdown of findings from 23 working memory (A), 24 spatial learning and memory (B), 27 nonspatial learning and memory (C), and 14 fear-motivated learning and memory (D) experiments.

(Fig. 10C). Thus the majority of studies found no effects on nonspatial learning and memory. There appears to be no precise reason for the observed impairments, as studies with almost identical methods produced no effects in other cases. There does appear to be a lesser rate of impairments in Sprague-Dawley rats than in the other strain/species. In all, the evidence suggests that MDMA likely has no effect on nonspatial learning and memory, but the reasons for occasional impairments are ambiguous.

The effects of MDMA on fear-motivated learning and memory were examined in 14 studies using the PA and FC tasks (Table 5). Of these 14 studies, one found memory enhancements, one found memory enhancements at doses of 2.5 and 5 mg/kg and no effects at doses of 1 and 10 mg/kg, 5 found no effects, 1 found no effects with postdrug training and memory impairments with on-drug training, and 6 found memory impairments only (Fig. 10D). Here, on-drug training always impaired memory acquisition, but only high doses of 10–20 mg/kg were tested. Administration of MDMA between training and testing enhanced or had no effect on memory consolidation at doses of 2.5–10 mg/kg and impaired or had no effect on memory consolidation at doses of 10–20 mg/kg. Postdrug training and testing most often resulted in no effects. In all, the effects of MDMA on fear-motivated learning and memory are mixed but appear to be highly dependent on dose and when the drug is administered.

Overall, this review reveals that MDMA likely has no effect on working memory and nonspatial learning and memory and may or may not impair spatial learning and memory and fear-motivated learning and memory. The reasons for these ambiguous findings may be revealed through further analyses.

B. Findings by Dose

With respect to typical, occasional users of MDMA and its potential for therapeutic use, an examination of the impact of low, clinically and community-relevant dosing is essential. To examine the role of dose in the cognitive effects of MDMA, we divided all experiments into four groups by dose of MDMA administered—less than 3, 3–6, 7.5–10, and 15–30 mg/kg. Given the average human weight of 70 kg, these levels correspond to less than 210, 210–420, 525–700, and 1050–2100 mg. Of the studies reviewed here, 15 experiments administered doses of less than 3 mg/kg, 31 experiments administered doses of 3–6 mg/kg, 50 experiments administered doses of 7.5–10 mg/kg, and 31 experiments administered doses of 15–30 mg/kg (note: some experiments used a range of doses, and the totals above account for experiments that administered doses from multiple levels). Figure 11 illustrates the breakdown of findings by these dose categories. Of these it is important to note that only the lowest dose range (<3 mg/kg) seems to reflect the doses taken by most recreational MDMA users (i.e., 1 to 2 mg/kg), and it is likely that any potential therapeutic dosing would be even lower. Although there are several studies in this dose range, there are very few that examine microdosing (e.g., <1 mg/kg). At these doses, MDMA may have high therapeutic value and will almost certainly pose even less risk. Therefore, we suggest more studies, both human and animal, to examine MDMA at microdose ranges (e.g., <1 mg/kg).

Perhaps the most important finding from this review is that there is no evidence that doses below 3 mg/kg MDMA, the doses that people ordinarily take, produce cognitive impairments in animals, even when the animals are on-drug (Fig. 11A). Doses of 0.1, 0.3, 0.32, 0.75, 1.0, 1.25, 1.7, 1.75, 1.8, 2.0, and 2.25 mg/kg produced no effects on working memory when animals were tested on-drug on the DMS (LeSage et al., 1993; Frederick et al., 1995a,b; Harper et al., 2005), OST (Hawkey et al., 2014), DA (Young et al., 2005), RAM (Braida et al., 2002; Kay et al., 2010), or MWM (Galizio et al., 2014) tasks. Doses of 0.3, 0.75, 1.0, 1.25, 1.7, and 2.25 mg/kg produced no effects on spatial learning and memory when animals were tested on-drug on the

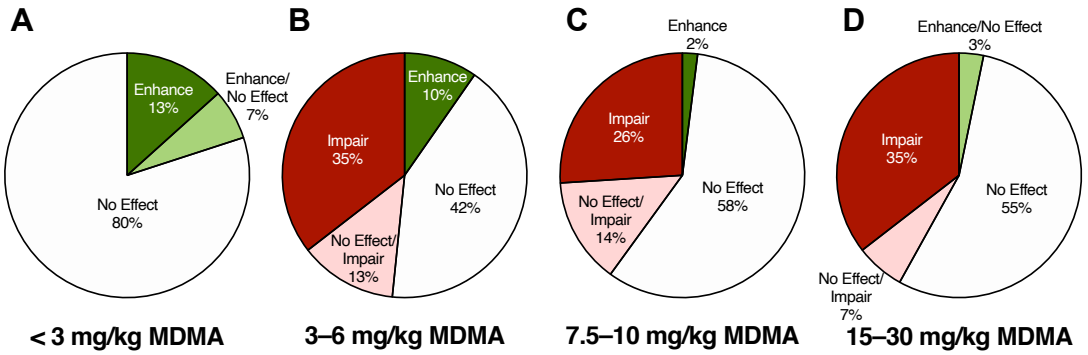

Fig. 11. Breakdown of findings from experiments that administered MDMA at doses of less than 3 mg/kg [(A); n = 15], 3–6 mg/kg [(B); n = 31], 7.5–10 mg/kg [(C); n = 50], and 15–30 mg/kg [(D); n = 31]. There is no evidence that MDMA produces cognitive impairments at doses below 3 mg/kg (A), and the evidence regarding doses of 3–30 mg/kg is mixed (B–D).

MWM (Galizio et al., 2014), RAM (Kay et al., 2010), or SD (Young et al., 2005) tasks. A dose of 1 mg/kg produced recognition memory enhancements when administered immediately after training / before on-drug testing on the SR task (Pompei et al., 2002). A dose of 1 mg/kg had no effect on memory and a dose of 2.5 mg/kg produced memory enhancements when administered between training and testing on the PA task (Jahanshahi et al., 2013; Budzynska et al., 2017). Evidently, doses of less than 3 mg/kg MDMA only led to no effects or memory enhancements in the studies reviewed here.

The majority of the studies reviewed here used unrealistically high MDMA doses of 3 mg/kg or greater. Doses of 10, 15, and 20 mg/kg were very common, as 69 of the 90 experiments studied the effects of one of these three doses. In an average human of 70 kg, 10 mg/kg is equivalent to 700 mg, 15 mg/kg is equivalent to 1050 mg, and 20 mg/kg is equivalent to 1400 mg. Given that the average human MDMA dose is about 1 to 2 mg/kg, these animal doses are 5–20 times greater than the doses taken by typical human users. Alternatively, this can be regarded as taking up to 20 MDMA tablets (each tablet = about 1 mg/kg) at one time, overdoses that would likely cause shock and alarm even among heavy users. In 48 of the 69 high-dose (≥3 mg/kg) experiments, doses of 10, 15, or 20 mg/kg were administered multiple (2–4) times per day. Twice daily administration of these doses is comparable to taking 20, 30, or 40 MDMA tablets in 1 day. Cohen et al. (2005), Able et al. (2006), Skelton et al. (2008), Vorhees et al. (2011), and Viñals et al. (2012), gave rodents extreme treatments of 60 mg/kg per day, which is equivalent to a human taking 4200 mg of MDMA or 60 MDMA tablets per day. The most extreme MDMA treatment was delivered by Murnane et al. (2012), who gave rodents a total of 80 mg/kg per day, which is equivalent to a human taking 5600 mg of MDMA or 80 MDMA tablets per day. These doses clearly do not reflect typical MDMA use in humans, and the validity of high-dose findings, outside of understanding very heavy users, should be of concern. It is somewhat misleading to portray the "typical" toxic effects of a drug based on what is essentially a 5–20× overdose. If those criteria were applied to other drugs, many existing therapeutics would be regarded as very unsafe, even lethal, including all of the statins, most antihypertensives, selective serotonin reuptake inhibitors, and even acetaminophen (paracetamol) (see Larson et al. (2005) for more about the high incidence and seriousness of acetaminophen overdose).

Regardless of the extreme MDMA treatments given to animal subjects, the findings on the cognitive effects of high-dose MDMA remains somewhat unconvincing. Of the 31 experiments that gave doses of 3–6 mg/kg, 11 found impairments, 4 found a mix of impairments and no effects depending on task parameters, 13 found no effects, and 3 found enhancements (Fig. 11B). Of the 50 experiments that gave doses of 7.5–10 mg/kg, 13 found impairments, seven found a mix of impairments and no effects depending on task parameters, 29 found no effects, and one found enhancements (Fig. 11C). Of the 31 experiments that gave doses of 15–30 mg/kg, 11 found impairments, 2 found a mix of impairments and no effects depending on task parameters, 17 found no effects, and 1 found a mix of no effects and enhancements depending on task parameters (Fig. 11D). Thus the administration of 3–30 mg/kg MDMA led to cognitive impairments in only less than half of experiments. Overall, the most compelling evidence of high-dose (≥3 mg/kg) MDMA-induced impairments is in spatial reference memory (assessed via the MWM probe and the RAM) and fear-motivated memory acquisition (assessed via on-drug PA training); high doses did not consistently lead to impairments in any other cognitive domain (e.g., nonspatial learning and memory).

In all, we found no evidence that low, clinically and community-relevant doses of MDMA (<3 mg/kg) produce cognitive impairments in animals. The findings

Note: Fig. 11 has been replaced to correct an error in the original publication. Fig. 11 as above reflects the *Erratum* in *Pharmacological Reviews*, **73**, 729.

regarding higher doses (≥3 mg/kg) are mixed yet led to cognitive impairments in less than half of experiments, which were primarily in the cognitive domains of spatial and fear-motivated learning and memory. Across all experiments, we did not find differences in effects based on route or frequency of administration. While heavy MDMA users, which account for only a small fraction of users, may use potentially memory-impairing doses (≥3 mg/kg), typical recreational and therapeutic doses lie below this range and did not produce cognitive deficits in any animal study.

C. Findings by When the Drug Was Administered

Here, we consider the effects of MDMA on learning and memory (all experiments except those on attention or working memory) with respect to when the drug was administered. Findings are categorized by whether MDMA was administered during training, between training and testing, during testing, or entirely prior to training and testing.

The effects of MDMA on memory acquisition are determined by on-drug training. Findings from the five experiments that conducted on-drug training (and then off-drug testing) reveal a clear dose-dependent effect of MDMA on memory acquisition. Doses of 0.3, 1, and 1.7 mg/kg had no effect on memory acquisition (Galizio et al., 2014), while doses of 3–20 mg/kg impaired memory acquisition (Barrionuevo et al., 2000; Moyano et al., 2004, 2005; Galizio et al., 2014; Taghizadeh et al., 2016).

The effects of MDMA on memory consolidation are determined by administering the drug between training and testing (typically immediately after training). Findings from the six experiments that administered MDMA after training yet before off-drug testing again present a dose-dependent effect on memory consolidation. Doses of 2.5, 5, and 10 mg/kg enhanced memory consolidation (Jahanshahi et al., 2013; Budzynska et al., 2017), while doses of 1 and 10 mg/kg also had no effect on memory consolidation (Shortall et al., 2013; Budzynska et al., 2017). Higher doses of 10 and 20 mg/kg impaired memory consolidation (Ros-Simó et al., 2013; Shariati et al., 2014).

The effects of MDMA on memory retrieval are determined by on-drug testing. Findings from the four experiments that conducted on-drug testing (but off-drug training) again exhibit a dose-dependent effect of MDMA on memory retrieval. Doses of 0.75, 1.25, 2.25, and 5 mg/kg had no effect on memory retrieval (Young et al., 2005; Kay et al., 2010), while doses of 3 and 4 mg/kg impaired memory retrieval (Kay et al., 2010; Kay et al., 2011; Harper et al., 2013). Different rat strains (Sprague-Dawley vs. Wistar) may account for the contradictory effects of doses in the 3–5 mg/kg range (specifically, the 5 mg/kg outlier).

Pompei et al. (2002) administered MDMA immediately after training, and testing took place 2 hours later on-drug. In this design, both memory consolidation and retrieval could be influenced by MDMA. Doses of 1 and 5 mg/kg enhanced memory consolidation/retrieval, while a dose of 10 mg/kg had no effect on memory consolidation/retrieval. Additionally, Shortall et al. (2013) administered MDMA before training and conducted testing 2 hours later so both training and testing occurred on-drug. In this case, MDMA could influence memory acquisition, consolidation, and retrieval. A dose of 10 mg/kg impaired memory acquisition/consolidation/retrieval.

Experiments in which memory acquisition and testing are performed completely postdrug treatment measure the persistent, long-term effects of exposure to MDMA. Most of the learning and memory studies reviewed here were performed in this manner, a total of 51 experiments, and all tested doses of 3 mg/kg or greater. Only 15 experiments found that MDMA consistently produced postdrug impairments in learning and memory, and another 10 experiments found impairments under specific task parameters only. Most of the experiments, a total of 36, found that MDMA produced no postdrug impairments in learning and memory under all/some task parameters. The reasons for occasional impairments, however, are ambiguous; there appears to be no clear pattern in terms of experimental methods.

Overall, the on-drug effects of MDMA on learning and memory appear to be dose-dependent, with lower doses producing no effects or enhancements and higher doses producing impairments. The threshold for impaired acquisition and retrieval appears to be approximately 3 mg/kg or more, which corresponds to the doses that are considered atypically high in human users. The dose threshold for impaired consolidation appears to be higher, at about 10 or more mg/kg, and there is even evidence that doses of 2.5–10 mg/kg can enhance consolidation. The postdrug effects of MDMA on learning and memory were negligible in most experiments, even given that these effects were assessed only at doses of 3 mg/kg or greater.

D. Findings by Species, Strain, Age, and Sex

To analyze findings by the species tested in each experiment, we focus on the five experiments in monkeys, the 19 experiments in mice, and the 65 experiments in rats [pigeons were only used in one study (LeSage et al., 1993), and no cognitive effects were found]. Of the five experiments in monkeys, four found no effects and one found impairments while on-drug but no post-drug effects. Of the 19 experiments in mice, one found a mix of enhancements and no effects (depending on dose), eight found no effects, five found a mix of no effects and impairments (depending on treatment/task parameters), and five found impairments only. Of the 65 experiments in rats, 3 found a mix of enhancements and no effects (depending on treatment/task parameters),

33 found no effects, 11 found a mix of no effects and impairments (depending on treatment/task parameters), and 18 found impairments only. In all three species, the majority of experiments found that MDMA has no cognitive effects. About 74% of the experiments in mice, about 72% of the experiments in rats, and all experiments in monkeys found negligible effects at some/all parameters.

The studies reviewed here tested a wide variety of rat and mouse strains. Except for the slight trends mentioned previously (in sections VI.A and VI.C), there appears to be no notable systematic differences in findings between the strains used in the present studies. Animal age (adolescents and/or adults) also did not appear to impact the findings. Of the 23 experiments that trained and/or tested adolescent rodents, one found a mix of enhancements and no effects, 13 found no effects, 2 found a mix of no effects and impairments, and 7 found impairments. This pattern of findings regarding adolescent animals generally mirrors that of all experiments (see Fig. 9). The majority of experiments tested male animals, but of the five experiments that included female animals, four found no effects and one found impairments. Although this suggests that MDMA may have less cognitive risk in females than males, there are not enough mixed-sex studies to have any confidence in this conclusion.

In all, there appears to be no differences in the cognitive effects of MDMA between rats and mice, and if anything, a less pronounced effect in monkeys. We also did not find any major differences in effects based on strain, age, or sex.

VII. High Doses and Neurotoxicology of Drugs of Abuse

An abundance of studies have reported neurotoxicity of MDMA and amphetamines, and as has been reviewed elsewhere, many of these studies exclusively used high doses (McCann and Ricaurte, 2004). Fundamentally, toxicology depends on the proper selection of doses relevant to those used by people, as even commonly consumed vitamins are readily toxic at high doses. For example, high doses of vitamin A are readily neurotoxic and cause birth defects, but we rarely hear calls that it be controlled or outlawed. Likewise, botulinum toxin is the most lethal substance known, but is used readily and safely at appropriate doses (Rietjens and Alink, 2006). In neurotoxicological research on drugs of abuse, there is an incentive to find neurotoxicological effects; these kinds of findings lead to more grants and more publications, while a lack of effects often leads to neither (Edwards and Roy, 2017). It is therefore natural to use high doses that are more likely to yield toxic effects. With a drug like MDMA that has no established medical use and is arguably a public health menace, there may seem to be little cost to arguing it causes brain damage rather than arguing it does not. However, when a previously maligned drug is argued to have new medical value, a proper assessment of its true toxicology is essential. Even with these factors, we found that a majority of experiments did not find evidence of MDMA-induced cognitive deficits in animals, even at high doses of 3 mg/kg or greater (Fig. 11). A careful consideration of the overall findings suggests that the preclinical literature on MDMA behavioral toxicity may only be relevant to certain, atypical, habitual users of high doses, rather than the typical recreational user; those findings are probably even less relevant to proposed therapeutic uses, where the drug may be given at low doses and only a few times.

VIII. Conclusions

This systematic review highlights that doses of less than 3 mg/kg MDMA, which we believe are appropriate to model typical human MDMA consumption, do not seem to impair cognition in animals. At doses of 3 mg/kg or greater, which model atypical, heavy MDMA use, the cognitive effects are unclear, as some findings suggest that these doses produce cognitive impairments while the slight majority suggest that they still do not influence cognition. The on-drug effects of MDMA on cognition have been assessed across a wide range of doses and appear to be dose-dependent. The postdrug effects of doses below 3 mg/kg have not yet been studied, but studies on doses of 3 mg/kg or greater reveal mixed findings that trend toward insignificance. After analyzing almost 25 years of findings with respect to methodology, we believe that the preclinical evidence of MDMA-induced cognitive deficits is relatively weak.

Previous neurotoxicity evidence suggests that rats, mice, and non-human primates exhibit vast differences in sensitivity to MDMA, with non-human primates showing the highest sensitivity and mice showing the lowest sensitivity to MDMA-induced serotonergic deficits. These differences are believed to arise from species differences in MDMA metabolism (Green et al., 2003, 2009, 2012a). Conversely, the present review suggests that rats and mice do not exhibit differences in sensitivity to MDMA-induced cognitive impairments, and that non-human primates are possibly less sensitive than rodents to these impairments. There is also some evidence of MDMA-induced cognitive impairments in rats and mice at doses lower than those necessary to produce neurotoxicity (20 mg/kg in rats, 50 mg/kg in mice). Together, this evidence suggests that MDMA-induced neurotoxicity and cognitive impairments may be unrelated, and active metabolites may not be responsible for the cognitive effects of MDMA.

Our analyses reveal that MDMA may have no effect on working memory or nonspatial learning and memory, but the potential to impair spatial learning and memory and/or fear-motivated learning and memory.

The most convincing impairments were those induced by high doses (3–20 mg/kg) in spatial reference memory and passive avoidance memory acquisition; however, visuospatial short-term and long-term memory deficits have not been consistently found in heavy MDMA users (Laws and Kokkalis, 2007). Our review also suggests that MDMA has no effect on working memory in animals across a range of doses, but retrospective studies have regularly found working memory deficits in MDMA users (Murphy et al., 2009, 2012; Nulsen et al., 2010). Human studies use nonrandom assignment and often test extremely heavy users; these deficits could have been present prior to MDMA use or may have been the result of very heavy atypical use. Since low doses (i.e., 1 to 2 mg/kg) of pure MDMA produce similar pharmacokinetic, pharmacological, and psychoactive effects in animals and humans (Baumann et al., 2007, 2009; Green et al., 2009, 2012a) and do not produce cognitive impairments in animals, we suspect that low doses of pure MDMA also do not impair cognition in humans.

To date, most evidence of MDMA-induced neurotoxicity and cognitive dysfunction has resulted from extreme animal dosing or heavy recreational use. While we agree that atypical heavy MDMA use may lead to some neural and behavioral toxicity, there is insufficient evidence that typical (i.e., low to moderate) MDMA use is detrimental to brain structure/function. Factors such as polydrug use, adulterants, hyperthermia, and hyponatremia can still increase the potential for adverse effects and are often involved in recreational MDMA use (Green et al., 2003; Baumann et al., 2007). Nevertheless, it is unlikely that less than 3 mg/kg of pure MDMA poses significant danger to neurological health if administered infrequently and in a controlled setting. Given that MDMA is administered in this manner during clinical investigations (Mithoefer et al., 2016), the therapeutic value of MDMA should not be dismissed due to potential neurological risks. However, it is critical to note that the margin between current therapeutic doses (1 to 2 mg/kg) and potentially memory-impairing doses ($\geq$3 mg/kg) is narrow. Therefore, 3 mg/kg should be considered the absolute limit for therapeutic dosing, and we recommend exploring even lower doses ($<$1 mg/kg).

We strongly suggest that preclinical MDMA researchers become more concerned with the critical aspect of proper animal dosing. There is considerable pessimism regarding the validity of allometric scaling in MDMA research (Baumann et al., 2007, 2009; Green et al., 2009, 2012a). Accordingly, the administration of excessively high doses of MDMA to animal subjects is not appropriate for determining potential toxic effects in typical MDMA users. Even at high doses, evidence of MDMA-induced cognitive deficits is relatively inconsistent. Future studies should aim to examine the effects of low-dose MDMA to reliably model typical human consumption and to evaluate any potential therapeutic value.

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Authorship Contributions

Performed data analysis: Pantoni.

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Chapter 3, in full, is a reprint of the material as it appears in Cognitive effects of MDMA in laboratory animals: a systematic review focusing on dose. *Pharmacological Reviews,* 71, 413–449. Pantoni, M. M., and Anagnostaras, S. G. (2019). DOI: 10.1124/pr.118.017087. Reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics. All rights reserved. The dissertation author was the primary investigator and author of this paper.

CHAPTER 4

MDMA and memory, addiction, and depression: dose-effect analysis

Abstract

±3,4-methylenedioxymethamphetamine (MDMA) is a widely abused recreational drug that shows substantial promise as a psychotherapeutic agent. Given its seemingly unique prosocial effects, MDMA has the potential to augment and enhance the effectiveness of psychotherapy for various psychiatric disorders or even improve social behavior as a stand-alone treatment. Nonetheless, the drug has considerable adverse effects such as amnesia and evidence is unclear as to whether or not its beneficial effects can be dissociated from its adverse effects, for example, by dose. We reviewed previous animal behavioral studies and concluded the likely dose required to produce amnesia is around 3 mg/kg (Pantoni and Anagnostaras, 2019). In the present study, we systematically examined the effects of a wide range of MDMA doses (0.01–10 mg/kg, i.p.) in mice on learning and memory, addiction-related behaviors, and depressive-like behavior. Low doses of MDMA (≤ 1 mg/kg) had no effect on these behaviors, while high doses of MDMA (≥ 3 mg/kg) produced memory impairments, some evidence of an addictive potential, and antidepressant effects. These findings demonstrate that careful selection of dose is critical. High-dose MDMA (≥ 3 mg/kg) should likely be avoided for its amnesic effects and addictive potential, but low-dose MDMA, which has been administered in recent clinical studies (approximately 1–2 mg/kg), is unlikely to produce amnesia and addiction. MDMA may even have remarkable therapeutic effects and a preferable safety profile at ultra-low doses (i.e., microdoses) and this should be investigated in future studies. In all, we believe that the potential adverse effects of MDMA should be considered within the framework of its therapeutic application, with particular orientation to the use of low doses.

Introduction

±3,4-methylenedioxymethamphetamine (MDMA) is a widely abused recreational drug that shows substantial promise as a psychotherapeutic agent (Sessa and Nutt, 2015; Feduccia et al., 2018; UNODC, 2020). MDMA targets various brain receptors and transporters with marked and preferential effects on the serotonergic system; it increases extracellular levels of serotonin (5-HT), norepinephrine (NE), and dopamine (DA) by reversing their transporters (SERT, NET, and DAT) and also exhibits some affinity for 5-HT, DA, muscarinic, histamine, and adrenergic receptors (Shulgin et al., 1986; Battaglia et al., 1988; Rudnick and Wall, 1992; Rothman et al., 2001; Torres et al., 2003). MDMA is classified chemically as a methamphetamine derivative, but behaviorally it is considered a stimulant-psychedelic by its detractors and an empathogen-entactogen by its proponents (Nichols, 1986; Liechti, 2015). It is these latter behavioral effects — increased empathy, trust, extroversion, and sociality — that distinguish MDMA from other related drugs (e.g., psychostimulants, psychedelics) and are of significant interest (Nichols, 1986; Hysek et al., 2014; Schmid et al., 2014; Kamilar-Britt and Bedi, 2015; Liechti, 2015; Bershad et al., 2016; Dolder et al., 2018; Holze et al., 2020). Given these unique prosocial effects, MDMA has the potential to augment and enhance the effectiveness of psychotherapy for psychiatric conditions such as social anxiety and autism spectrum disorders (Danforth et al., 2018) or even to improve social behavior as a stand-alone treatment (Heifets and Malenka, 2016). Recent Phase 2 clinical studies also reveal that MDMA-assisted psychotherapy is an effective therapeutic for treatment-resistant post-traumatic stress disorder (Bouso et al., 2008; Mithoefer et al., 2011, 2013, 2018; Oehen et al., 2013; Ot'alora et al., 2018) that may

outperform approved pharmacotherapies (i.e., paroxetine and sertraline) in terms of efficacy (Feduccia et al., 2019). Nevertheless, there is some concern regarding the behavioral toxicity of MDMA (Schenk and Newcombe, 2018), such as its potential to elicit memory impairments, addiction, and depressed mood, which warrants additional investigation.

The effects of low and high doses of the same drug can vary dramatically. For example, psychostimulants (e.g., amphetamine, methylphenidate, cocaine, modafinil) are highly effective cognitive enhancers at ultra-low and low doses but are highly addictive and cognitively impairing at high doses (for review, see Wood et al., 2014). We previously explored the role of dose in the cognitive effects of MDMA in a systematic review of existing literature (Pantoni and Anagnostaras, 2019) and found no preclinical evidence that MDMA impairs memory at low doses (< 3 mg/kg) but mixed results regarding cognitive effects at high doses (≥ 3 mg/kg). There have been few attempts to explore the effects of MDMA across a wide range of doses within the same study and even fewer investigations of low-dose MDMA (≤ 1 mg/kg). The current study aims to expand the known behavioral profile of MDMA across a wider range of doses (0.01–10 mg/kg). This range captures doses from one-tenth to ten times those used in recent clinical studies (approximately 1–2 mg/kg MDMA; Bouso et al., 2008; Mithoefer et al., 2011, 2013, 2018; Oehen et al., 2013; Danforth et al., 2018; Ot'alora et al., 2018). Generally, we have argued that doses should be scaled between animals and humans directly by body weight unless specific evidence (e.g., actual exposure data) justifies some specific kind of alternative scaling (see Carmack et al., 2014, Wood et al., 2014, and Pantoni and Anagnostaras, 2019). Low-dose MDMA

(about 1 to 2 mg/kg) produces equivalent increases in plasma drug concentration and monoamine release in humans (oral administration) and rodents (parenteral administration) (Baumann et al., 2007; Green et al., 2012), but time of peak drug exposure is shorter in rodents (10 to 45 min; Baumann et al., 2009) than in humans (about 145 min; Kolbrich et al., 2008). This data justifies temporal scaling but not dose scaling between rodent and human MDMA studies.

Here, we examined the cognitive effects of a wide range of MDMA doses using Pavlovian fear conditioning, a simple and efficient tool for modeling drug effects on learning and memory in rodents (Anagnostaras et al., 2000, 2010; Maren, 2001; Carmack et al., 2014). In this task, an initially neutral conditioned stimulus (CS; e.g., a tone or a context) is paired with an aversive unconditioned stimulus (US; e.g., a footshock). When learning occurs as a result of this pairing, either CS alone will elicit a conditioned response (CR; e.g., fear). In rodents, fear memory is typically quantified by measuring freezing behavior in response to a CS. Both context and tone fear memory are amygdala-dependent while contextual fear memory is also hippocampus-dependent (Maren et al., 1998; Anagnostaras et al., 1999, 2000, 2001, 2010; Gale et al., 2004). Psychostimulants modulate fear learning and memory dose-dependently: they enhance long-term memory at low, clinically relevant doses (0.005–0.05 mg/kg d-amphetamine; 0.01 and 1 mg/kg methylphenidate; 0.1 mg/kg cocaine; 0.75 mg/kg modafinil) but impair long-term memory at high, abused doses (4 and 8 mg/kg d-amphetamine; 10 mg/kg methylphenidate; 15 mg/kg cocaine; 75 mg/kg modafinil) (Wood et al., 2007; Shuman et al., 2009; Wood and Anagnostaras, 2009; Carmack et al., 2014). Citalopram, a highly selective serotonin

reuptake inhibitor, also impairs fear memory at high doses (10 mg/kg) but has no effect at low doses (0.01–1 mg/kg) (Carmack et al., 2014). Additional evidence suggests that psychostimulant-induced memory enhancement requires the combination of both DAT and NET inhibition (see Carmack et al., 2014 and Pantoni et al., 2020), but the impact of SERT inhibition when combined with DAT and NET inhibition is unclear.

We also evaluated the addictive potential[1] of MDMA using behavioral sensitization, conditioned place preference, and conditioned responding (Robinson and Berridge, 1993, 2003, 2008; Anagnostaras and Robinson, 1996; Anagnostaras et al., 2002; Carmack et al., 2017). Behavioral sensitization is a progressive increase in response following repeated administration of a drug and models the transition from casual drug use to compulsive drug taking. Conditioned place preference is the preference for a context that has been paired with a drug and models the rewarding effects of a drug, as well as instrumental drug seeking. Conditioned responding after repeated environment-drug (CS-US) pairings is a drug-like CR to a drug-paired context and models associative learning thought to elicit craving. The effects of psychostimulants on these behaviors are also dose-dependent: low, memory-enhancing doses (0.005 mg/kg d-amphetamine; 1 mg/kg methylphenidate; 0.15 mg/kg cocaine; 0.75 mg/kg modafinil) show no evidence of an addictive potential while high, memory-impairing doses (1.5 mg/kg d-amphetamine; 10 mg/kg methylphenidate; 15 mg/kg cocaine; 75 mg/kg modafinil) show evidence of a high

[1] In this article, we refer to "addictive potential" rather than "abuse potential" because even acute recreational use of MDMA is considered abuse. The existence of an illicit market means that, at present, any amount of MDMA is considered abused. Rather we are referring to the potential to develop addiction.

addictive potential (Shuman et al., 2012; Carmack et al., 2014). The action of high-dose psychostimulants at DAT and the ensuing increase in extracellular DA levels are largely responsible for the addictive potential of psychostimulants (Volkow et al., 1999, 2002; Koob and Volkow, 2010). Evidence suggests that drugs with strong activity at DAT (i.e., high binding affinity, high dose) are likely to produce addiction but drugs with weak activity at DAT (i.e., low binding affinity such as bupropion, low dose such as Adderall) are *not* likely to produce addiction (Carmack et al., 2014; Pantoni et al., 2020).

Lastly, we explored the effects of MDMA on depressive-like behavior using the forced swim test, one of the leading models used to screen for antidepressant drugs in rodents (Porsolt et al., 1977). In this test, animals are placed into a tank filled with water and time spent mobile (i.e., animal is active as it attempts to escape the stressful environment) versus immobile (i.e., "behavioral despair," animal is passive as it loses hope to escape the stressful environment) is measured. Several classes of antidepressant drugs, including selective serotonin reuptake inhibitors (SSRIs), dopamine reuptake inhibitors (DRIs), norepinephrine reuptake inhibitors (NRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAO-Is), and other atypical antidepressants decrease time spent immobile, which is believed to reflect their efficacy in reducing depressive-like behaviors (Cryan et al., 2005a; Petit-Demouliere et al., 2005). The effects of many antidepressants on the forced swim test are also dose-dependent: high doses (15 mg/kg fluoxetine; 15 mg/kg moclobemide; 60 mg/kg reboxetine) reveal antidepressant effects after acute or subacute administration (1–3 days) whereas low, clinically relevant doses (2–5 mg/kg fluoxetine or desipramine; 2.5

mg/kg moclobemide; 10 mg/kg reboxetine) require chronic administration (1–2 weeks) to show maximal efficacy (Detke et al., 1997; Vázquez-Palacios et al., 2004; Cryan et al., 2005a, 2005b). As such, the effects of low, clinically relevant doses on the rodent forced swim test are a better reflection of the delayed onset of antidepressant effects in patients.

In the present study, we found that MDMA modulated behavior dose-dependently. Low doses of MDMA (≤ 1 mg/kg) had no effect on memory, addiction-related behaviors, or depressive-like behavior, while high doses of MDMA (3 and 10 mg/kg) produced memory impairments, some evidence of an addictive potential (at 10 mg/kg only), and antidepressant effects. We conclude that MDMA is safest when used at low doses (< 3 mg/kg) and that higher doses should likely be avoided. We discuss the possible mechanisms underlying the behavioral effects of MDMA and its potential as a psychotherapeutic.

Methods

Subjects. 184 hybrid C57BL/6Jx129S1/SvImJ (129B6; Jackson Laboratory, West Sacramento, CA, USA) male (n = 91) and female (n = 93) mice were used. Mice were weaned at 3 weeks of age and group housed (2–5 mice per same sex cage) with unrestricted access to food and water. The animal colony was maintained on a 14:10-h light/dark schedule and all testing occurred during the light phase. Mice were at least 10 weeks old and handled for 3 days (1 min/day) prior to testing. All 184 mice were used for fear conditioning; of these mice, 45 (24 males and 21 females) were used 6 weeks later for conditioned place preference and behavioral sensitization, and 79 (33 males and 46

females) were used 8 weeks later for the forced swim test. All animal care and experimental procedures were approved by the UCSD IACUC and compliant with the NRC Guide for the Care and Use of Laboratory Animals.

Drugs. 3,4-MDMA HCl (CAS No. 64057-70-1; Cayman Chemical, Ann Arbor, MI, USA) was dissolved in 0.9 % physiological saline and given intraperitoneally (i.p.) in a volume of 10 mL/kg. A range of MDMA doses were selected: 0, 0.01, 0.05, 0.1, 0.5, 1, 3, and 10 mg/kg (salt weight).

Fear Conditioning. The VideoFreeze system (Med Associates Inc., St. Albans, VT, USA) and fear conditioning protocol were used as described previously (Anagnostaras et al., 2000, 2010; Shuman et al., 2009; Wood and Anagnostaras, 2011; Carmack et al., 2014; Pantoni et al., 2020). Four mice were tested concurrently in individual conditioning chambers (32 cm × 25 cm × 25 cm) that consisted of stainless-steel sidewalls and rod floors, white acrylic back walls, and clear polycarbonate front and top walls. Each chamber was transformed across multiple sensory dimensions to create two distinct contexts: a training context, which was used for training and context testing, and an alternate context, which was used for tone testing. For the training context, chambers were cleaned and scented with 7 % isopropanol, and illuminated with moderate (80 lx) white light and near-infrared light (980 nm). For the alternate context, chambers were outfitted with a black plastic, triangular teepee and white acrylic floors, cleaned and scented with a 5 % vinegar solution, and illuminated with only near-infrared light to create a dark environment. VideoFreeze software (Med Associates Inc.) used digital video to score freezing behavior and locomotor activity (Anagnostaras et al., 2010).

184 mice were randomly assigned to groups by dose of MDMA administered: 0 (n = 35), 0.01 (n = 20), 0.05 (n = 20), 0.1 (n = 30), 0.5 (n = 20), 1 (n = 20), 3 (n = 20), or 10 (n = 19) mg/kg. Groups were counterbalanced by sex and conditioning chamber. Mice were given an injection of MDMA or saline 30 min before a 10-min training session. A delay of 30 min was selected due to its temporal proximity to peak drug exposure, locomotor activity (from pilot work in our lab), core temperature, and behavioral effects following intraperitoneal MDMA in mice (Fantegrossi et al., 2008; for review, see Pantoni and Anagnostaras, 2019). Training began with a 3-min baseline period followed by a single tone-shock pairing, which consisted of a 30-s pure tone (2.8 kHz, 85 dBA) presented through a speaker in the chamber sidewall that co-terminated with a 2-s scrambled, AC constant current footshock (0.75 mA, RMS) delivered through the rod floor. Ninety seconds after the tone-shock pairing, mice underwent a 5-min post-shock test. Locomotor activity during the baseline period and during the footshock was used to measure on-drug baseline locomotion and shock reactivity, respectively, while freezing behavior during the post-shock test was used to measure on-drug short-term memory.

Seven days after training, mice were returned to the training context, off drug, for a 5-min context test. Freezing behavior during the test was used to measure long-term context memory. One day after context testing, mice were brought to the alternate context, off drug, for a 5-min tone test. Tone testing consisted of a 2-min baseline period, followed by the presentation of 3, 30-s tones identical to the training tone each separated by 30-s. Freezing behavior during the tone presentations was used to measure long-term tone memory.

Conditioned Place Preference and Behavioral Sensitization. Eight mice were tested concurrently in individual place preference chambers (Med Associates Inc., St. Albans, VT, USA) as described previously (Carmack et al., 2013, 2014; Pantoni et al., 2020). Each chamber (43 cm × 43 cm × 31 cm) consisted of two sides — a drug-paired side and an unpaired side — separated by a black wall with a removable insert. The two sides were visually and tactilely distinct as they differed by flooring (stainless steel rods or wire mesh) and walls (white and decorated with stickers or undecorated clear polycarbonate). Chambers were counterbalanced by flooring and wall combinations and by paired versus unpaired side assignments. Each chamber was cleaned with 10 % glass cleaner (Zep Inc., Atlanta, GA, USA) between trials. Activity Monitor software (Med Associates Inc.) used the interruption of infrared beams to identify mouse position and score locomotion (distance), stereotypy (counts), and verticality (counts).

45 mice were randomly assigned to new groups by dose of MDMA administered: 0 (n = 12), 0.1 (n = 10), 1 (n = 11), or 10 (n = 12) mg/kg. Groups were counterbalanced by sex and testing chamber. Mice were habituated to the testing chamber, off drug, for 30 min per side per day for 2 consecutive days prior to training (with the order of side placement counterbalanced). Four days after habituation, mice were trained for seven alternating days. On each training day, mice were injected with saline before being placed into the unpaired side for 15 min, then injected with MDMA before being placed into the paired side for 15 min. Locomotor, stereotyped, and vertical activity on the paired side was scored and behavioral sensitization was calculated as the difference between average activity on Day 7 versus Day 1.

Twenty-four hours after the last training day, mice were tested off drug for conditioned place preference. The inserts that previously separated the two sides of the chambers were removed. Mice were placed into the entryway between the two sides of the chamber (with the direction of entry counterbalanced) and allowed access to both sides for 15 min. Locomotor activity and time spent on each side was scored and place preference was calculated as the difference between responses on the paired side versus the unpaired side.

Forty-eight hours after the last training day, mice received two back-to-back challenge tests: one with saline and one with a high dose of MDMA (10 mg/kg). Mice were injected with saline and immediately placed into the paired side for 15 min and then removed and injected with 10 mg/kg MDMA and immediately returned to the paired side for 45 min. Locomotor, stereotyped, and vertical activity was scored to evaluate the presence of conditioned responding to the drug-paired side (saline challenge) and/or sensitized responding to the high dose of 10 mg/kg MDMA (high dose challenge). One mouse trained with 10 mg/kg MDMA died during the high dose challenge and its data was excluded from that test only.

Forced Swim Test. The forced swim test procedure was adapted from existing protocols (Porsolt et al., 2001; Castagné et al., 2011; Can et al., 2012; Yankelevitch-Yahav et al., 2015). Five mice were tested concurrently in individual cylindrical beaker-like glass tanks (10 cm diameter x 24 cm height) that were visually separated by white opaque dividers. Each tank was filled with water (24 °C ± 0.5 °C) to a depth of 15 cm. Mice were tested in bright light (approximately 80 lx) and immobility was measured using an HD

USB video camera and behavioural tracking software (ANY-Maze, Wood Dale, IL, USA; minimum immobility time = 2000 ms, immobility sensitivity = 75 %).

79 mice were randomly assigned to new groups by dose of MDMA administered: 0 (n = 14), 0.1 (n = 13), 0.5 (n = 13), 1 (n = 13), 3 (n = 13), or 10 (n = 13) mg/kg. Groups were counterbalanced by sex and testing tank. Mice were given an injection of MDMA or saline 30 min before testing. Mice were placed into the water for a 6-min test and the time spent immobile was scored during the last 4 minutes to evaluate potential antidepressant effects (reduced immobility).

Statistical Analyses. Data were analyzed using univariate or multivariate analyses of variance (ANOVAs) to identify overall group differences. Post-hoc comparisons were performed following significant ANOVAs using Fisher's Least Significant Difference (LSD) tests against the saline control group. With the exception of **Figure 4.3**, data from male and female mice were merged as we found no other statistically significant sex differences that meaningfully influenced these findings (p values > 0.05).

Results

Fear Conditioning. The effects of MDMA (0–10 mg/kg, i.p.) on fear learning and memory were examined using Pavlovian fear conditioning. Mice were trained on drug with a single tone-shock pairing. Freezing was scored during an on-drug post-shock test and one week later during an off-drug context test and an off-drug tone test to evaluate short- and long-term memory. MDMA weakly dose-dependently modulated locomotor activity during the training baseline period [$F(7, 176) = 2.08$, $p = 0.05$; **Figure 4.1A, lower line**].

Only mice given 3 mg/kg MDMA showed significantly increased baseline locomotion compared to saline controls ($p = 0.001$; all other p values > 0.07). The shock elicited a large activity burst unconditioned response that did not significantly differ between groups [$F(7, 176) = 0.43$, $p = 0.88$; **Figure 4.1A, upper line**]. MDMA dose-dependently modulated freezing during the on-drug post-shock [$F(7, 176) = 5.24$, $p < 0.001$; **Figure 4.1B**], off-drug context [$F(7, 176) = 7.17$, $p < 0.001$; **Figure 4.1C**], and off-drug tone [$F(7, 176) = 3.98$, $p < 0.001$; **Figure 4.1D**] tests. Compared to saline controls, only mice given 10 mg/kg MDMA exhibited reduced freezing during the post-shock test ($p < 0.001$; all other p values > 0.05), and only mice previously given 3 or 10 mg/kg MDMA exhibited reduced freezing during the context (p values ≤ 0.03; all other p values > 0.1) and tone (p values ≤ 0.01; all other p values > 0.06) tests.

Conditioned Place Preference and Behavioral Sensitization. The effects of MDMA (0–10 mg/kg, i.p.) on addiction-related behaviors were examined using conditioned place preference and behavioral sensitization. Mice were trained for 7 days in a two-sided chamber; on each day, mice were injected with saline and placed into the unpaired side and then injected with MDMA and placed into the paired side. Locomotor, stereotyped, and vertical activity on the drug-paired side was measured. Significant group differences in activity were not observed on Day 1 [locomotion: $F(3, 41) = 1.77$, $p = 0.17$; stereotypy: $F(3, 41) = 1.06$, $p = 0.38$; verticality: $F(3, 41) = 0.43$, $p = 0.74$; **Figures 4.2A, 4.2D, 4.2G, left**], but were observed on Day 7 [locomotion: $F(3, 41) = 11.85$, $p < 0.001$; stereotypy: $F(3, 41) = 7.54$, $p < 0.001$; verticality: $F(3, 41) = 6.82$, $p < 0.001$; **Figures 4.2A, 4.2D, 4.2G, right**]. Compared to saline controls, only mice receiving 10 mg/kg MDMA

showed significantly increased locomotor ($p < 0.001$; all other p values > 0.7), stereotyped ($p = 0.001$; all other p values > 0.3), and vertical ($p < 0.001$; all other p values > 0.6) activity on Day 7.

There were also significant main effects of group [locomotion: $F(3, 41) = 11.23$, $p < 0.001$; stereotypy: $F(3, 41) = 7.21$, $p < 0.001$; verticality: $F(3, 41) = 3.64$, $p = 0.02$] and group-by-day interactions [locomotion: $F(18, 246) = 3.6$, $p < 0.001$; stereotypy: $F(18, 246) = 2.51$, $p < 0.001$; verticality: $F(18, 246) = 3.51$, $p < 0.001$] on average daily activity across the seven days of training (**Figures 4.2B, 4.2E, 4.2H**). Compared to saline controls, only mice receiving 10 mg/kg MDMA showed significantly increased locomotor ($p < 0.001$; all other p values > 0.8), stereotyped ($p = 0.001$; all other p values > 0.5), and vertical ($p = 0.007$; all other p values > 0.6) activity, and these effects were observed on the last five days (locomotion: p values ≤ 0.002; stereotypy: p values ≤ 0.02; verticality: p values ≤ 0.01) but not the first two days of training (locomotion: p values > 0.07; stereotypy: p values > 0.09; verticality: p values > 0.1). Lastly, there were significant group differences in the development of sensitization as measured by the difference in average activity on Day 7 versus Day 1 [locomotion: $F(3, 41) = 4.42$, $p = 0.009$; stereotypy: $F(3, 41) = 3.57$, $p = 0.02$; verticality: $F(3, 41) = 4.32$, $p = 0.01$; **Figures 4.2C, 4.2F, 4.2I**]. Only mice receiving 10 mg/kg MDMA exhibited a significant increase in locomotor ($p = 0.002$; all other p values > 0.5), stereotyped ($p = 0.03$; all other p values > 0.3), and vertical ($p = 0.04$; all other p values > 0.1) activity from Day 1 to Day 7 when compared to saline controls.

We found statistically significant sex differences in the effects of MDMA on locomotor activity during training. There was a main effect of sex that trended towards

significance [$F(1, 37) = 3.06$, $p = 0.09$] and a significant group-by-sex interaction [$F(3, 37) = 9.99$, $p < 0.001$] on Day 1 (**Figure 4.3A, left**). There was also a significant main effect of sex [$F(1, 37) = 5.76$, $p = 0.02$] and a significant group-by-sex interaction [$F(3, 37) = 3.27$, $p = 0.03$] on Day 7 (**Figure 4.3A, right**). Sex differences were observed only in mice receiving 10 mg/kg MDMA (Day 1: $p < 0.001$, all other p values > 0.2; Day 7: $p < 0.001$, all other p values > 0.3). Compared to saline controls of the same sex, female ($p < 0.001$) but not male ($p = 0.17$) mice receiving 10 mg/kg MDMA showed significantly increased locomotion on Day 1, and both female ($p < 0.001$) and male ($p = 0.01$) mice receiving 10 mg/kg MDMA showed significantly increased locomotion on Day 7.

There was also a significant main effect of sex [$F(1, 37) = 7.61$, $p = 0.009$] and a significant group-by-sex interaction [$F(3, 37) = 11.8$, $p < 0.001$] on average daily locomotion across the seven days of training (**Figure 4.3B**). Sex differences were observed only in mice receiving 10 mg/kg MDMA ($p < 0.001$; all other p values > 0.6). Compared to saline controls of the same sex, female ($p < 0.001$) but not male ($p = 0.09$) mice receiving 10 mg/kg MDMA showed significantly increased locomotion across the seven days of training. In female mice, there was a significant main effect of group [$F(3, 17) = 39.12$, $p < 0.001$] and a significant group-by-day interaction [$F(18, 102) = 2.07$, $p = 0.01$]. Compared to female saline controls, only female mice receiving 10 mg/kg showed significantly increased locomotion ($p < 0.001$; all other p values > 0.8) and this effect was observed on all seven days of training (p values ≤ 0.002). In male mice, there was no significant main effect of group [$F(3, 20) = 1.63$, $p = 0.21$] but there was a significant group-by-day interaction [$F(18, 120) = 2.02$, $p = 0.01$]. Compared to male saline controls,

only male mice receiving 10 mg/kg showed significantly increased locomotion on the last three days (p values ≤ 0.04) but not the first four days (p values > 0.2) of training. Despite significant sex differences in the acute effects of MDMA on locomotion, no main effect of sex [$F(1, 37) = 0.71$, $p = 0.41$] or group-by-sex interaction [$F(3, 37) = 0.19$, $p = 0.9$] was observed for the development of sensitization as measured by the difference in average locomotion on Day 7 versus Day 1 (**Figure 4.3C**).

Twenty-four hours after the last training day, mice were tested off drug for conditioned place preference. Mice were allowed free access to both sides and place preference was measured by the difference in distance traveled and time spent on the drug-paired side versus the unpaired side. There were no significant group differences in distance traveled [$F(3, 41) = 0.48$, $p = 0.7$; **Figure 4.4A**] or time spent [$F(3, 41) = 0.18$, $p = 0.91$; **Figure 4.4B**] between sides. Additionally, none of the groups exhibited place preference in locomotor activity [one sample two-tailed t-test against hypothesized $\mu = 0$; 0 mg/kg, $t(11) = 0.09$, $p = 0.93$; 0.1 mg/kg, $t(9) = 0.7$, $p = 0.5$; 1 mg/kg, $t(10) = 0.43$, $p = 0.68$; 10 mg/kg, $t(11) = 0.81$, $p = 0.43$] or time spent [0 mg/kg, $t(11) = 0.65$, $p = 0.53$; 0.1 mg/kg, $t(9) = 0.74$, $p = 0.48$; 1 mg/kg, $t(10)= 0.58$, $p = 0.57$; 10 mg/kg, $t(11) = 1.39$, $p = 0.19$].

Forty-eight hours after the last training day, mice were challenged with saline and then a high dose of MDMA (10 mg/kg) on the paired side. Locomotor, stereotyped, and vertical activity in response to the saline challenge and the high dose challenge was scored to evaluate conditioned and sensitized responding, respectively. There were significant group differences in locomotion following the saline [$F(3, 41) = 4.31$, $p = 0.01$; **Figure**

4.5A, left] and high dose MDMA [$F(3, 40) = 13.14$, $p < 0.001$; **Figure 4.5A, right**] challenges. Compared to saline controls, only mice trained with 10 mg/kg MDMA exhibited a CR as measured by increased locomotion following the saline challenge ($p = 0.008$; all other p values > 0.5) or sensitization as measured by increased locomotion following the high dose MDMA challenge ($p < 0.001$; all other p values > 0.5]. The same pattern of effects was observed for stereotypy (group differences: p values ≤ 0.02; 10 mg/kg versus saline: p values ≤ 0.03) but not verticality (group differences: p values > 0.2) (data not depicted).

Forced Swim Test. The effects of MDMA (0–10 mg/kg, i.p.) on depressive-like behavior were examined using the forced swim test. Mice underwent a 6-min on-drug test and time spent immobile was scored during the last 4 minutes of testing. MDMA dose-dependently modulated immobility [$F(5, 73) = 13.13$, $p < 0.001$; **Figure 4.5B**]. Only mice given 3 or 10 mg/kg MDMA exhibited reduced immobility relative to saline controls (p values < 0.001; all other p values > 0.5).

Discussion

The present study provides further evidence for the critical role of dose selection in the behavioral effects of MDMA. Specifically, we found that high doses of MDMA produced memory impairments (at 3 and 10 mg/kg), some evidence of an addictive potential (at 10 mg/kg), and antidepressant effects (at 3 and 10 mg/kg), while low doses of MDMA (≤ 1 mg/kg) did not. Frequent high-dose MDMA (≥ 3 mg/kg) should likely be avoided for its amnesic effects and addictive potential but low-dose MDMA, which has

been administered in recent clinical studies (approximately 1–2 mg/kg MDMA; for review, see Feduccia et al., 2018), is likely safe in terms of the behaviors analyzed herein. It appears that MDMA has a narrow viable therapeutic window and lowering dose should remain an important consideration in clinical use.

Our earlier systematic review (Pantoni and Anagnostaras, 2019) questioned concerns that therapeutic use of MDMA would cause memory problems, as there was no preclinical evidence that MDMA impairs cognition at low, clinically relevant doses (< 3 mg/kg) but results regarding higher doses (≥ 3 mg/kg) were mixed. The present dose-effect analysis provides further evidence that 3 mg/kg MDMA appears to be the threshold for memory impairments. Using a Pavlovian fear conditioning paradigm, 10 mg/kg MDMA impaired short-term memory (on drug), 3 and 10 mg/kg MDMA impaired long-term context and tone memory (off drug), and 0.01 to 1 mg/kg MDMA did not impair memory. These memory impairments were not confounded by effects on nociception, as demonstrated by lack of group differences in shock reactivity, nor by effects on locomotor activity, as the short-term memory-impairing dose of 10 mg/kg MDMA had no effect on baseline locomotion and the long-term memory tests were conducted off drug. We did not detect any MDMA-induced fear memory enhancements even though psychostimulants enhance memory at low, clinically relevant doses (Wood et al., 2007; Shuman et al., 2009; Wood and Anagnostaras, 2009; Carmack et al., 2014) and there is only sparse evidence that MDMA may sometimes enhance cognition (for review, see Pantoni and Anagnostaras, 2019). Instead, MDMA produced dose-dependent effects that were similar to that of the SSRI citalopram (i.e., no effects at low, clinically relevant doses; impairments at high

doses; Carmack et al., 2014). It is possible that MDMA does not act strongly enough at DAT and NET to enhance memory or that drug action at SERT interferes with memory enhancement. Enhanced memory reconsolidation and fear extinction has been proposed as a potential therapeutic mechanism of MDMA-assisted psychotherapy for post-traumatic stress disorder (Feduccia and Mithoefer, 2018). While we did not detect changes in fear learning at low, clinically relevant doses of MDMA, high-dose MDMA (7.8 mg/kg) has been shown to enhance fear memory extinction (Young et al., 2015, 2017), and further research should investigate the effects of low-dose MDMA on fear extinction.

The addictive potential of high-dose psychostimulants is reflected in their propensity to elicit acute locomotor stimulation, behavioral sensitization, conditioned responding, and conditioned place preference (Robinson and Berridge, 1993, 2003, 2008; Anagnostaras and Robinson, 1996; Anagnostaras et al., 2002; Shuman et al., 2012; Carmack et al., 2014, 2017). We found that treatment with low, clinically relevant doses of 0.01 and 1 mg/kg MDMA did not lead to any addiction-related behaviors, even following the 10 mg/kg MDMA high dose challenge. Treatment with a high, memory-impairing dose of 10 mg/kg MDMA did lead to behavioral sensitization and conditioned responding, but not acute locomotor stimulation or conditioned place preference. There were also interesting sex differences in the effects of 10 mg/kg MDMA on acute locomotor activity, as only females showed increased locomotion starting on the first day of training. This may be related to findings that females are more sensitive than males to the psychological effects of MDMA (for review, see Liechti et al., 2001 and Allott and Redman, 2007). However, both sexes similarly developed sensitization. Other drug-pairing

procedures have also been found to occasion behavioral sensitization or conditioned responding in the absence of conditioned place preference (Hemby et al., 1992; Brown and Fibiger, 1993; Rowlett et al., 1994; Seymour and Wagner, 2008; Carmack et al., 2013). These findings suggest that repeated use of MDMA at high (but not low) doses may lead to compulsive drug taking and drug-cue elicited craving, although MDMA may be less rewarding and less likely to provoke drug seeking than psychostimulants and other drugs that induce conditioned place preference (for reviews, see Tzschentke, 2007 and Carmack et al., 2017).

There are opposing views regarding how MDMA modulates depressive symptoms — one view holds that MDMA exacerbates mood problems including depression (for review, see Morgan, 2000), while the other holds that MDMA has antidepressant properties that are implicated in its therapeutic effects (Yazar-Klosinski and Mithoefer, 2017; Thal and Lommen, 2018). Recent clinical studies report both depression symptom improvement as a secondary outcome and depressed mood as a treatment-emergent adverse event following MDMA-assisted psychotherapy (Mithoefer et al., 2019). Using the forced swim test, we detected acute MDMA-induced antidepressant effects at high, memory-impairing doses of 3 and 10 mg/kg but not at lower doses of 0.1, 0.5, and 1 mg/kg. Drugs that induce acute locomotor stimulation can lead to a false positive result in the forced swim test (Porsolt et al., 1978). This is a common concern with psychostimulants; however, there is clinical data suggesting that psychostimulants do indeed alleviate depressive symptoms and thus the term “false positive” may be misleading (Candy et al., 2008; Castagné et al., 2011). It is unlikely that locomotor stimulation was responsible for decreased immobility

in the present study as we found little evidence that a single dose of 3 or 10 mg/kg MDMA acutely stimulates locomotor activity when averaged across both sexes. Since we found no acute antidepressant effects at low, clinically relevant doses, it is possible that low-dose MDMA requires chronic administration to reduce depressive-like behavior as do low-dose SSRI, NRI, TCA, and MAO-I antidepressants (Detke et al., 1997; Vázquez-Palacios et al., 2004; Cryan et al., 2005a, 2005b). Low, non-amnesic doses of MDMA may also have other therapeutic effects, such as increased sociality or openness, that facilitate the clinical improvements observed following MDMA-assisted psychotherapy (Heifets and Malenka, 2016; Wagner et al., 2017).

There is increasing evidence that the therapeutic effects of MDMA are mediated by the serotonergic system whereas its memory-impairing and addiction-related effects are mediated by the dopaminergic system. Young et al. (2017) demonstrated that the action of MDMA at SERT and subsequent 5-HT2A receptor activation plays an important role in its enhancement of fear memory extinction. Similarly, Heifets et al. (2019) demonstrated that the action of MDMA at SERT and subsequent 5-HT1B receptor activation within the nucleus accumbens is necessary and sufficient for its prosocial effects, whereas MDMA binding at DAT and the consequent increase in DA release is required for its rewarding effects. Risbrough et al. (2006) revealed that the DA receptor subtypes have differential modulatory roles in MDMA-induced hyperactivity; specifically, D1 receptor activation modifies the type of activity (linear versus circumscribed) whereas D2 receptor activation contributes to repetitive circling behavior. Squire et al. (2020) found that MDMA may indirectly impair memory via overstimulation of D1 receptors, which challenges the

assumption that its acute memory effects are predominantly due to serotonergic mechanisms. The effects of MDMA on the serotonergic versus dopaminergic systems are also dose-dependent. At low doses (< 3 mg/kg), MDMA stimulates 5-HT release and little to no DA release, whereas at high doses (≥ 3 mg/kg), MDMA stimulates both 5-HT and DA release (Kankaanpää et al., 1998; Baumann et al., 2005, 2007). In accordance with these findings, we detected MDMA-induced memory impairments and addiction-related behaviors at high doses that correlate with substantial DA release. Additional evidence suggests that the R(-) enantiomer of MDMA retains the therapeutic effects but not the adverse effects of racemic MDMA because of its significantly decreased potency as a DA releaser (Curry et al., 2018; Pitts et al., 2018). It is plausible that low-dose racemic MDMA or another drug that preferentially induces 5-HT release may promote prosocial behavior without impairing memory or producing addiction.

Our findings suggest that therapeutic use of MDMA below 3 mg/kg is unlikely to produce significant adverse cognitive effects. While psychostimulants have the potential for addiction and toxicity at high doses, they are effective and safe cognitive enhancers that are prescribed at low doses for extended periods of time (for review, see Wood et al., 2014). Similarly, MDMA is showing great promise as a psychotherapeutic, and low doses seem to pose little risk of memory impairments, addiction, or depressed mood. Since the dose threshold for potential memory impairments and addiction (3 mg/kg MDMA) is close to the doses used in recent clinical studies (approximately 1–2 mg/kg MDMA; for review, see Feduccia et al., 2018), future studies should consider exploring even lower doses. MDMA may have remarkable therapeutic effects and a preferable safety profile at ultra-

low doses (i.e., microdoses), as do psychostimulants (e.g., Wood and Anagnostaras, 2009) and possibly psychedelics such as LSD and psilocybin (Kuypers et al., 2019). In all, we believe that the potential adverse effects of MDMA should be considered within the framework of its therapeutic application.

Chapter 4, in full, is currently being prepared for submission for publication of the material. Pantoni, M. M., Kim, J. L., Van Alstyne, K. R., and Anagnostaras, S. G. The dissertation author was the primary investigator and author of this paper.

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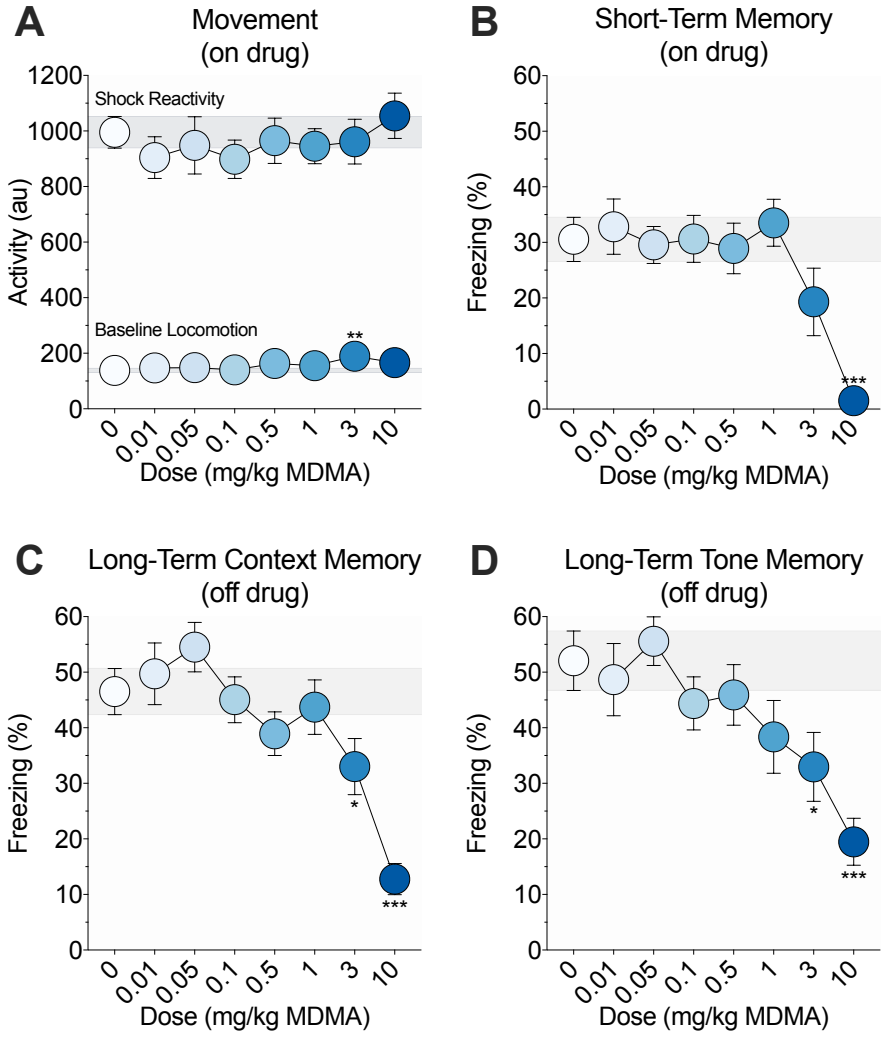

Figure 4.1 Effects of MDMA on fear learning and memory. (A) On-drug activity during the 3-min training baseline period and the 2-s footshock. Mice given 3 mg/kg MDMA showed increased baseline locomotion relative to saline controls. There were no group differences in shock reactivity. **(B)** Short-term memory as measured by percent freezing during the on-drug post-shock test. Mice given 10 mg/kg MDMA showed impaired short-term memory relative to saline controls. **(C)** Long-term context memory as measured by percent freezing during the off-drug context test, one week after training. Mice previously given 3 or 10 mg/kg MDMA showed impaired long-term context memory relative to saline controls. **(D)** Long-term tone memory as measured by percent freezing during the off-drug tone test, one day after context testing. Mice previously given 3 or 10 mg/kg MDMA showed impaired long-term tone memory relative to saline controls. Each point represents the mean ± 1 standard error. The grey bar indicates standard error range for the comparison saline control group. Asterisks identify significant comparisons against the saline control group using Fisher's LSD (*P < 0.05, **P < 0.01, and ***P< 0.001).

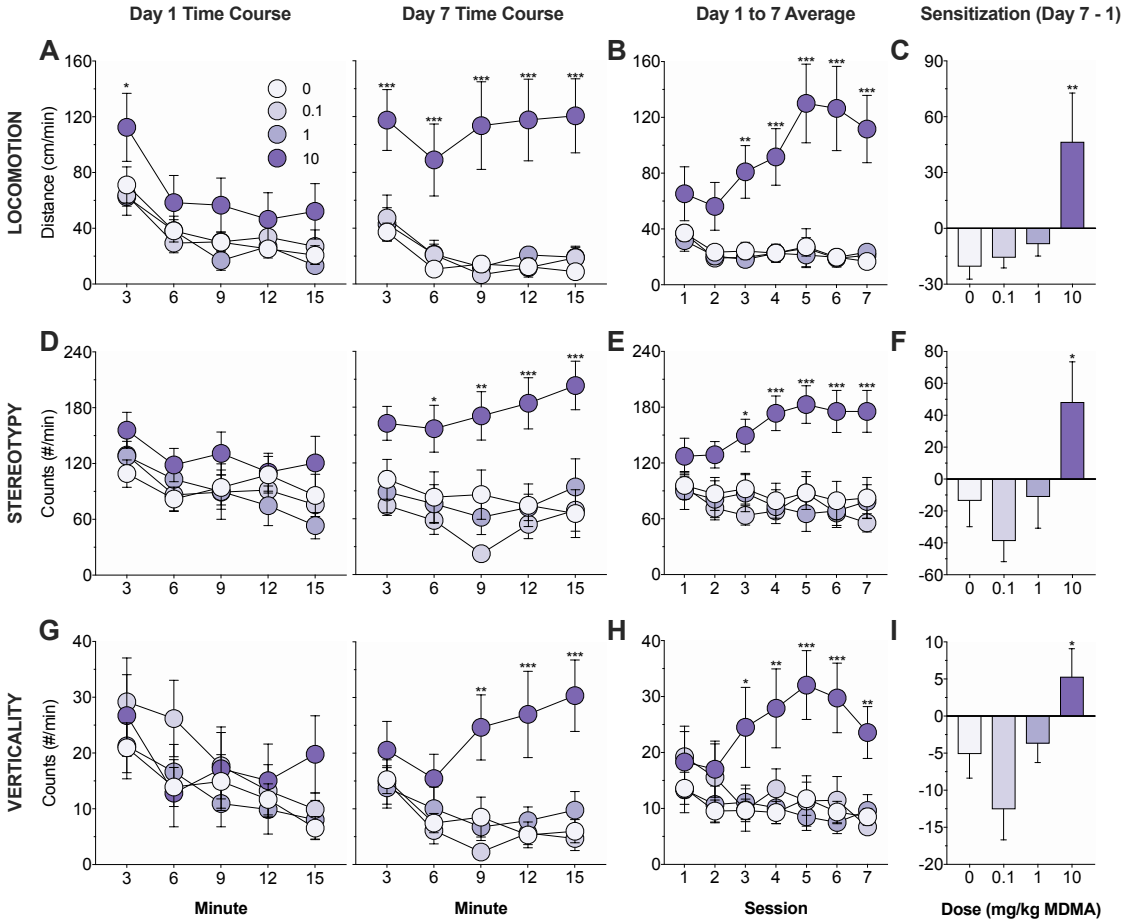

Figure 4.2 Effects of MDMA on behavioral sensitization. Mice were trained for 7 days and locomotion **(A–C)**, stereotypy **(D–F)**, and verticality **(G–I)** on the drug-paired side was measured. **(A, D, G)** Time course of activity on Day 1 **(left)** and Day 7 **(right)** of training. There were no group differences on Day 1, but on Day 7, mice receiving 10 mg/kg MDMA exhibited increased locomotion **(A)**, stereotypy **(D)**, and verticality **(G)** relative to saline controls. **(B, E, H)** Average activity on each of the seven days of training. Mice receiving 10 mg/kg MDMA exhibited increased locomotion **(B)**, stereotypy **(E)**, and verticality **(H)** relative to saline controls from Day 3 to Day 7. **(C, F, I)** Development of sensitization as measured by the difference in average activity on Day 7 versus Day 1. Mice receiving 10 mg/kg MDMA exhibited a greater increase in locomotion **(C)**, stereotypy **(F)**, and verticality **(I)** from Day 1 to Day 7 relative to saline controls. Asterisks identify significant comparisons against the saline control group at the same time point.

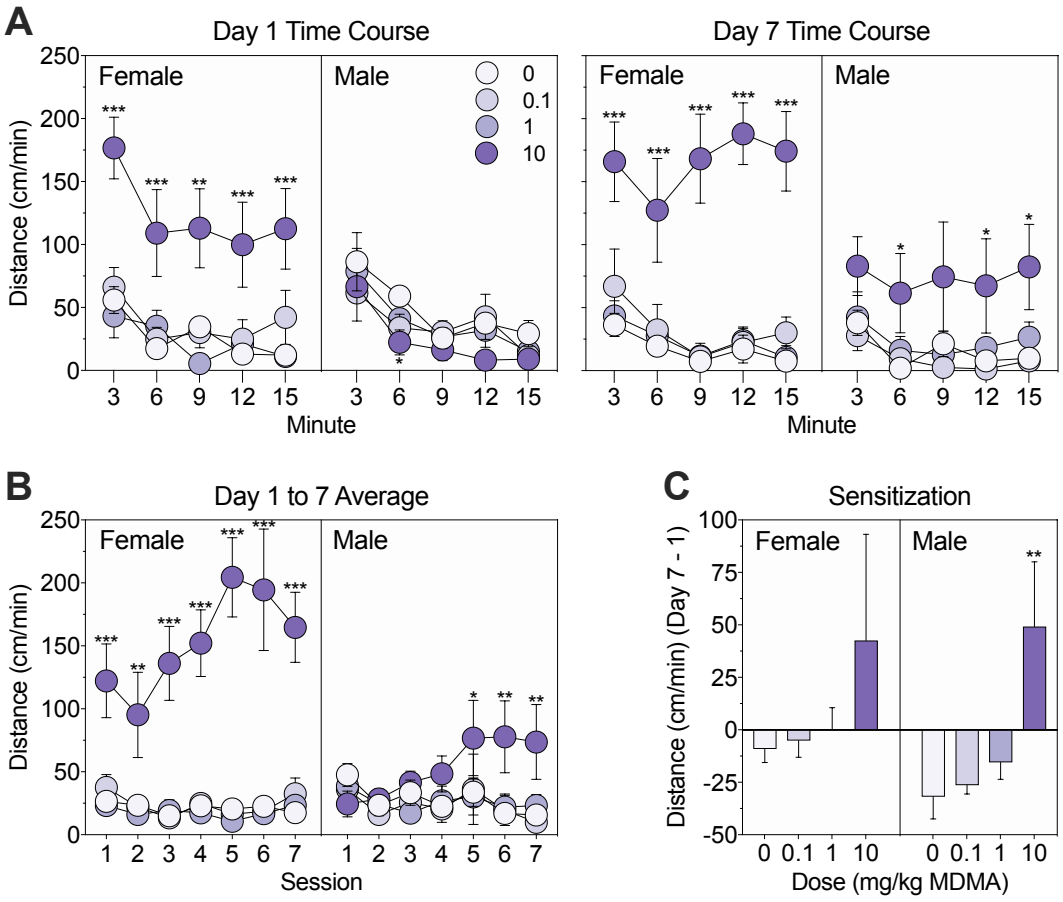

Figure 4.3 Sex differences in effects of MDMA on locomotion. Data from Figure 4.2 divided by female **(left)** and male **(right)** mice. **(A)** Time course of locomotion on Day 1 **(left)** and Day 7 **(right)** of training. Female mice receiving 10 mg/kg MDMA exhibited increased locomotion relative to female saline controls on Days 1 and 7. Male mice receiving 10 mg/kg MDMA exhibited increased locomotion relative to male saline controls on Day 7 only. **(B)** Average locomotion on each of the seven days of training. Female mice receiving 10 mg/kg MDMA exhibited increased locomotion relative to female saline controls on all seven days. Male mice receiving 10 mg/kg MDMA exhibited increased locomotion relative to male saline controls from Day 5 to Day 7 only. **(C)** Development of sensitization as measured by the difference in average locomotion on Day 7 versus Day 1. There was no main effect of sex or group-by-sex interaction. Asterisks identify significant comparisons against the saline control group of the same sex and at the same time point.

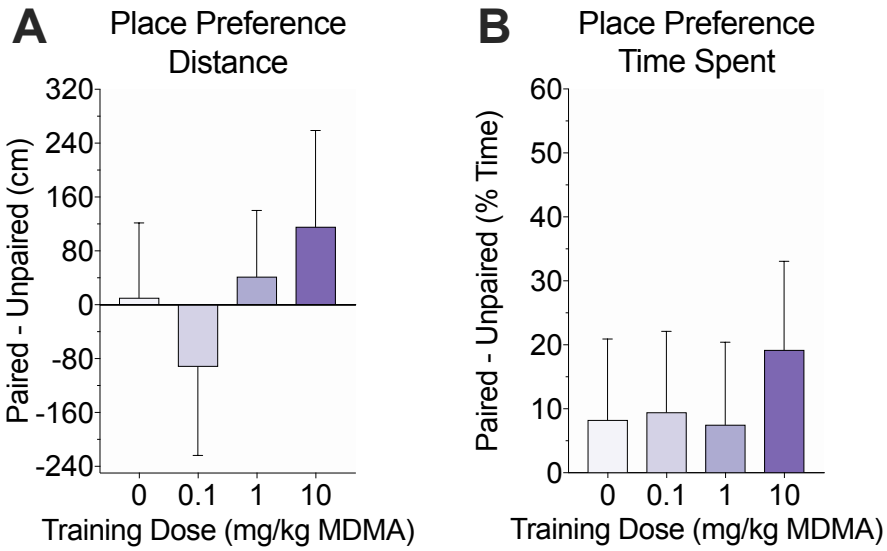

Figure 4.4 Effects of MDMA on conditioned place preference. Following 7 days of training, mice were tested off drug for place preference, which was measured by the difference in distance traveled **(A)** and time spent **(B)** on the drug-paired side versus the unpaired side. There were no significant group differences and none of the groups exhibited a significant preference for either side.

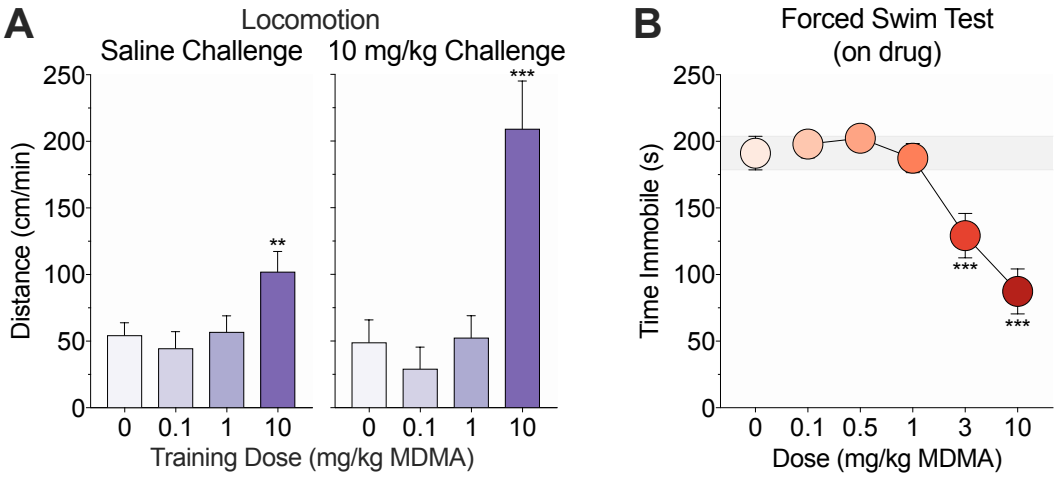

Figure 4.5 Effects of MDMA on conditioned and sensitized responding, and depressive-like behavior. (A) Following training and place preference testing, mice underwent saline **(left)** and high dose MDMA **(right)** challenge tests on the paired side and locomotion was scored to evaluate conditioned and sensitized responding, respectively. Mice trained with 10 mg/kg MDMA showed increased locomotion relative to saline controls following both challenge injections. **(B)** A separate cohort of mice underwent a 6-min on-drug forced swim test and time spent immobile was measured during the last 4 minutes of testing. Mice given 3 or 10 mg/kg MDMA exhibited reduced immobility relative to saline controls.

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CHAPTER 5

Quantifying the acoustic startle response in mice using standard digital video

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
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Quantifying the Acoustic Startle Response in Mice Using Standard Digital Video

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The startle response is an unconditional reflex, characterized by the rapid contraction of facial and skeletal muscles, to a sudden and intense startling stimulus. It is an especially useful tool in translational research for its consistency across species, simple neural circuitry, and sensitivity to a variety of experimental manipulations. The rodent acoustic startle response is commonly used to study fundamental properties of the central nervous system, including habituation, sensitization, classical conditioning, fear and anxiety, sensorimotor gating, and drug effects. The rodent startle response is typically assessed in stabilimeter chambers, and while these systems are excellent at measuring startle, they are designed only for this sole purpose. In the present study, we used the VideoFreeze system—a widely used tool for studying Pavlovian fear conditioning—to assess the acoustic startle response in freely moving mice. We validated the use of this system to quantify startle response amplitude and prepulse inhibition of startle. This is the first demonstration to date of using standard video in the automated assessment of the acoustic startle response in rodents. We believe that researchers already using the VideoFreeze system will benefit from the additional ability to assess startle without the purchase of new equipment.

Keywords: startle, prepulse inhibition, video, methods, rodent models, phenotyping, fear conditioning, neuropsychiatric disorders

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INTRODUCTION

The startle response is an unconditional reflex, characterized by the rapid contraction of facial and skeletal muscles, to a sudden and intense startling stimulus, such as a noise burst, air puff, or light flash (Landis and Hunt, 1939; Koch and Schnitzler, 1997; Berg and Balaban, 1999; Swerdlow et al., 1999). It is an especially useful tool in translational research for its consistency across species (Landis and Hunt, 1939; Bullock, 1984; Davis, 1984; Swerdlow et al., 1999), simple neural circuitry (Davis et al., 1982; Lingenhohl and Friauf, 1994; Yeomans and Frankland, 1995; Koch and Schnitzler, 1997), and sensitivity to a variety of experimental manipulations (Koch and Schnitzler, 1997; Koch, 1999; Fendt and Koch, 2013). The rodent acoustic startle response is commonly used to study fundamental properties of the central nervous system, including habituation, sensitization,

classical conditioning, fear and anxiety, sensorimotor gating, and drug effects (Groves and Thompson, 1970; Davis, 1980, 1986, 2006; Davis et al., 1982, 1993; Swerdlow et al., 1992; Pilz and Schnitzler, 1996; Koch, 1999). One important phenomenon that is used to model sensorimotor gating is prepulse inhibition (PPI), the suppression of the startle response when a weak prestimulus precedes the strong startling stimulus (Graham, 1975; Swerdlow et al., 2000; Li et al., 2009). Deficits in sensorimotor gating are important features of many neuropsychiatric disorders (e.g., schizophrenia, obsessive compulsive disorder, Huntington's disease, Tourette syndrome) (see review by Kohl et al., 2013), and thus PPI of the rodent acoustic startle response has become a leading tool for studying the pathophysiology, pharmacology, and genetics of these disorders (Swerdlow and Geyer, 1998; Swerdlow et al., 2000, 2016; Geyer et al., 2001, 2002; Powell et al., 2011; Fendt and Koch, 2013).

Assessing the startle response in rodents can be challenging given its extremely brief duration. The latency of the rodent acoustic startle response is estimated to be between 5 and 12 ms among different muscle groups (e.g., neck, hindlimb) (Ison et al., 1973; Willott et al., 1979; Davis et al., 1982; Cassella et al., 1986; Parham and Willott, 1988; Lingenhohl and Friauf, 1994; Yeomans and Frankland, 1995; Pilz and Schnitzler, 1996; Koch and Schnitzler, 1997; Carlson and Willott, 1998). Because of this challenge, the rodent startle response is typically assessed in small stabilimeter chambers that constrain animal movement (Geyer and Swerdlow, 1998; Geyer and Dulawa, 2003). This testing process can be stressful and unpleasant for animals and requires extensive habituation and calming procedures (Geyer and Swerdlow, 1998). Moreover, this chamber is designed only to measure this single behavior. Thus, the ability to measure rodent startle intensity using alternative methods such as standard video in a Skinner-type conditioning chamber could be exceptionally valuable.

In the present study, we validate the use of the VideoFreeze system (Med-Associates Inc., Georgia, VT, USA) to assess the acoustic startle response and detect PPI of this response in freely moving mice. This system was designed for the automated assessment of freezing behavior and locomotor activity using digital video (see Anagnostaras et al., 2010). Animal movement within the digital video stream is quantified using a motion index, which is generated using a proprietary motion analysis algorithm that compares successive video frames while controlling for baseline video noise on a pixel-by-pixel basis. VideoFreeze is quite sensitive in scoring rodent movements of any kind, including ultra-fine movements such as respiration. VideoFreeze samples video at 30 Hz, and at face value, it may seem that the acoustic startle response is too fast to capture using standard digital video. However, the VideoFreeze system time locks stimulus presentation with the timing of video frame acquisition, and the exposure time per frame is relatively long. Thus, it is plausible that the 30 Hz video stream would capture frames just before, during, and immediately after the startle response, and then could be used to score startle intensity. Indeed, we found that the VideoFreeze system accurately measured the startle response and PPI of this response in mice. Although the traditional floor deflection potentiometer startle systems are excellent at measuring startle responses, they are also complex, specialized only for startle, expensive, and take up lab space. We suggest this advancement could be useful for labs that already own VideoFreeze systems and may want to evaluate startle.

MATERIALS AND METHODS

Subjects

16 (8 males, 8 females) hybrid C57BL/6Jx129S1/SvImJ (Jackson Laboratory, West Sacramento, CA, USA) mice were used. Mice were weaned at 3 weeks of age and group-housed (2–5 mice per cage) with unrestricted access to food and water. The animal colony was kept on a 14:10-h light/dark schedule and all testing occurred during the light phase. Mice were at least 10 weeks old and handled for 3 days (1 min/day) prior to testing. All animal care and experimental procedures were approved by the UCSD IACUC and in compliance with the NRC 8th *Guide for the Care and Use of Laboratory Animals.*

VideoFreeze System

The VideoFreeze system (Med-Associates Inc., Georgia, VT, USA; see Anagnostaras et al., 2010) was used to assess acoustic startle. For all experiments, four mice were tested concurrently in individual chambers (32 × 25 × 25 cm), which consisted of stainless-steel side walls and rod floors, white acrylic back walls, and clear polycarbonate front and top walls. Testing chambers were illuminated with white and near-infrared light and were cleaned with 7% isopropyl alcohol. Each chamber was encased in a sound-attenuated box, and background noise (65 to 70 dB) was produced by internal ventilation fans. A broad band white noise signal generated by VideoFreeze was rerouted through a consumer amplifier (80W RMS per speaker; Denon DRA-395) and sent to consumer speakers (2.75-inch woofer, 0.5-inch tweeter; Yamaha NS-AP1400S) placed inside each chamber. Testing sessions were video recorded at a rate of 30 Hz by a standard digital camera mounted in front of each chamber and connected to a Windows computer running the VideoFreeze software (Med Associates Video Freeze Software, RRID:SCR_014574, SOF-843). VideoFreeze used this video stream to quantify animal movement via a motion index (see Motion Scoring section below).

Input/Output Function

A protocol adapted from Valsamis and Schmid (2011) was used to generate an input/output (i/o) function for our hybrid mouse colony, which represents the relationship between acoustic stimulus intensity and startle response amplitude. Mice were habituated to both the testing chambers and the acoustic stimuli twice prior to i/o function testing. The acoustic stimuli were 200 ms white noise bursts with 0 ms rise times that varied in decibel intensities. Testing began with a 4-min baseline period, followed by the presentation of one noise burst every 20 s, which started at 75 dB and increased between each presentation by 5 dB until reaching 120 dB. The 75 dB noise burst was presented four times and all other noise bursts were presented only once.

High-Speed Video

A separate observation of the startle response was conducted using a high-speed imaging system to observe the response with greater temporal resolution. A MotionBLITZ EoSens mini camera (Mikrotron, Munich, Germany) was used to record video at 1,000 Hz. Video acquisition was triggered by an output from VideoFreeze using a 28-volt to TTL converter (SG-231, Med Associates) so that the high-speed video could be correlated with the timing of startle stimulus presentation. The VideoFreeze system was running simultaneously so that the videos and data from the high-speed imaging system and the VideoFreeze system could be compared. The primary purpose of this was to ensure the startle response we were recording accords well with that recorded in standard startle chambers.

Prepulse Inhibition

A protocol adapted from Valsamis and Schmid (2011) was used to assess prepulse inhibition (PPI) of the acoustic startle response. Based on the i/o function (see Results section, **Figure 1**), the 105 dB noise burst produced significant startle and the 75 and 85 dB noise bursts produced little to no startle. Accordingly, 105 dB white noise bursts (200 ms duration, 0 ms rise time) were used as pulse stimuli and 75 or 85 dB white noise bursts (4 ms duration, 0 ms rise time) were used as prepulse stimuli.

PPI testing began with a 5-min baseline, followed by a habituation phase and then a PPI phase. The habituation phase consisted of the presentation of 30 pulses, each 20 s apart. The PPI phase consisted of 50 trials—pulse-only trials (10) and prepulse/pulse trials (40)—each 20 s apart. In the prepulse/pulse trials, the prepulse was presented prior to the pulse at an inter-stimulus interval (ISI) of 50 or 100 ms. The 50 trials were pseudorandomized into five conditions: (1) No prepulse (pulse-only), (2) 75 dB prepulse and 50 ms ISI, (3) 85 dB prepulse and 50 ms ISI, (4) 75 dB prepulse and 100 ms ISI, or (5) 85 dB prepulse and 100 ms ISI.

An additional prepulses-only experiment was conducted to determine the effect of the prepulses alone on startle. This experiment began with a 5-min baseline, followed by 20 prepulse-only trials, each 20 s apart, that alternated between 75 and 85 dB.

Motion Scoring

VideoFreeze uses a proprietary motion analysis algorithm (see (Anagnostaras et al., 2010) for full description) to calculate a motion index (in arbitrary units [au]) for each frame of video, which measures the number of changed pixels between successive video frames while ignoring pixel changes caused by video noise (primarily jitter and compression artifacts). A reference video sample is taken before an animal is placed in the conditioning chamber in order to establish the amount of baseline noise inherent to the video signal. This approach determines the number of pixels in which the intensity value is changing from frame to frame under baseline (no animal present) conditions. Once the animal is placed in the chamber, the number of pixels in which the intensity value is changing from frame to frame is compared against the baseline noise reference. The motion index represents the number of pixels that are changing from frame to frame above the baseline noise level. Consequently, a frame in which a large movement occurs results in a high motion index, and because the camera accumulates exposure across each shutter interval, this movement appears as blur in the video still image.

The maximum motion index value within a specified time frame was used to score startle amplitude, as this measure captures rapid yet significant alterations in movement that occur in response to the onset of a noise burst. The maximum motion index during the time frame of interest (i.e., during the noise burst) was normalized to the maximum motion index during a baseline period (i.e., immediately prior to the noise burst). Despite the brevity of the mouse startle response (see Introduction), it is advised to measure whole-body startle over a relatively long interval (e.g., 100 to 200 ms) after stimulus onset (Cassella et al., 1986). For the i/o experiment, normalized startle amplitude was calculated as the maximum motion index during the 200 ms *after* the onset of the noise burst minus the maximum motion index during the 200 ms *before* the onset of the noise burst. For the PPI experiment, normalized startle amplitude was calculated as the maximum motion index during the 200 ms *after* the onset of the pulse minus the maximum motion index during the 200 ms *before* the onset of the prepulse (300 to 100 ms before the onset of the pulse). For the prepulses-only experiment, normalized startle amplitude was calculated as the maximum motion index during the 200 ms *after* the onset of the prepulse minus the maximum motion index during the 200 ms *before* the onset of the prepulse.

A motion index was also calculated for each frame of the high-speed video stream. Here, each video frame (a region of interest containing the mouse) was compared to the background video (same sized region, but no mouse) on a pixel-by-pixel basis and expressed as an overall ratio, such that a motion index of 1 represents animal motion that is similar to the background level of video noise. The pseudocoloring of the video frame pixels in **Figure 3** is scaled according to how much each pixel varies from the background video signal, with brighter colors (i.e., yellow) indicating more animal motion in that region.

Statistical Analyses

Data were analyzed using repeated measures univariate analyses of variance (ANOVAs) to identify overall group differences. *Post-hoc* comparisons were performed following significant ANOVAs using Fisher's Least Significant Difference (LSD) tests against a control condition (75 dB noise burst in the i/o experiment; pulse-only, pulse/prepulse at the same intensity, and prepulse-only at the same intensity in the PPI experiment). The level of significance was set at $p \leq 0.05$ for all analyses.

RESULTS

We first explored the potential to elicit and measure the acoustic startle response using the Video Freeze system. Mice were presented with white noise burst stimuli of increasing intensities and movement was quantified via motion index scores derived from the video signal. **Figure 1** displays the i/o function for our hybrid mice, which established the average normalized startle amplitude in response to acoustic stimuli of increasing

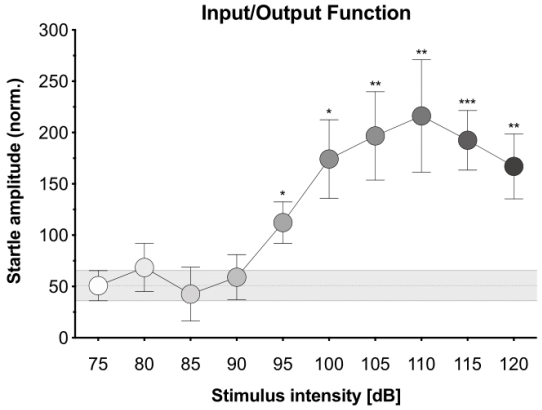

FIGURE 1 | Input/output function. The relationship between acoustic stimulus intensity and startle response amplitude for our hybrid mice. Acoustic stimuli were white noise bursts (200 ms with 0 ms rise time) of increasing intensities (four trials at 75 dB then single trials at 80–120 dB). Normalized startle amplitude was significantly enhanced at the 95, 100, 105, 110, 115, and 120 dB noise bursts relative to the 75 dB noise burst. Each point represents the mean ± 1 standard error. The gray bar indicates standard error range for the comparison 75 dB noise burst. Data points with asterisk identify significant comparisons against the 75 dB noise burst using Fisher's LSD (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

intensities (see **Supplementary Data 1** for corresponding data sheet and **Supplementary Figure 1** for scatterplot of individual animal data). Normalized startle amplitude differed significantly across stimulus intensities [$F_{(3.894,\ 58.41)} = 5.325$, $p = 0.001$]. The 95, 100, 105, 110, 115, and 120 dB noise bursts led to significantly higher normalized startle amplitudes than the 75 dB noise burst (p-values ≤ 0.012). The 80, 85, and 90 dB noise bursts had no effect on normalized startle amplitude relative to the 75 dB noise burst (p-values ≥ 0.524). To address concerns regarding the robustness of these measures in freely moving mice, we analyzed the effect of animal orientation on startle measurements. Animals were grouped by whether they were oriented forward, backward, or sideways during the 105, 110, and 115 dB noise bursts. Normalized startle amplitude did not significantly differ between animal orientations at all three stimulus intensities [see **Supplementary Figure 2**; 105 dB, $F_{(2,\ 13)} = 0.079$, $p = 0.925$; 110 dB, $F_{(2,\ 13)} = 0.2$, $p = 0.821$; 115 dB, $F_{(2,\ 13)} = 0.976$, $p = 0.403$].

To confirm that the startle amplitude increases produced by the higher-intensity noise bursts in the i/o experiment accurately reflect the mouse startle response, we analyzed startle video recordings from: (1) VideoFreeze (i/o experiment), and (2) a high-speed camera (a separate experiment). **Figure 2** is a frame-by-frame exhibition of a mouse startle response to a 105 dB, 200 ms noise burst, as recorded by VideoFreeze at 30 Hz during i/o testing (see **Supplementary Movie 1**). Each frame represents 33.33 ms of standard digital video and the VideoFreeze motion index for each frame is indicated. The 200 ms *before* (top six frames) and the 200 ms *after* (bottom six frames) the onset of the noise burst ($t = 0$ ms) are shown. Before the noise burst, baseline activity (i.e., walking) was captured by motion indexes of ≤ 93 au. The startle response was observed at $t = 66.67$ ms after the onset of the noise burst and was characterized by a rapid recoil of the head and ears, hunching of the back, and extension of the tail. Because the camera accumulates exposure across each shutter interval, this appears as a blur which is scored as a large movement by the VideoFreeze algorithm. Accordingly, at this same time point ($t = 66.67$ ms), there was a large spike in the motion index to 485 au. Nearly all of the startle response was captured within this 1 video frame except for some tail movement that was observed at $t = 100$ ms. In all, the startle response was clearly reflected by a large increase in the maximum motion index (485 au) relative to baseline (93 au), resulting in a normalized startle amplitude of 392 au.

In a separate experiment, a high-speed camera that samples video at 1,000 Hz was used alongside VideoFreeze to observe the startle response with greater temporal resolution. **Figure 3A** is a frame-by-frame exhibition of a mouse startle response to a 105 dB, 200 ms noise burst, as recorded by the high-speed imaging system (see **Supplementary Movie 2**). Each frame represents 1 ms of digital video, and every fifth frame from 20 ms before to 200 ms after the onset of the noise burst ($t = 0$ ms) is shown. Animal motion is represented by pseudocoloring of the pixels, which was scaled according to how much the pixels varied from the background video signal, such that brighter colors indicate more movement in that region. Before the noise burst, very little movement was observed. The startle response was observed from $t = 5$ ms to $t = 105$ ms and was characterized by the same nose, ear, back, and tail movements observed in **Figure 2**, which progressed from rostral to caudal. **Figure 3B** presents the motion index calculated from the high-speed video of every 1 ms from 100 ms before to 200 ms after the onset of the noise burst. **Figure 3C** presents the motion index calculated by VideoFreeze of every 33.33 ms from 100 ms before to 200 ms after the onset of the noise burst. Both the high-speed (**Figure 3B**) and VideoFreeze (**Figure 3C**) motion indexes sharply increased following the onset of the noise burst ($t = 0$ ms) and remained elevated throughout the duration of the 200 ms noise burst. These responses also coincide with the startle response observed in **Figure 3A**. In short, the high-speed (**Figure 3B**) and VideoFreeze (**Figure 3C**) motion indexes captured the startle response in a similar manner—they both rose sharply and remained elevated during the 200 ms noise burst. The motion index reported by VideoFreeze was relatively larger than that reported by high-speed camera; this is likely because most of the motion that was resolved on a millisecond basis in individual frames in the high-speed recording was captured as motion blur in a single frame in VideoFreeze. Overall, the startle response that was captured using high-speed video was also captured using standard video rates and quantified using the VideoFreeze motion index.

Lastly, we explored the potential to capture prepulse inhibition (PPI) of the startle response using the VideoFreeze system. Mice were presented with pulse stimuli (200 ms, 105 dB) alone or preceded by a prepulse stimulus (4 ms, 75 or 85 dB, 50 or 100 ms prior to the pulse). In a separate

experiment, mice were presented with prepulse stimuli (4 ms, 75 or 85 dB) alone. The pulse and prepulse intensities were selected because the 105 dB noise burst produced significant startle and the 75 and 85 dB noise bursts produced little to no startle during i/o testing (see **Figure 1**). **Figure 4** displays the average normalized startle amplitude elicited by the pulse-only, prepulse/pulse, and prepulse-only stimuli (see **Supplementary Data 2** for corresponding data sheet and **Supplementary Figure 3** for scatterplot of individual animal data). Normalized startle amplitude differed significantly across

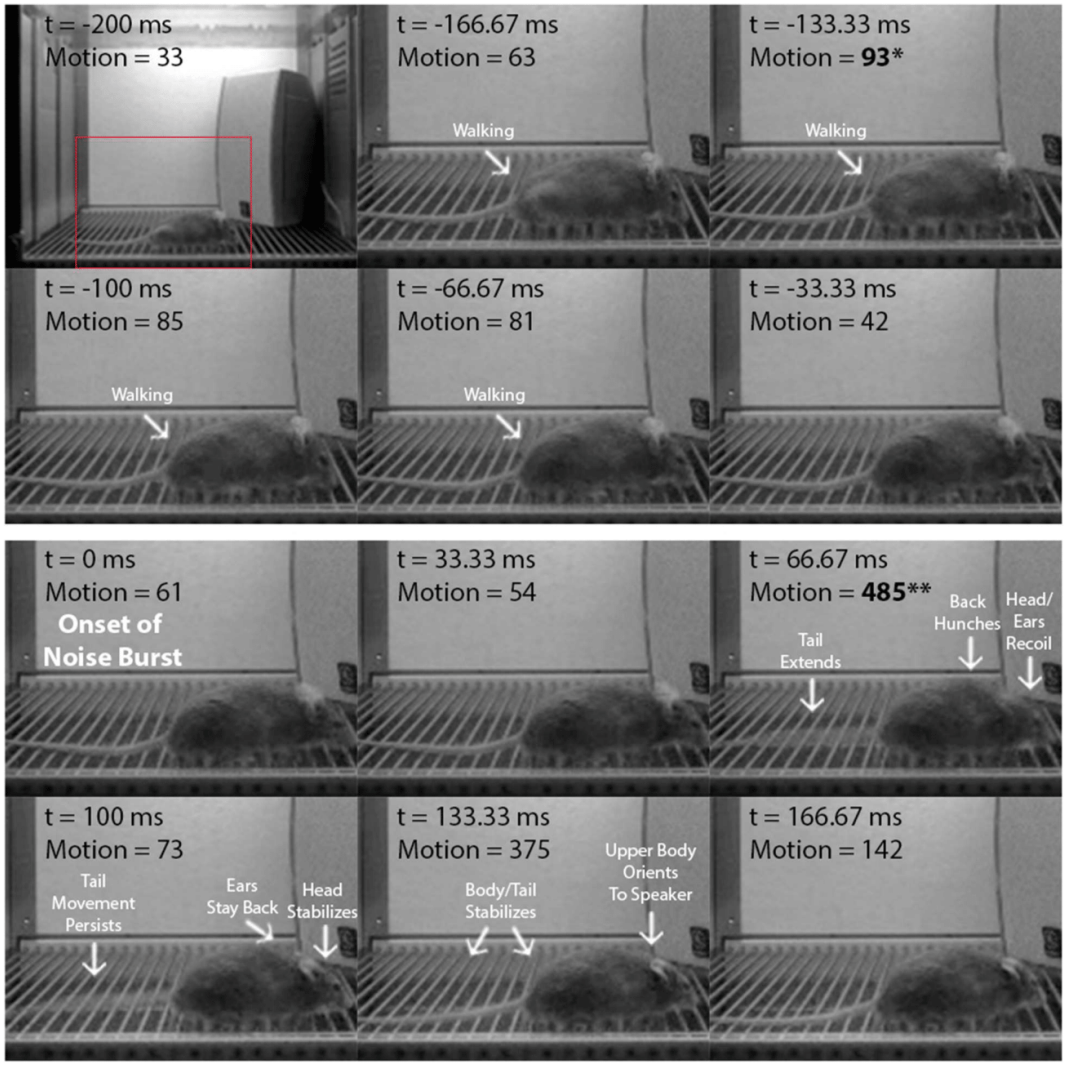

FIGURE 2 | The startle response as captured by VideoFreeze. Frame-by-frame video still images showing a mouse from 200 ms before to 200 ms after the onset of the 105 dB, 200 ms white noise burst in the i/o experiment. Digital video was recorded at 30 Hz, so each frame represents 33.33 ms of video. Time (t) is relative to the onset of the noise burst ($t = 0$ ms). Motion represents the motion index score calculated by the VideoFreeze system. Top six frames include the 200 ms before the onset of the noise burst (maximum motion index = 93 au, indicated by asterisk) and bottom six frames include the 200 ms after the onset of the noise burst (maximum motion index = 485 au, indicated by double-asterisk). For this example, normalized startle amplitude (maximum motion index after minus before onset) = 392 au. Before the noise burst, baseline activity was captured by motion indexes of ≤ 93 au. At $t = 66.67$ ms, there was a large spike in the motion index to 485 au, and the startle response was observed and characterized by significant head, ear, back, and tail movements (body parts in motion appear blurry in image). At $t = 100$ ms, the motion index decreased to 73 au, as movement was observed in the tail only. The startle response concluded by $t = 133.33$ ms.

stimuli conditions [$F_{(5.12,\ 814)} = 8.789$, $p < 0.001$]. Compared to the pulse-only condition, the presentation of a prepulse immediately prior to the pulse significantly reduced normalized startle amplitude (p-values ≤ 0.007). Within the two 75 dB prepulse/pulse conditions, the 100 ms ISI led to a significantly higher normalized startle amplitude than the 50 ms ISI ($p = 0.047$). There were no significant differences between the other prepulse/pulse conditions (p-values ≥ 0.198). Normalized startle amplitude was significantly higher at the 75 dB, 100 ms ISI (but not 50 ms ISI) prepulse/pulse condition relative to the 75 dB prepulse-only condition ($p < 0.001$) and at both 85 dB prepulse/pulse conditions relative to the 85 dB prepulse-only condition (p-values ≤ 0.03). Normalized startle amplitude did not differ between the 75 dB, 50 ms ISI prepulse/pulse condition and the 75 dB prepulse-only condition ($p = 0.124$).

DISCUSSION

Here, we demonstrate the ability to use the VideoFreeze system to elicit and measure the acoustic startle response and PPI of this response in freely moving mice. Mice were first presented with 200 ms white noise bursts of increasing intensities and exhibited no startle responses to lower-intensity stimuli (75 dB to 90 dB) but significant startle responses to higher-intensity stimuli (95 to 120 dB) (**Figure 1**). We quantified startle amplitude using VideoFreeze's automated assessment of animal movement. Specifically, the maximum motion index during the noise burst was normalized against the maximum motion index immediately prior to the noise burst, which captured rapid yet substantial increases in movement relative to a moving baseline. Similar to previous reports (Valsamis

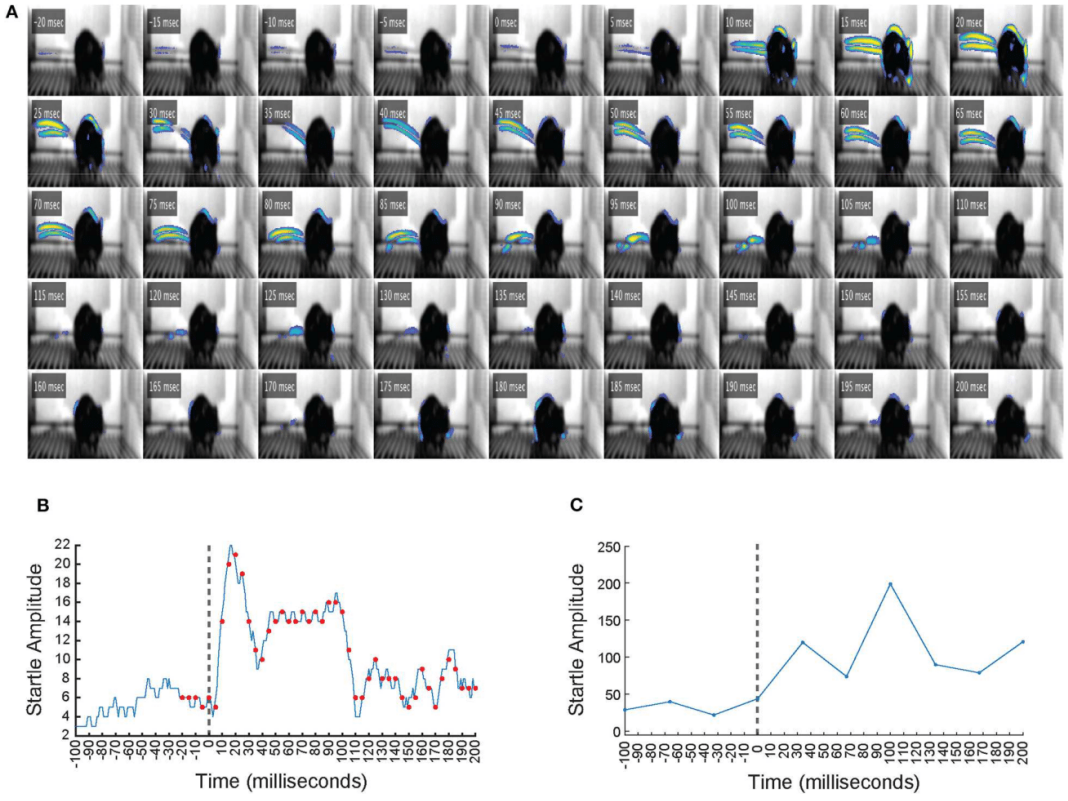

FIGURE 3 | The startle response as captured by a high-speed imaging system. **(A)** Frame-by-frame video still images showing a mouse from 20 ms before to 200 ms after the onset of a 105 dB, 200 ms white noise burst. Digital video was recorded at 1,000 Hz, so each frame represents 1 ms of video. Time (t) indicated is relative to the onset of the noise burst ($t = 0$ ms). Animal motion is represented by pseudocoloring of the pixels, such that brighter colors indicate more movement in that region. The startle response was observed from $t = 5$ ms to $t = 105$ ms and was characterized by significant nose, ear, back, and tail movements that progressed from rostral to caudal. **(B)** The motion index calculated from the high-speed video of every 1 ms from 100 ms before to 200 ms after the onset of the noise burst. Vertical dashed line indicates the onset of the white noise burst at $t = 0$ ms. Red dots represent the time points of the video frames displayed in **(A)**. Startle amplitude showed a large increase between $t = 0$ ms and $t = 200$ ms. **(C)** The motion index calculated by VideoFreeze of every 33.33 ms from 100 ms before to 200 ms after the onset of the noise burst. Vertical dashed line indicates the onset of the white noise burst at $t = 0$ ms. Startle amplitude showed a large increase between $t = 0$ ms and $t = 200$ ms.

and Schmid, 2011), mice began to startle at 95 dB, and startle amplitude increased with increasing stimulus intensity until reaching a plateau of maximum startle amplitude at 110 dB. The mouse startle response was characterized by significant nose, ear, back, and tail movements that were observed using standard video of 30 Hz and captured quantitively by the normalized startle amplitude (**Figure 2**, **Supplementary Movie 1**). In the video still images, the startle response appears as motion blur because the VideoFreeze standard camera temporally integrates all of the motion that occurs over a single frame of 33.33 ms. We believe it is precisely because of this motion blur that VideoFreeze is able to capture and quantify the startle response.

The mouse startle response was also observed using high-speed video of 1,000 Hz, which appeared similar to the response captured by standard video, yet the progression of movement from rostral to caudal was more evident (**Figure 3A**, **Supplementary Movie 2**). We compared motion indexes from the high-speed video (**Figure 3B**) and from VideoFreeze (**Figure 3C**), and found that despite the differences in video rates, both measures captured the intensity of the mouse startle response observed in **Figure 3A**. Specifically, the startle response was reflected by a sharp increase in the high-speed and VideoFreeze motion indexes during the startling stimulus. This comparison to high-speed video serves to reinforce that the signal measured in VideoFreeze is in-line with what one would expect based on the higher temporal resolution imaging signal. In addition to observing and quantifying the mouse startle response, we demonstrated the ability to capture prepulse inhibition of acoustic startle response using VideoFreeze. Normalized startle amplitude in response to a strong pulse (200 ms, 105 dB white noise burst) was significantly reduced when the pulse was preceded by a weak prepulse (4 ms, 75 dB or 85 dB white noise burst; 50 ms or 100 ms ISI) (**Figure 4**).

There can be unexpected variability in motion index scores between individual animals or between trials, however in our experience, a sample size of 16 mice with 1 trial per i/o condition and 10 trials per PPI condition was sufficient for averaging out this variability and detecting startle and PPI (see **Supplementary Figures 1, 3** for scatterplots of individual animal data). Future experiments with different parameters (e.g., animal strain, age, size) may introduce more variability and require larger sample sizes and/or more trials.

While this is the first demonstration of using VideoFreeze to quantify the startle in mice, Kirshenbaum et al. (2019) validated the use of VideoFreeze to track and quantify startle and modifications of startle (e.g., PPI and habituation) in zebrafish. Other than this, there are relatively few previous reports of using video to measure the startle response. High-speed video has been used to capture the startle response in various species of fish (Wieland and Eaton, 1983; Hale, 2000; Rice et al., 2011; Chicoli et al., 2014; Hale et al., 2016). High-speed video (Derakhshani and Lovelace, 2010; Bernard et al., 2013) and standard video (Essex et al., 2003; Vousdoukas et al., 2012; Cosić et al., 2016) have also been used in the automated analysis of eye blinks in response to startling stimuli in humans. High-speed video has also been used in conjunction with a piezoelectric startle plate to measure the acoustic startle response in mice (Grimsley et al., 2015), and standard video has been used to detect but not quantify the acoustic startle response in rats (Tovote et al., 2005). Thus, this is the first demonstration of using standard video in the automated assessment of the acoustic startle response in rodents.

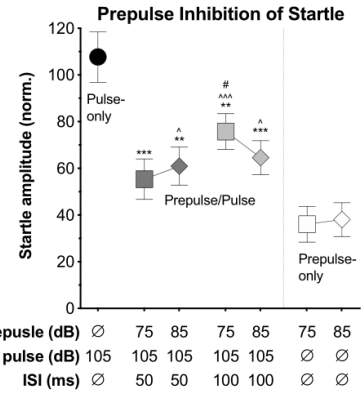

FIGURE 4 | Prepulse inhibition (PPI). Average normalized startle amplitude elicited by the pulse alone, the pulse when preceded by a prepulse, or the prepulse alone. The pulses were 200 ms, 105 dB white noise bursts with a 0 ms rise time. The prepulses were 4 ms, 75 or 85 dB white noise bursts with a 0 ms rise time. The inter-stimulus intervals (ISI) between the prepulse and pulse on prepulse/pulse trials were either 50 or 100 ms. Normalized startle amplitude was significantly lower at all prepulse/pulse conditions relative to the pulse-only condition. Within the two 75 dB prepulse/pulse conditions, normalized startle amplitude was significantly higher at the 100 ms ISI relative to the 50 ms ISI. Normalized startle amplitude was significantly higher at the 75 dB, 100 ms ISI prepulse/pulse condition relative to the 75 dB prepulse-only condition and at both 85 dB prepulse/pulse conditions relative to the 85 dB prepulse-only condition. Starred data points identify significant comparisons against the pulse-only condition (**$P < 0.01$, ***$P < 0.001$), the prepulse/pulse condition at the same intensity (dB) (#$P < 0.05$), and the prepulse-only condition at the same intensity (dB) (^$P < 0.05$, and ^^^$P < 0.001$) using Fisher's LSD.

The VideoFreeze system is a versatile behavioral testing apparatus that is used extensively to study Pavlovian fear conditioning in rodents (Anagnostaras et al., 2010), and as shown here, may also be a valuable tool for studying startle response. In addition to the capabilities already described, the VideoFreeze system is equipped to present the sound, light, and footshock stimuli required in various startle paradigms (e.g., fear-potentiated startle). Dedicated equipment using stabilimeters still may be more precise than VideoFreeze in assessing startle, and may be a better option for certain experiments such as those requiring high temporal resolution. Nevertheless, we believe that researchers already using the VideoFreeze system will benefit from the additional ability to assess startle in a freely behaving animal without the purchase of new equipment.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee, University of California San Diego.

AUTHOR CONTRIBUTIONS

MP, GH, and SA contributed to the conception and design of the study as well as data interpretation. MP, GH, and KV contributed to data acquisition. MP and GH contributed to data analysis. MP wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2020.00083/full#supplementary-material

Supplementary Movie 1 | The startle response as captured by VideoFreeze.

Supplementary Movie 2 | The startle response as captured by a high-speed imaging system.

Supplementary Data 1 | Data sheet corresponding to **Figure 1** (Input/output function) in the article.

Supplementary Data 2 | Data sheet corresponding to **Figure 4** (Prepulse inhibition) in the article.

Supplementary Figure 1 | Input/output function with individual animal data.

Supplementary Figure 2 | Startle amplitude by animal orientation.

Supplementary Figure 3 | Prepulse inhibition (PPI) with individual animal data.

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Chapter 5, in full, is a reprint of the material as it appears in Quantifying the acoustic startle response in mice using standard digital video. *Frontiers in Behavioral Neuroscience,* 14, 83. Pantoni, M. M., Herrera, G. M., Van Alstyne, K. R., and Anagnostaras, S. G. (2020). DOI: 10.3389/fnbeh.2020.00083. The dissertation author was the primary investigator and author of this paper.

CHAPTER 6

Looking beyond the classical psychedelics,
entactogens, and stimulants in neuropsychiatry

Neuropsychiatric drug development is facing an ongoing crisis that has previously been discussed (Paul et al., 2010; Brady and Insel, 2011; Chandler, 2013; Hyman, 2013). Neuropsychiatric disorders are the leading cause of disability in the U.S. and the third leading cause of disability worldwide (US Burden of Disease Collaborators, 2013; Whiteford et al., 2013). The National Institute of Mental Health estimates that the economic burden associated with serious mental illness is $317 billion per year (Insel, 2008). Although one in six U.S. adults takes at least one psychotherapeutic agent (MEPS, 2018), people suffering from neuropsychiatric disorders still face serious unmet medical needs due to existing treatments being inadequate, having serious adverse effects, or lacking entirely (Scavone et al., 2019). For example, unsuccessful treatment of bipolar disorder contributes to a rate of suicide that is 15 to 30 times higher than the general population (Bauer et al., 2018); up to 50 to 60 percent of depressed individuals do not achieve adequate response following antidepressant treatment (Fava, 2003); and one- to two-thirds of children with attention deficit hyperactivity disorder (ADHD) cannot adhere to psychostimulant treatment because of intolerable side effects or poor access to controlled medications (Charach and Gajaria, 2008). Lastly, other core symptoms remain completely untreated such as social deficits in autism or cognitive deficits in schizophrenia (Gray and Roth, 2007; Ghosh et al., 2013). Although the pharmaceutical industry has invested tremendous amounts of time and money into neuropsychiatric drug development, there have been few meaningful advances over the last three decades (Paul et al., 2010; Hyman, 2013). As such, in recent years, most of the leading pharmaceutical companies have significantly or entirely cut back from this therapeutic area (Miller, 2010; Abbott, 2011;

Pankevich et al., 2013; Kesselheim et al., 2015). It is very troubling that neuropsychiatry's skyrocketing disease burden is paralleled by plummeting drug development efforts (Chandler, 2013). Innovative approaches are critically needed to transform this field and fulfill demands for safe and effective psychotherapeutic drugs (Insel et al., 2013; Pankevich et al., 2013).

Monoaminergic-based drugs have historically been a primary focus of neuropsychiatric drug discovery efforts. The earliest monoaminergic psychotherapeutics — e.g., amphetamine (a psychostimulant), reserpine (an antidepressant), imipramine (an antidepressant), and chlorpromazine (an antipsychotic) — resulted from serendipitous clinical findings in the mid-20th century (Bradley, 1937; Kline, 1954; Lehmann and Hanrahan, 1954; Kuhn, 1958). These successes were followed by many "me-too" structural analog drugs that offered minor improvements in side effect profiles without real improvements in efficacy (Bokhari and Fournier, 2013; Pereira and Hiroaki-Sato, 2018; Aronson and Green, 2020). While this approach led to a plethora of drugs that are useful today, their mechanisms of action remain poorly understood (Hyman, 2012). A greater understanding of the mechanisms underlying the therapeutic and adverse effects of existing drugs could facilitate the development of new and improved drugs among a "gold mine" that is presumed to have run dry.

Our lab has taken a systematic approach to better understanding the mechanisms of monoaminergic drugs to advance neuropsychiatric drug development. Specifically, we have tested a range of inhibitors and reversers of the dopamine (DAT), norepinephrine (NET), and serotonin (SERT) transporters at both low and high doses on various behavioral

outcomes in mice (**Table 6.1**). By comparing drugs that non-selectively target the monoamine transporters (e.g., the classical psychostimulants of amphetamine, methylphenidate, and cocaine) to more selective inhibitors of DAT, NET, and SERT (e.g., bupropion, atomoxetine, and citalopram, respectively), it is evident that both DAT and NET inhibition is likely responsible for the therapeutic, memory-enhancing effects of low-dose psychostimulants, and that high affinity DAT inhibition is likely responsible for the adverse, reinforcing effects of high-dose psychostimulants (Carmack et al., 2014). Using a combination of bupropion and atomoxetine, we tested and confirmed our hypothesis that a combination of a low affinity DAT inhibitor and a high affinity NET inhibitor would produce long-term memory enhancement but not reinforcement (Pantoni et al., 2020; see Chapter 2). This is just one example of how a systematic approach can lead to novel, optimized leads that retain or lack specific therapeutic or adverse effects.

One of the largest challenges in neuropsychiatric drug development has been the development of brand-new drugs for untreated indications. Most current psychotherapeutics were discovered serendipitously or through a "me-too" approach that utilized existing therapeutic drugs as templates (Fibiger, 2012). Yet, a long history of recreational drug use also provides a wealth of valuable information for developing novel neuropsychiatric drugs. Indeed, the border between legal, therapeutic drugs and illicit, recreational drugs has been fluid throughout history (Sneader, 2005). We are currently in the midst of one such tipping point recognized as a "psychedelic renaissance" (Sessa, 2012, 2018; Kelly et al., 2019; Nutt, 2019). Scientific interest in the therapeutic benefits of psychedelics, entactogens, and other illicit compounds is booming (Rucker et al., 2018;

Andersen et al., 2020; Nutt and Carhart-Harris, 2020; Vollenweider and Preller, 2020; Inserra et al., 2021). A growing assembly of scientists, clinicians, patients, and investors project that these compounds may revolutionize neuropsychiatric care as we know it today (Pollan, 2019), although their approach to drug selection has been opportunistic rather than systematic.

Psychedelics such as psilocybin, LSD (lysergic acid diethylamide), and DMT (N,N-dimethyltryptamine) are drugs that produce changes in perceived reality and an apparent expansion of consciousness (Leary et al., 1966). Theories suggest that psychedelics enhance neural plasticity and allow the revision of entrenched patterns of thought and behaviors that maintain psychopathological conditions (Carhart-Harris and Friston, 2019). As such, psychedelics may be especially useful for treating general treatment-resistance in various conditions such as major depressive disorder, addiction, and psychological sequelae in terminal illness (Rucker et al., 2018). Empathogen-entactogens such as MDMA (3,4-methylenedioxymethamphetamine) are commonly referred to as psychedelics, but in fact are a categorically distinct class of drugs that primarily increase feelings of empathy, social connectedness, and benevolence towards others (Greer and Tolbert, 1986; Nichols, 1986). Because of their seemingly unique prosocial effects, entactogens may be especially useful as an adjunct to psychotherapy or for treating disorders in which social behavior is impaired such as autism spectrum disorder, social anxiety, and certain personality disorders (Decety and Moriguchi, 2007; Heifets and Malenka, 2016). Together, psychedelics and entactogens have potential to tackle some of the most critical unmet medical needs in neuropsychiatry.

A few psychedelic and entactogenic compounds are currently in various stages of the U.S. Food and Drug Administration (FDA) drug approval process for an array of severe and poorly treated conditions (ClinicalTrials.gov, 2021). Psilocybin and LSD are being investigated as psychotherapy adjuncts or stand-alone treatments in numerous Phase 2 clinical trials, including psilocybin for unipolar and bipolar depressive disorders, cluster headache, anorexia nervosa, body dysmorphic disorder, and alcohol or cocaine use disorders, as well as LSD for major depressive disorder, anxiety disorders, cluster headache, and anxiety in severe somatic diseases. MDMA is also being investigated as a psychotherapy adjunct in Phase 2 clinical trials for anorexia nervosa and binge-eating disorder, social anxiety in autistic adults, and anxiety associated with life-threatening illnesses including cancer, as well as in Phase 3 clinical trials for post-traumatic stress disorder. Other Phase 2 clinical trials include DMT (a rapid-acting psychedelic) for depression and ibogaine (a psychedelic with dissociative properties) for methadone detoxification and alcoholism. To date, the first and only psychedelic-like compound to have reached the psychiatric market is esketamine, a dissociative hallucinogen and S(+) enantiomer of ketamine (Kim et al., 2019). Esketamine (Spravato) was approved by the FDA for treatment-resistant depression in 2019 and for major depressive disorder with accompanying suicidal ideation in 2020 (Janssen Pharmaceuticals, 2020). In fact, this NMDA receptor antagonist was the first mechanistically novel psychiatric drug that had been developed in over 30 years (Potter et al., 2020).

The “psychedelic renaissance” has primarily been focused on the following four drugs: psilocybin, LSD, MDMA, and ketamine. Despite these compounds demonstrating

improved efficacy relative to existing psychotherapeutics (e.g., Feduccia et al., 2019), they too pose their own challenges. LSD is extremely potent and may have a narrow range of safe and effective doses (Nichols, 2018). Psilocybin, while less potent, is a naturally occurring prodrug that is difficult and expensive to cultivate and extract in reliable quantities and also challenging to chemically synthesize (Milne et al., 2020). MDMA can produce substantial cardiac, neural, hepatic, and hyperpyrexic toxicity, even acutely (Kalant, 2001). Ketamine and esketamine often produce states of severe dissociation and adverse patient experiences (Gastaldon et al., 2019), and potentially produce diffuse brain damage (Wang et al., 2013). The reason why we have landed on these few drugs seems random but is likely because of their early identification, enthusiastic case reports of their therapeutic utility, and now, knowledge gained by their widespread recreational use (Sessa, 2016). Nevertheless, there is no reason to limit our search for candidate psychotherapeutics to these few well-known compounds.

Hundreds of related compounds have been discovered since the introduction of psilocybin, LSD, and MDMA (for examples, see **Figure 6.1** and **Figure 6.2**). Alexander (Sasha) Shulgin and David Nichols have been two key leaders in the fields of psychedelic research and rational drug design. Shulgin is known for the discovery, synthesis, and personal bioassay of over 230 psychoactive drugs for their psychedelic and entactogenic potential. Similarly, Nichols is known for the synthesis and reporting of a number of important psychedelics, stimulants, and entactogens. The books PiHKAL and TiHKAL ("Phenethylamines and Tryptamines I Have Known And Loved"), written by Shulgin and his wife, systematically detail hundreds of the compounds discovered by him or the Nichols

lab (Shulgin and Shulgin, 1991, 1997). Together, these books provide an especially valuable menu of potential psychotherapeutics. Still, little is known about these compounds beyond what is published in Shulgin's books, despite that many have recently become trending illicit "designer" drugs (Sexton et al., 2020). It is quite possible that these largely unexplored compounds are therapeutically valuable, and thus, they should be explored further.

Most of the compounds discovered by Shulgin and Nichols are monoaminergic phenethylamine, tryptamine, or lysergamide derivatives with varying degrees of psychedelic, entactogen, and/or stimulant properties. Depending on their behavioral properties, they may be useful for different clinical purposes — psychedelic effects for treatment-resistance, entactogenic effects for social dysfunction, and stimulant effects for cognitive dysfunction. Indeed, many neuropsychiatric disorders are characterized by varying degrees of these symptoms. **Figure 6.3** is a speculative model that we created to depict the possible clinical uses of various classical and novel monoaminergic drugs. The inner triangle ranks the degree to which various known drugs produce psychedelic, entactogen, and/or stimulant effects. The outer triangle ranks the degree to which various neuropsychiatric conditions produce treatment-resistance, social dysfunction, and/or cognitive dysfunction. Depending on the core symptoms of a given condition, patients may benefit from pure or mixed psychedelic, entactogen, and/or stimulant effects. Potential uses for pure effects may include: psychedelics for treatment-resistance in terminal illnesses or suicidal ideation, entactogens for social dysfunction in several personality disorders (e.g., schizoid, paranoid, antisocial, narcissistic) or social anxiety, and stimulants for cognitive

dysfunction in ADHD or learning disorders. Potential uses for mixed effects may include: psychedelic-entactogens for treatment-resistance and social dysfunction in emotional disturbance or intermittent explosive disorder, entactogen-stimulants for social and cognitive dysfunction in autism or conduct disorders, and stimulant-psychedelics for cognitive dysfunction and treatment-resistance in depression or addiction.

Although many pure psychedelics (e.g., LSD, psilocybin) and stimulants (e.g., amphetamine, methylphenidate) have been investigated as therapeutics, there may be even safer or more effective drugs in these classes. Pure entactogens, on the other hand, have been studied very little. Even MDMA is not a pure entactogen, as it has some psychedelic and stimulant properties (Nichols, 1986). There are a range of potential entactogens with little to no psychedelic or stimulant properties that may target social dysfunction more precisely. The potential clinical applications of these alternative entactogens have already been reviewed (Oeri, 2020). Besides MDMA, other mixed-effect drugs have been studied very little, though this concept is not novel. The first Phase 1 clinical trial examining LSD and MDMA co-administration recently began at the University Hospital in Basel, Switzerland (ClinicalTrials.gov, 2021, NCT04516902). It is possible that a single drug with both psychedelic and entactogen properties may elicit these same desired effects but lack the complications that may arise from administering two drugs in combination. Some mixed-effect compounds that appear especially promising are a part of Shulgin's self-rated most important phenethylamine compounds, the so-called "magical half dozen" (i.e., mescaline, DOM, 2C-B, 2C-E, 2C-T-2, 2C-T-7; Shulgin and Shulgin, 1991). It is likely that there are many more drugs in these broad classes that have yet to be discovered.

A broad effort to systematically analyze the drugs in **Figure 6.3** in a similar manner to how we analyzed the drugs in **Table 6.1** is critically needed. Such information is especially needed for the classical drugs (e.g., psilocybin, LSD, MDMA) that skipped the preclinical stage of drug development and have not been particularly well characterized in animals (Murnane, 2018). We have already begun to backfill this information for MDMA. Our systematic review of existing literature (Pantoni and Anagnostaras, 2019; see Chapter 3) and in-house experiments (see Chapter 4) revealed that *dose* critically mediates the adverse behavioral effects of MDMA in animal models. Specifically, preclinical evidence suggests that high-dose MDMA (≥ 3 mg/kg) impairs cognition and may have a high addictive potential, while low- to moderate-dose MDMA (< 3 mg/kg), which has been administered in recent clinical studies (approximately 1–2 mg/kg; Feduccia et al., 2018), does not. A practical next step could be to identify a compound that mimics the therapeutic effects of low-dose MDMA but not the adverse effects of high-dose MDMA, similar to how we found that a combination of atomoxetine and bupropion can mimic the memory-enhancing but not the reinforcing effects of psychostimulants (Pantoni et al., 2020; see Chapter 2). The R(-) enantiomer of MDMA may be one such candidate (Fantegrossi, 2008; Curry et al., 2018; Pitts et al., 2018).

The systematic analysis of the classical psychedelics and entactogens should then be extended to the novel, largely unexplored compounds in **Figure 6.3**. Additional compounds (e.g., psychedelics, entactogens, stimulants), drug targets (e.g., serotonin, dopamine, and adrenergic receptors), and behavioral effects (e.g., sociality, cognitive flexibility) can be added as needed. Together, these efforts will facilitate an unprecedented

understanding of the therapeutic and adverse effects of these drugs as well as the pharmacological mechanisms underlying their behavioral effects. Such knowledge can then be used to identify therapeutic candidates or to discover novel, optimized drugs. The anti-migraine triptan medications resulted from a similar, mechanistic analysis of psychedelic-induced migraine relief. Sumatriptan (Imitrex), for example, is a derivative of DMT that reduces migraines by selectively binding to serotonin 5-HT1D receptors, but it does not produce hallucinations as it does not bind to serotonin 5-HT2A receptors (see **Figure 6.2**; Cameron and Olson, 2018; National Center for Biotechnology Information, 2021). In all, this bottom-up approach critically mirrors the typical drug discovery and development process, minimizes risk, and maximizes the likelihood of successful FDA approval (Lipsky and Sharp, 2001).

The novel compounds that appear to be most promising and should be prioritized include: Shulgin's "magical half dozen" (mescaline, DOM, 2C-B, 2C-E, 2C-T-2; 2C-T-7 is excluded because of reported dangerous effects), the pure entactogens (MBDB, MDEA, MDAI, 5-IAI, α-ET), the entactogen-stimulant cathinones (ethylone, methylone, butylone), a less intense LSD-like drug (LSM-775, AL-LAD, or MiPLA), as well as 6-APB, 4-FA, and 2-FMA (see **Figure 6.1** and **Figure 6.2**). Likewise, the neuropsychiatric conditions that have been most neglected and should be prioritized include: the personality disorders, autism, emotional disturbance, intermittent explosive disorder, conduct/oppositional defiant disorders, depersonalization, and suicidal ideation. The importance of including a wide range of doses in these studies must also be emphasized. Drugs that produce therapeutic effects at doses far below those that produce serious adverse

effects will by far have the strongest clinical potential, especially in sensitive patient populations.

Overall, psychedelics, entactogens, and related compounds are showing great promise as therapeutics for some of the most critical unmet medical needs in neuropsychiatry, including treatment-resistance and social dysfunction. While the current "psychedelic renaissance" is focused on psilocybin, LSD, and MDMA, there are hundreds of largely unexplored monoaminergic drugs with psychedelic, entactogen, and/or stimulant properties and some may have similar or even greater therapeutic potential. As such, this abundant menu of compounds offers hopeful prospects amidst the current neuropsychiatric drug development crisis. A broad effort to systematically analyze these compounds could lead to a robust pipeline of new drugs for a range of untreated and poorly treated neuropsychiatric disorders.

Chapter 6, in full, is currently being prepared for submission for publication of the material. Pantoni, M. M., and Anagnostaras, S. G. The dissertation author was the primary investigator and author of this paper.

Table 6.1 Binding affinities and behavioral effects of monoamine transporter inhibitors and reversers (Revisited).

[a]Actions of methylphenidate, cocaine, atomoxetine, bupropion, and citalopram as transporter inhibitors and of d-amphetamine and MDMA as transporter reversers are previously reviewed (Kristensen et al., 2011).

[b]Published K_i values are shown for methylphenidate, d-amphetamine, cocaine, bupropion, citalopram (Richelson and Pfenning, 1984), atomoxetine (Wong et al., 1982), and MDMA (Rothman et al., 2001) in the rat brain. Please note low K_i values indicate high affinity. Binding affinities of combined atomoxetine/bupropion are represented symbolically: (+) low affinity, (++) high affinity, (–) negligible affinity.

[c](↑) The drug elevates locomotor activity at the specified dose; (↓) the drug decreases locomotor activity; (–) no effect; (?) the drug effect is not known; (†) the drug effect is inconclusive.

[d](↑) The drug increases addictive potential at the specified dose; (–) no known addictive potential; (?) the drug effect is not known; (†) the drug effect is inconclusive.

[e](↑) The drug enhances memory at the specified dose; (↓) the drug impairs memory; (–) no effect; (?) the drug effect is not known.

[f](↓) The drug has antidepressant efficacy at the specified dose; (?) the drug effect is not known.

[g]Methylphenidate's locomotor, reinforcing, and memory effects are previously published (Figs. 1 and 2 in Carmack et al., 2014).

[h]d-Amphetamine's locomotor, reinforcing, and memory effects are previously published (Figs. 1 and 3 in Wood and Anagnostaras, 2009; Fig. 4 in Carmack et al., 2014).

[i]Cocaine's locomotor, reinforcing, and memory effects are previously published (Figs. 1 and 3 in Wood et al., 2007; Fig. 4 in Carmack et al., 2014).

[j]Atomoxetine's locomotor, reinforcing, and memory effects are previously published (Figs. S1 and S2 in Carmack et al., 2014); its locomotor and memory effects are also depicted in Fig. 2.1.

[k]Bupropion's locomotor and memory effects are previously published (Fig. S1 in Carmack et al., 2014) and also depicted in Fig. 2.1; its reinforcing and antidepressant effects are reported in Wellbutrin's FDA approved labeling (GlaxoSmithKline, 2011).

[l]Citalopram's locomotor and memory effects are previously published (Fig. S1 in Carmack et al., 2014); its reinforcing and antidepressant effects are reported in Celexa's FDA approved labeling (Forest Laboratories, 2011).

[m]Combined atomoxetine/bupropion's locomotor, reinforcing, and memory effects are depicted in Figs. 2.2 and 2.4.

[n]MDMA's locomotor, reinforcing, memory, and antidepressant effects are depicted in Figs. 3.11 and 4.1–4.5. Note that high-dose MDMA elevated locomotor activity in females only and produced behavioral sensitization but not conditioned place preference.

		Binding affinity (K_i)[b]				Behavior			
Drug	Action[a]	DAT (nM)	NET (nM)	SERT (nM)	Dose	Locomotion[c]	Reinforcement[d]	Memory[e]	Depression[f]
Methylphenidate[g]	Inhibit	160	40	22,000	Low	–	–	↑	?
					High	↑	↑	↓	?
D-Amphetamine[h]	Reverse	82	50	1840	Low	–	–	↑	?
					High	↑	↑	↓	?
Cocaine[i]	Inhibit	270	155	180	Low	↑	–	↑	?
					High	↑	↑	↓	?
Atomoxetine[j]	Inhibit	1600	1.9	750	Low	–	–	–	?
					High	↓	↑	–	?
Bupropion[k]	Inhibit	630	2300	15,600	Low	–	–	–	↓
					High	–	?	↓	?
Citalopram[l]	Inhibit	28,000	4000	1.3	Low	–	–	–	↓
					High	–	?	↓	?
Atomoxetine/ Bupropion[m]	Inhibit	+	++	–	Low	–	–	↑	?
					High	?	?	?	?
MDMA[n]	Reverse	1572	462	238	Low	–	–	–	–
					High	†	†	↓	↓

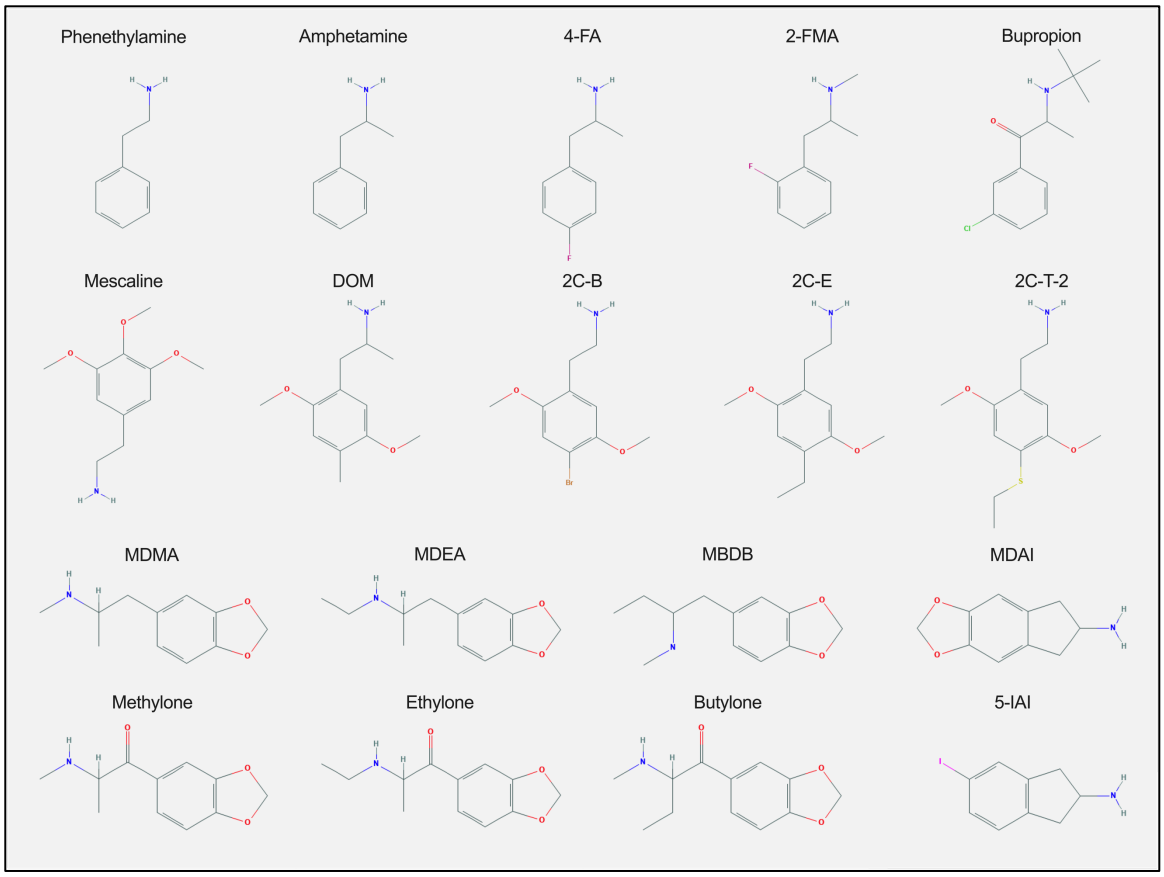


Figure 6.1 Chemical structures of select phenethylamines. DOM, 2,5-dimethoxy-4-methylamphetamine; MBDB, N-methyl-1,3-benzodioxolylbutanamine; MDAI, 5,6-methylenedioxy-2-aminoindane; MDEA, 3,4-methylenedioxy-N-ethylamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; 2-FMA, 2-fluoromethamphetamine; 2C-B, 4-bromo-2,5-dimethoxyphenethylamine; 2C-E, 2,5-dimethoxy-4-ethylphenethylamine; 2C-T-2, 2,5-dimethoxy-4-ethylthiophenethylamine; 4-FA, 4-fluoroamphetamine; 5-IAI, 5-iodo-2-aminoindane. (https://pubchem.ncbi.nlm.nih.gov/, *Open Source*).

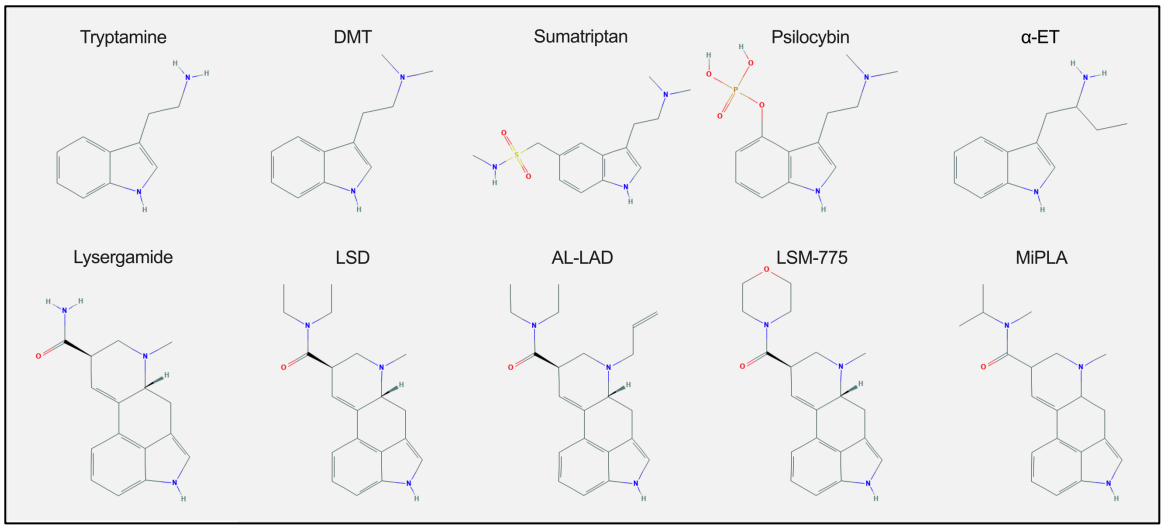
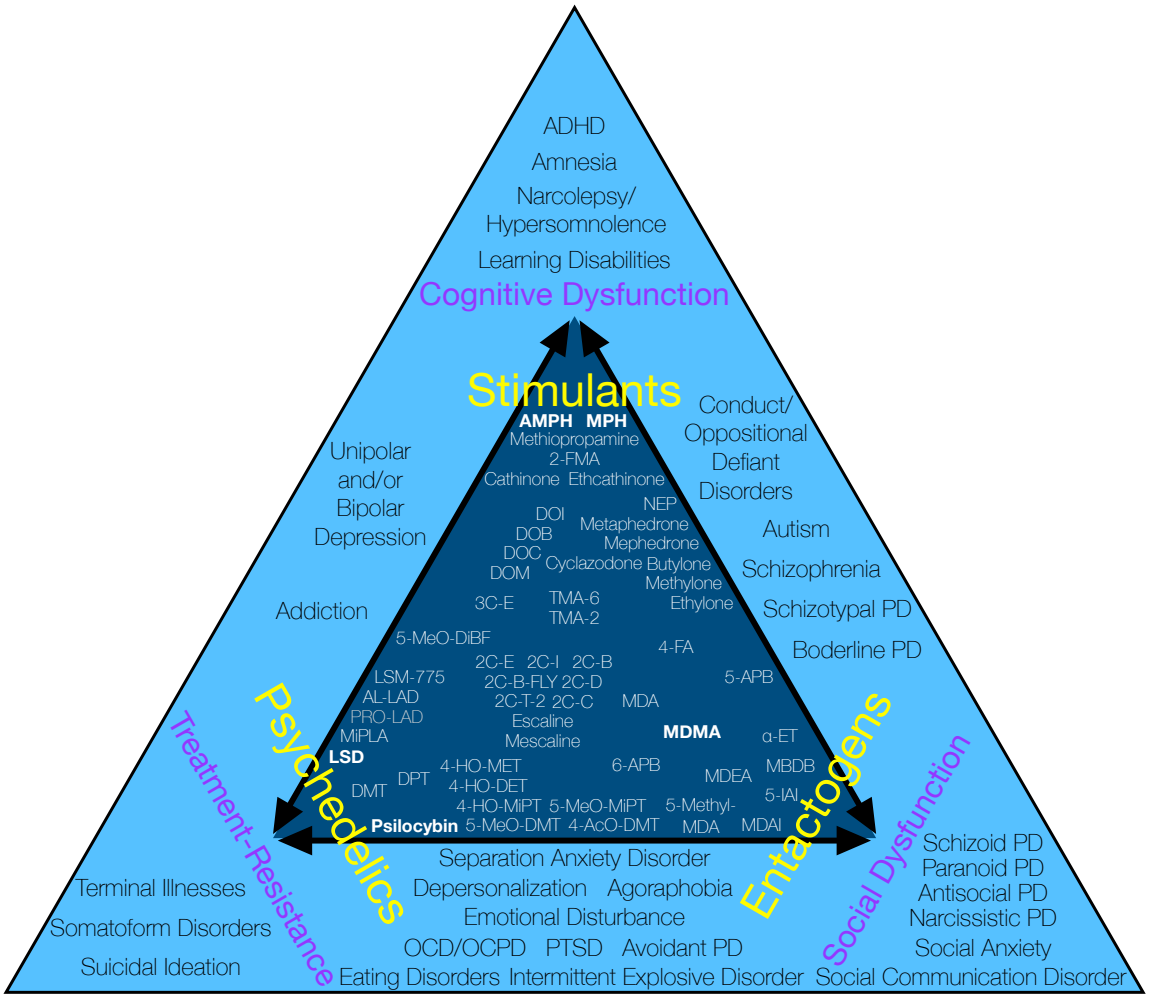


Figure 6.2 Chemical structures of select tryptamines and lysergamides. AL-LAD, N-allyl-nor-LSD; DMT, N,N-dimethyltryptamine; LSD, lysergic acid diethylamide; LSM-775, lysergic acid morpholide; MiPLA, methylisopropyllysergamide; α-ET; alpha-ethyltryptamine. (https://pubchem.ncbi.nlm.nih.gov/, *Open Source*).

Figure 6.3 Speculative model depicting the possible clinical uses of classical and novel monoaminergic drugs.
Inner triangle: approximate degree to which various known drugs produce psychedelic, entactogen, and/or stimulant effects. AL-LAD, N-allyl-nor-LSD; AMPH, amphetamine; DMT, N,N-dimethyltryptamine; DOB, 4-bromo-2,5-dimethoxyamphetamine; DOC, 4-chloro-2,5-dimethoxyamphetamine; DOI, 2,5-dimethoxy-4-iodoamphetamine; DOM, 2,5-dimethoxy-4-methylamphetamine; DPT, N,N-dipropyltryptamine; LSD, lysergic acid diethylamide; LSM-775, lysergic acid morpholide; MBDB, N-methyl-1,3-benzodioxolylbutanamine; MDA, 3,4-methylenedioxyamphetamine; MDAI, 5,6-methylenedioxy-2-aminoindane; MDEA, 3,4-methylenedioxy-N-ethylamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; MiPLA, methylisopropyllysergamide; MPH, methylphenidate; NEP, N-ethyl-nor-pentedrone; PRO-LAD, 6-propyl-6-nor-LSD; TMA-2, 2,4,5-trimethoxyamphetamine; TMA-6, 2,4,6-trimethoxyamphetamine; 2-FMA, 2-fluoromethamphetamine; 2C-B, 4-bromo-2,5-dimethoxyphenethylamine; 2C-B-FLY, 8-bromo-2,3,6,7-benzo-dihydro-difuran-ethylamine; 2C-C, 4-chloro-2,5-dimethoxyphenethylamine; 2C-D, 2,5-dimethoxy-4- methylphenethylamine; 2C-E, 2,5-dimethoxy-4-ethylphenethylamine; 2C-I, 2,5-dimethoxy-4-iodophenethylamine; 2C-T-2, 2,5-dimethoxy-4-ethylthiophenethylamine; 3C-E, 3,5-dimethoxy-4-ethoxyamphetamine; 4-AcO-DMT, 4-acetoxy-DMT; 4-FA, 4-fluoroamphetamine; 4-HO-DET, 4-hydroxy-N,N-diethyltryptamine; 4-HO-MET, 4-hydroxy-N-methyl-N-ethyltryptamine; 4-HO-MiPT, 4-hydroxy-N-methyl-N-isopropyltryptamine; 5-APB, 5-(2-aminopropyl)benzofuran; 5-IAI, 5-iodo-2-aminoindane; 5-MeO-DiBF, 5-methoxy-N,N-diisopropylbenzofuranethylamine; 5-MeO-DMT, 5-methoxy-DMT; 5-MeO-MiPT, 5-methoxy-N-methyl-N-isopropyltryptamine; 6-APB, 6-(2-aminopropyl)benzofuran; α-ET; alpha-ethyltryptamine.
Outer triangle: approximate degree to which various neuropsychiatric conditions produce treatment-resistance, social dysfunction, and/or cognitive dysfunction. ADHD, attention deficit hyperactivity disorder; OCD, obsessive compulsive disorder; OCPD, obsessive compulsive personality disorder; PD, personality disorder; PTSD, post-traumatic stress disorder.


ADHD
Amnesia
Narcolepsy/
Hypersomnolence
Learning Disabilities
Cognitive Dysfunction
Stimulants
AMPH MPH
Methiopropamine
2-FMA
Cathinone Ethcathinone
NEP
DOI
Metaphedrone
DOB
Mephedrone
DOC
Cyclazodone Butylone
DOM
Methylone
3C-E
TMA-6
TMA-2
Ethylone
5-MeO-DiBF
4-FA
2C-E 2C-I 2C-B
2C-B-FLY 2C-D
5-APB
LSM-775
AL-LAD
2C-T-2 2C-C
MDA
PRO-LAD
Escaline
MiPLA
Mescaline
MDMA
α-ET
LSD
6-APB
MBDB
4-HO-MET
DPT
MDEA
DMT
4-HO-DET
5-IAI
4-HO-MiPT 5-MeO-MiPT
5-Methyl-
Psilocybin 5-MeO-DMT 4-AcO-DMT
MDA
MDAI
Unipolar
and/or
Bipolar
Depression
Addiction
Conduct/
Oppositional
Defiant
Disorders
Autism
Schizophrenia
Schizotypal PD
Boderline PD
Psychedelics
Entactogens
Treatment-Resistance
Social Dysfunction
Separation Anxiety Disorder
Depersonalization Agoraphobia
Emotional Disturbance
OCD/OCPD PTSD Avoidant PD
Eating Disorders Intermittent Explosive Disorder
Terminal Illnesses
Somatoform Disorders
Suicidal Ideation
Schizoid PD
Paranoid PD
Antisocial PD
Narcissistic PD
Social Anxiety
Social Communication Disorder

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