UC San Diego

UC San Diego Electronic Theses and Dissertations

Title

The contribution of dopamine and norepinephrine transporters to psychostimulant-induced memory enhancement /

Permalink

https://escholarship.org/uc/item/3x93q1nv

Author

Carmack, Stephanie Ann

Publication Date

2014

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA, SAN DIEGO

The contribution of dopamine and norepinephrine transporters to psychostimulant-induced memory enhancement

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

in

Psychology

by

Stephanie Ann Carmack

Committee in charge:

Professor Stephan Anagnostaras, Chair
Professor Robert Clark
Professor Michael Gorman
Professor Mark Mayford
Professor John Wixted

2014

©

Stephanie Ann Carmack, 2014

All rights reserved.

The Dissertation of Stephanie Ann Carmack is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

_____

_____

_____

Chair

University of California, San Diego

2014

DEDICATION

To my parents, Michael and Debra Carmack.

EPIGRAPH

It’s not null, it’s clear. Crispy clear.

- Stephan Anagnostaras

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

Thank you to my advisor Stephan Anagnostaras for your guidance and you humor. I have benefited enormously from your encyclopedic knowledge and I will miss working with you. I hope that one day I can inspire students as much as you have inspired me. And thank you for accepting me into your lab, even though I didn't own a yacht.

Thank you to Michael Gorman for your science and your poodle, Gali.

Thank you to the incredible group of scientists who have served as my dissertation committee members: Robert Clark, Ronald Kuczenski, Michael Gorman, Mark Mayford, and John Wixted. Your advice has been invaluable.

Thank you to Kristin Howell (mentee and friend) and Suzanne Wood (mentor and friend) for being the most awesome lab mates, ever.

Thank you to the amazing friends that I have made in graduate school: Anna Byers, Mandy Damaggio, Katherine Stravropolous; and a huge thank you to Liz Harrison, for being there every step of the way. Thank you to the amazing friends who saw me through grad school: Samantha Set, Leah Segal, and Christine Chen.

Thank you to the undergraduates and staff that I was fortunate enough to work with: Carina Block, Sarah D'Angelo, Laila Davis, Erin McNalley, Alexandra Cifelli, Monette Leyva, Lauren Sawyer, Vanessa Wu, Michelle Autrey, Kleou Rasaei, Monica Kullar, Leila Oueini, Daniel Ma, Jonathan Sun, Antonio Mora, and Pierre.

Thank you to Norman Anderson for your constant support of graduate students.

Thank you to my family for your encouragement, support, and patience, especially to my parents Mike and Debbie; my siblings, Michelle and Michael; and my best friends Jordan and Joey, for everything. I'm finally done! I'm so lucky to have all of you in my life.

Chapter 2, in full, is a reprint of the material as it appears in Amphetamine and extinction of cued fear. *Neuroscience Letters*, 468, 18-22. Carmack SA, Wood SC & Anagnostaras SG (2014). The dissertation author was the primary investigator and author of this paper.

Chapter 3, in full, is a reprint of the material as it appears in Animal model of methylphenidate's long-term memory-enhancing effects. *Learning & Memory*, 21, 82-89. Carmack SA, Howell KK, Rasaei K, Reas ET & Anagnostaras SG (2014). The dissertation author was the primary investigator and author of this paper.

Chapter 4, in full, is a reprint of the material as it appears in Methylphenidate enhances acquisition and retention of spatial memory. *Neuroscience Letters*, 567, 45-50. Carmack SA, Block CL, Howell KK & Anagnostaras SG (2014). The dissertation author was the primary investigator and author of this paper.

Chapter 5, in full, is currently being prepared for submission for publication of the material**.** Carmack SA, Scudder SL, Howell KK, Harrison EM, Patrick GN, Gu HH & Anagnostaras SG (2014). The dissertation author was the primary investigator and author of this paper.

VITA

2014	Doctor of Philosophy, Psychology, University of California, San Diego
2009	Master of Arts, Psychology, University of California, San Diego
2008	Bachelor of Science, Neuroscience, Brown University

PUBLICATIONS

Carmack SA, Block CL, Howell KK & Anagnostaras SG (2014) Methylphenidate enhances acquisition and retention of spatial memory. *Neuroscience Letters,* 468 (1): 18-22.

Carmack SA, Howell KK, Wood SC, Rasaei K, Reas ES & Anagnostaras SG (2014) Animal model of methylphenidate's long-term memory-enhancing effects. *Learning & Memory*, 21 (2): 82-89.

Howell KK, Monk BR, Carmack SA, Mrowczynski OD, Clark RE & Anagnostaras SG (2014) Inhibition of PKC disrupts addiction-related memory. *Frontiers in Behavioral Neuroscience*, 8 (70).

Carmack SA, Kim JS, Sage JR, Thomas AW, Skillicorn KN & Anagnostaras SG (2013) The competitive NMDA receptor antagonist CPP disrupts cocaine-induced conditioned place preference, but spares behavioral sensitization. *Behavioural Brain Research*, 239: 155-163.

Carmack SA, Wood SC & Anagnostaras SG (2010) Amphetamine and extinction of cued fear. *Neuroscience Letters*, 468(1): 18-22.

ABSTRACT OF THE DISSERTATION

The contribution of dopamine and norepinephrine transporters to psychostimulant-induced memory enhancement

by

Stephanie Ann Carmack

Doctor of Philosophy in Psychology

University of California, San Diego, 2014

Professor Stephan Anagnostaras, Chair

The psychostimulants methylphenidate and amphetamine enhance monoaminergic neurotransmission by acting on reuptake transporters. Together, they form the cornerstone of treatment for attention-deficit hyperactivity disorder, the most common psychiatric disorder in children, because of their ability to improve learning at low doses. At high doses, they are subject to abuse that can lead to addiction and cognitive dysfunction. Current theories posit that methylphenidate and amphetamine exert their therapeutic effects by acting on the norepinephrine transporter (NET) and produce their reinforcing effects by acting on the dopamine transporter (DAT). The studies in this dissertation were specifically aimed at identifying the contributions of NET and DAT to stimulant-induced memory enhancement. While stimulant effects on memory have typically been interpreted as the result of changes in high-level functions like impulsivity and executive function, here we present evidence that stimulants can also

improve memory directly (Chapters 2-4). Furthermore, memory enhancement is independent of effects on locomotion, reinforcement, and anxiety (Chapter 3). In comparing the effects of agonists with varying affinities for DAT and NET on memory, we conclude that action at DAT and perhaps NET is required for these memory enhancements (Chapter 3). To clarify this mechanism, we used triple point mutant knockin mice of the gene coding DAT or NET that hinder the binding of methylphenidate and reduce the efficiency of the transporters (Chapter 5). We found that the mutations in DAT, but not NET, produced severe learning and memory defects across multiple memory domains. Together, these results indicate that stimulants enhance memory and we propose that DAT plays an obligatory role in memory.

Chapter 1

Introduction

Psychostimulants comprise a broad collection of sympathomimetic drugs that produce a wide range of behavioral effects [1]. For instance, the therapeutics methylphenidate (e.g. Ritalin, Concerta, Focalin) and amphetamine (e.g. Benzedrine, Adderall, Vyvanse) promote wakefulness and enhance cognition, including learning and memory [2–5]. They have been used to treat a variety of disorders, such as narcolepsy and depression [1,2,6–8], and form the cornerstone of treatment for attention deficit hyperactivity disorder (ADHD) [9,10]. At the same time, stimulants like cocaine and methamphetamine are often associated with abuse and addiction in vulnerable individuals [1,2]. Addiction to stimulants is strongly associated with cognitive dysfunction, particularly in measures of learning and memory, executive function, and attention [1,11–13]. Together, these observations raise the question: how does the same class of drugs enhance cognition in some individuals and impair cognition and produce addiction in others?

A preponderance of evidence indicates that the answer to this question is the dose of stimulant used [1,14,15]. Therapeutic effects are associated with low doses and slow routes of administration [1]. In contrast, addictive effects and cognitive deficits are related to high doses, fast routes of administration, and more potent stimulants [1,16]. As stimulants' effects on cognition and reinforcement are readily dissociable by dose, we sought to determine whether they are also dissociable at the synaptic level.

Psychostimulant action at the synapse

Generally, psychostimulants are thought to induce changes in behavior by increasing monoamine neurotransmission at the synapse [1]. Though each stimulant has unique effects on the cell [17,18], classical stimulants share the property of acting on monoamine reuptake transporters [1,15,16,19]. Methylphenidate is a high-affinity inhibitor of both the dopamine and norepinephrine transporters (DAT and NET,

respectively) [20–23]. By preventing reuptake, methylphenidate increases the duration of the dopamine and norepinephrine signal. Cocaine blocks DAT and NET, binding to a similar site as methylphenidate, and additionally inhibits the serotonin transporter (SERT) [1,19]. Amphetamine is a substrate at DAT, NET, and SERT and can both block and reverse transport; consequently, it increases both the duration of the signal and the amount of monoamine released into the extracellular space [21,24].

Psychostimulants have different affinities for each monoamine transporter in their binding profile [18,19]. Transporters have varying affinities for substrates and transporter expression levels vary across brain regions [18,25,26]. Therefore, stimulant actions and the resulting changes in monoamine neurotransmission can also be brain-region specific [26]. Further, recent evidence suggests that stimulants' actions at a single transporter can be dose-dependent. For example, it has been proposed that amphetamine's mechanism of action at DAT at low doses is primarily to inhibit the transporter, while much higher doses, such as those used by addicts, are required to reverse the transporter [27].

Role of DAT and NET in psychostimulants' dose-dependent behavioral effects

Relatively little research has investigated the molecular basis of stimulants' pro-cognitive effects [1,5,14,28]. Most theories posit that stimulants improve cognition by enhancing dopamine and norepinephrine transmission in the prefrontal cortex (PFC) [18,28–30]. In the PFC there is a very low density of DAT and a high density of NET [18,26], so stimulants would increase catecholamine transmission by acting at NET [26]. This proposal is largely based on the observation that stimulants can improve working memory and executive control, both of which require the PFC, in animals and humans [31,32]. It is important to note that these theories contain the caveat that there is an optimal level of catecholamine transmission for peak cognitive performance (inverted u-shaped function), so stimulants' effects on cognition are constrained both by the

baseline catecholamine levels in the subject and the dose of the stimulant administered [1,28,33].

By contrast to stimulants' effects on cognition, much work has investigated the molecular basis of stimulants' reinforcing effects and abuse potential. Enhanced dopamine in the nucleus accumbens is thought to produce addiction [34–36]. As noted above, transporter expression varies across brain regions and a high density of DAT, but not NET, is found in the nucleus accumbens [18]. Affinity for DAT has been strongly implicated in stimulants' reinforcing effects [34,35].

In support of the prevailing views on the roles for DAT and NET in stimulants' pro-cognitive versus reinforcing effects, methylphenidate, amphetamine, and cocaine have a higher affinity for NET than DAT [18,19]. Thus, at very low doses stimulants could act on NET in the PFC and improve cognition, without acting at DAT in the nucleus accumbens and producing reinforcement [22]. At very high doses, stimulants could act on DAT in the nucleus accumbens and produce reinforcement. Cognitive dysfunction in addiction would then be the result of increasing catecholamine transmission in the PFC beyond optimal levels. Following this, if stimulants' behavioral effects are dissociable by their requirement for DAT and NET, it is possible that one could create a drug that retains stimulants' cognitive-enhancing properties without the abuse potential.

Psychostimulants and long-term memory

The studies in this dissertation were aimed identifying the contributions of the dopamine (DAT) and norepinephrine transporters (NET) (Chapters 3 and 5) to psychostimulant-induced enhancements in long-term memory (Chapters 2-4). Enhanced memory as a result of stimulant use has typically been interpreted as the result of improved executive function and reduced impulsivity [37]. Consistent with this, at the cellular level, monoamines are traditionally considered modulatory, rather than

obligatory, for learning and memory. Monoamines can modulate the persistence of synaptic plasticity; thus it has been proposed that they control the entrance into and persistence of information in long-term memory based on high-level functions like motivation and reward [38–42]. An emerging literature, however, directly implicates stimulants in learning and memory [4,5,43–52].

Goals of the present experiments.

1. Determine if methylphenidate's memory-enhancing and reinforcing properties are dissociable.
2. Examine the role of DAT and NET in stimulant-induced memory enhancement

In Chapter 2 we compared amphetamine's ability to modulate fear conditioning, a PFC-independent task [53,54], with its ability to modulate fear extinction, a PFC-dependent task [55,56]. Fear conditioning is a leading model of memory in rats and mice with very modest attentional demands. In this task an animal is brought to a conditioning chamber and presented with an initially neutral tone (conditioned stimulus) that is immediately followed by an aversive shock to the foot (unconditioned stimulus). As the result of this pairing, the animal exhibits a learned fear response (freezing) in response to presentation of the tone or the conditioning context alone [53,54]. Context and tone fear acquisition require the amygdala, while context fear additionally requires the hippocampus [57,58].

In contrast, the PFC is not required for fear acquisition, but has an essential role in working memory and executive function and a limited role in fear extinction [55,56]. We predicted that if amphetamine enhances learning and memory via a PFC-dependent mechanism then doses able to facilitate fear acquisition (0.005 and 0.05 mg/kg, ip) [51] should enhance fear extinction, a weak form of new learning [59]. However, amphetamine did not affect fear extinction at any of the doses tested.

In Chapter 3 we examined the effects of a range of doses of methylphenidate (0.01 – 10 mg/kg, i.p.) on Pavlovian fear acquisition [60]. We predicted that pre-training methylphenidate would dose-dependently modulate fear conditioning based on previous work from our lab showing that low doses of amphetamine, modafinil, and cocaine enhanced acquisition of fear memory, while high doses impaired fear memory [50–52]. We found a clear long-term enhancement of memory by methylphenidate at doses similar to those prescribed for ADHD (0.01 – 1 mg/kg). Importantly, effects on locomotion, anxiety, or reinforcement did not confound this enhancement.

In Chapter 4 we determined whether methylphenidate-induced memory enhancements would generalize beyond fear learning [61]. We assessed the effects of methylphenidate on spatial memory using the Morris water maze [62]. We selected the doses of methylphenidate that maximally enhanced (1 mg/kg) or impaired (10 mg/kg) fear memory in Chapter 3 [60]. This study revealed that a much higher dose of methylphenidate (10 mg/kg) was required to enhance water maze acquisition and retention as compared to fear conditioning (0.01 – 1 mg/kg).

In Chapters 2 through 4 we were able to demonstrate that stimulants dose- and task-dependently modulate long-term memory. We next examined the contribution of DAT and NET inhibition in stimulant-induced memory enhancement using two approaches. In Chapter 3 we took a pharmacological approach and compared the effects on fear learning of several agonists with varying affinities for DAT and NET. Specifically, we administered drugs that have been used to treat ADHD: atomoxetine (high affinity NET inhibitor), bupropion (a low affinity DAT and NET inhibitor), and citalopram (high affinity SERT inhibitor) [16,19]. From these studies, we conclude that psychostimulant-induced memory enhancement is likely due to a combination of DAT and NET binding.

In Chapters 5 and 6 we used knock-in mice with three point mutations in the DAT (DAT^{CI} mutants) or NET (NET^{CI} mutants) that markedly reduce the binding of

methylphenidate and cocaine [63–65]. These mutants are ideal models for studying stimulant effects on behavior, as the mutations do not lead to dramatic compensation by other neurotransmitter systems, which are often observed in complete knockouts [63,66–69]. We anticipated that studying the effects of methylphenidate in these mice would reveal the contributions of DAT and NET inhibition in mediating stimulant-induced memory enhancement. For example, if NET inhibition were required for methylphenidate-induced memory enhancement, then we would predict this effect to be reduced or absent in NET^{CI} mice.

We were quite surprised to find stunning effects of the DAT and NET knockins on learning and memory, even in the absence of methylphenidate or cocaine. The DAT^{CI} mutation produced severe defects across multiple memory domains, which challenges the conception that DAT plays a minor, modulatory role in learning and memory (Chapter 5). In contrast, the NET^{CI} mutations led to enhanced memory, but also produced a marked anxiety phenotype. In Chapter 6 we give a theoretical account for the above findings and suggest that the dopamine transporter functions as critical mechanism for opening the temporal window of learning.

References

[1] S.C. Wood, J.R. Sage, T. Shuman, S.G. Anagnostaras, Psychostimulants and Cognition: A Continuum of Behavioral and Cognitive Activation, Pharmacol. Rev. 66 (2014) 193–221.

[2] N. Rasmussen, America's first amphetamine epidemic 1929-1971: a quantitative and qualitative retrospective with implications for the present., Am. J. Public Health. 98 (2008) 974–85.

[3] W. Somerville, The effect of benzedrine on mental or physical fatigue in soldiers, Can. Med. Assoc. Jouranl. 55 (1946) 470–476.

[4] S.M. Rhodes, D.R. Coghill, K. Matthews, Methylphenidate restores visual memory, but not working memory function in attention deficit-hyperkinetic disorder., Psychopharmacology (Berl). 175 (2004) 319–30.

[5] S.M. Rhodes, J. Park, S. Seth, D.R. Coghill, A comprehensive investigation of memory impairment in attention deficit hyperactivity disorder and oppositional defiant disorder., J. Child Psychol. Psychiatry. 53 (2012) 128–37.

[6] T.D. Challman, J.J. Lipsky, Methylphenidate: Its Pharmacology and Uses, Mayo Clin. Proc. 75 (2000) 711–721.

[7] A. Myerson, Effect of benzedrine sulfate on mood and fatigue in normal and in neurotic persons, Arch. Neurol. Psychiatry. 36 (1936) 816–822.

[8] W. Sargant, J.M. Blackburn, The effect of benzedrine on intelligence scores, Lancet. 228 (1936) 1385–1387.

[9] G. Polanczyk, L.A. Rohde, Epidemiology of attention-deficit/hyperactivity disorder across the lifespan., Curr. Opin. Psychiatry. 20 (2007) 386–92.

[10] R.C. Kessler, L. Adler, R.A. Barkley, J. Biederman, K. Conners, O. Demler, S.V. Faraone, L.L. Greenhill, M.J. Howes, K. Secnik, T. Spencer, B. Utsun, E.E. Walters, A.M. Zaslavsky, The prevalence and correlates of adult ADHD in the United States: results from the National Comorbidity Survey replication, Am. J. Psychiatry. 163 (2006) 716–723.

[11] A. Ardila, M. Rosselli, S. Strumwasser, Neuropsychological deficits in chronic cocaine abusers, Int. J. Neurosci. 57 (1991) 73–79.

[12] R.D. Rogers, B.J. Everitt, A. Baldacchino, a J. Blackshaw, R. Swainson, K. Wynne, N.B. Baker, J. Hunter, T. Carthy, E. Booker, M. London, J.F.W. Deakin, B.J. Sahakian, T.W. Robbins, Dissociable deficits in the decision-making cognition of chronic amphetamine abusers, opiate abusers, patients with focal damage to prefrontal cortex, and tryptophan-

depleted normal volunteers: evidence for monoaminergic mechanisms., Neuropsychopharmacology. 20 (1999) 322–39.

[13] S. T, S.L. Simon, C.P. Domier, K. Richardson, R. Rawson, W. Ling, Cognitive deficits among methamphetamine users with attention deficit hyperactivity disorder symptomology., J. Addict. Disord. 21 (2002) 75–89.

[14] A.F.T. Arnsten, Stimulants: Therapeutic actions in ADHD., Neuropsychopharmacology. 31 (2006) 2376–83.

[15] R. Kuczenski, D.S. Segal, Stimulant actions in rodents: implications for attention-deficit/hyperactivity disorder treatment and potential substance abuse., Biol. Psychiatry. 57 (2005) 1391–6.

[16] K.C.F. Fone, D.J. Nutt, Stimulants: use and abuse in the treatment of attention deficit hyperactivity disorder., Curr. Opin. Pharmacol. 5 (2005) 87–93.

[17] P. Seeman, B.K. Madras, Anti-hyperactivity medication: methylphenidate and amphetamine, Mol. Psychiatry. 3 (1998) 386–396.

[18] B.K. Madras, G.M. Miller, A.J. Fischman, The dopamine transporter and attention-deficit/hyperactivity disorder., Biol. Psychiatry. 57 (2005) 1397–409.

[19] E. Richelson, M. Pfenning, Blockade by antidepressants and related compounds of biogenic amine uptake into rat brain synaptosomes: most antidepressants selectively block norepinephrine uptake, Eur. J. Pharmacol. 104 (1984) 277–286.

[20] D.D. Han, H.H. Gu, Comparison of the monoamine transporters from human and mouse in their sensitivities to psychostimulant drugs., BMC Pharmacol. 6 (2006) 6.

[21] R. Kuczenski, D.S. Segal, Effects of methylphenidate on extracellular dopamine, serotonin, and norepinephrine: comparison with amphetamine., J. Neurochem. 68 (1997) 2032–7.

[22] R. Kuczenski, D.S. Segal, Exposure of adolescent rats to oral methylphenidate: preferential effects on extracellular norepinephrine and absence of sensitization and cross-sensitization to methamphetamine., J. Neurosci. 22 (2002) 7264–71.

[23] R. Chen, D.D. Han, H.H. Gu, A triple mutation in the second transmembrane domain of mouse dopamine transporter markedly decreases sensitivity to cocaine and methylphenidate., J. Neurochem. 94 (2005) 352–9.

[24] S.D. Robertson, H.J.G. Matthies, A. Galli, A closer look at amphetamine-induced reverse transport and trafficking of the dopamine and norepinephrine transporters., Mol. Neurobiol. 39 (2009) 73–80.

[25] B.J. Ciliax, C. Heilman, H.B. Niznik, L. Demchyshyn, I. Levey, O.M.T. R, The Dopamine Transporter: Localization in Brain Characterization, J. Neurosci. 15 (1995) 1714–1723.

[26] J. a Morón, A. Brockington, R.A. Wise, B.A. Rocha, B.T. Hope, Dopamine uptake through the norepinephrine transporter in brain regions with low levels of the dopamine transporter: evidence from knock-out mouse lines., J. Neurosci. 22 (2002) 389–95.

[27] E.S. Calipari, M.J. Ferris, Amphetamine mechanisms and actions at the dopamine terminal revisited., J. Neurosci. 33 (2013) 8923–5.

[28] A.F.T. Arnsten, Toward a new understanding of attention-deficit hyperactivity disorder pathophysiology: an important role for prefrontal cortex dysfunction., CNS Drugs. 23 Suppl 1 (2009) 33–41.

[29] T. Spencer, J. Biederman, T. Wilens, M. Harding, D. O'Donnell, S. Griffin, Pharmacotherapy of attention-deficit hyperactivity disorder across the life cycle., J. Am. Acad. Child Adolesc. Psychiatry. 35 (1996) 409–32.

[30] A.F. Arnsten, A.G. Dudley, Methylphenidate improves prefrontal cortical cognitive function through alpha2 adrenoceptor and dopamine D1 receptor actions: Relevance to therapeutic effects in Attention Deficit Hyperactivity Disorder., Behav. Brain Funct. 1 (2005) 2.

[31] C.W. Berridge, D.M. Devilbiss, M.E. Andrzejewski, A.F.T. Arnsten, A.E. Kelley, B. Schmeichel, C. Hamilton, R.C. Spencer, Methylphenidate preferentially increases catecholamine neurotransmission within the prefrontal cortex at low doses that enhance cognitive function., Biol. Psychiatrysychiatry. 60 (2006) 1111–20.

[32] M.A. Mehta, I.M. Goodyer, B.J. Sahakian, Methylphenidate improves working memory and set-shifting in AD/HD: relationships to baseline memory capacity., J. Child Psychol. Psychiatry. 45 (2004) 293–305.

[33] S. Vijayraghavan, M. Wang, S.G. Birnbaum, G. V Williams, A.F.T. Arnsten, Inverted-U dopamine D1 receptor actions on prefrontal neurons engaged in working memory., Nat. Neurosci. 10 (2007) 376–84.

[34] N. Volkow, Role of Dopamine, the Frontal Cortex and Memory Circuits in Drug Addiction: Insight from Imaging Studies, Neurobiol. Learn. Mem. 78 (2002) 610–624.

[35] N.D. Volkow, G.J. Wang, J.S. Fowler, J. Logan, S.J. Gatley, C. Wong, R. Hitzemann, N.R. Pappas, Reinforcing effects of psychostimulants in humans are associated with increases in brain dopamine and occupancy of D(2) receptors., J. Pharmacol. Exp. Ther. 291 (1999) 409–15.

[36] G.F. Koob, N.D. Volkow, Neurocircuitry of addiction., Neuropsychopharmacology. 35 (2010) 217–38.

[37] G. Lynch, L.C. Palmer, C.M. Gall, The likelihood of cognitive enhancement., Pharmacol. Biochem. Behav. 99 (2011) 116–29.

[38] S.J. Sara, The locus coeruleus and noradrenergic modulation of cognition., Nat. Rev. Neurosci. 10 (2009) 211–23.

[39] P. Waelti, A. Dickinson, W. Schultz, Dopamine responses comply with basic assumptions of formal learning theory., Nature. 412 (2001) 43–8.

[40] D. Shohamy, R.A. Adcock, Dopamine and adaptive memory., Trends Cogn. Sci. 14 (2010) 464–72.

[41] J.I. Rossato, L.R.M. Bevilaqua, I. Izquierdo, J.H. Medina, M. Cammarota, Dopamine controls persistence of long-term memory storage., Science. 325 (2009) 1017–20.

[42] J.E. Lisman, A.A. Grace, The hippocampal-VTA loop: controlling the entry of information into long-term memory., Neuron. 46 (2005) 703–13.

[43] K. Krauel, E. Duzel, H. Hinrichs, S. Santel, T. Rellum, L. Baving, Impact of emotional salience on episodic memory in attention-deficit/hyperactivity disorder: a functional magnetic resonance imaging study., Biol. Psychiatry. 61 (2007) 1370–9.

[44] S. Kempton, A. Vance, P. Maruff, E. Luk, J. Costin, C. Pantelis, Executive function and attention deficit hyperactivity disorder: stimulant medication and better executive function performance in children., Psychol. Med. 29 (1999) 527–38.

[45] S.M. Rhodes, D.R. Coghill, K. Matthews, Neuropsychological functioning in stimulant-naive boys with hyperkinetic disorder, Psychol. Med. 35 (2005) 1109–1120.

[46] S.M. Rhodes, D.R. Coghill, K. Matthews, Acute neuropsychological effects of methylphenidate in stimulant drug-naïve boys with ADHD II--broader executive and non-executive domains., J. Child Psychol. Psychiatry. 47 (2006) 1184–94.

[47] J.L. Rapoport, G. Inoff-Germain, Responses to methylphenidate in Attention-Deficit/ Hyperactivity Disorder and normal children: update 2002, J. Atten. Disord. 6 (2002) S57–60.

[48] J.L. Rapoport, M.S. Buchsbaum, H. Weingartner, T.P. Zahn, C. Ludlow, E.J. Mikkelsen, Dextroamphetamine: It's Cognitive and Behavioral Effects in Normal and Hyperactive Boys and Normal Men, Arch. Gen. Psychiatry. 37 (1980) 933–943.

[49] A.M.W. Linssen, E.F.P.M. Vuurman, A. Sambeth, W.J. Riedel, Methylphenidate produces selective enhancement of declarative memory consolidation in healthy volunteers., Psychopharmacology (Berl). 221 (2012) 611–9.

[50] S.C. Wood, J. Fay, J.R. Sage, S.G. Anagnostaras, Cocaine and Pavlovian fear conditioning: dose-effect analysis., Behav. Brain Res. 176 (2007) 244–50.

[51] S.C. Wood, S.G. Anagnostaras, Memory and psychostimulants: modulation of Pavlovian fear conditioning by amphetamine in C57BL/6 mice., Psychopharmacology (Berl). 202 (2009) 197–206.

[52] T. Shuman, S.C. Wood, S.G. Anagnostaras, Modafinil and Memory: Effects of Modafinil on Morris Water Maze Learning and Pavlovian Fear Conditioning, Behav. Neurosci. 123 (2009) 257–266.

[53] S.G. Anagnostaras, S.C. Wood, T. Shuman, D.J. Cai, A.D. Leduc, K.R. Zurn, J.R. Sage, G.M. Herrera, Automated assessment of pavlovian conditioned freezing and shock reactivity in mice using the video freeze system., Front. Behav. Neurosci. 4 (2010) 1–11.

[54] S. Maren, Pavlovian fear conditioning as a behavioral assay for hippocampus and amygdala function: cautions and caveats., Eur. J. Neurosci. 28 (2008) 1661–6.

[55] T.S. Braver, D.M. Barch, W.M. Kelley, R.L. Buckner, N.J. Cohen, F.M. Miezin, A.Z. Snyder, J.M. Ollinger, E. Akbudak, T.E. Conturo, S.E. Petersen, Direct comparison of prefrontal cortex regions engaged by working and long-term memory tasks., Neuroimage. 14 (2001) 48–59.

[56] M.A. Morgan, J.E. LeDoux, Differential contribution of dorsal and ventral medial prefrontal cortex to the acquisition and extinction of conditioned fear in rats., Behav. Neurosci. 109 (1995) 681–8.

[57] S.G. Anagnostaras, S. Maren, M.S. Fanselow, Temporally graded retrograde amnesia of contextual fear after hippocampal damage in rats: within-subjects examination., J. Neurosci. 19 (1999) 1106–14.

[58] G.D. Gale, S.G. Anagnostaras, B.P. Godsil, S. Mitchell, T. Nozawa, J.R. Sage, B. Wiltgen, M.S. Fanselow, Role of the basolateral amygdala in the storage of fear memories across the adult lifetime of rats., J. Neurosci. 24 (2004) 3810–5.

[59] S.A. Carmack, S.C. Wood, S.G. Anagnostaras, Amphetamine and extinction of cued fear., Neurosci. Lett. 468 (2010) 18–22.

[60] S.A. Carmack, K.K. Howell, K. Rasaei, E.T. Reas, S.G. Anagnostaras, Animal model of methylphenidate's long-term memory-enhancing effects, Learn. Mem. 21 (2014) 82–89.

[61] S.A. Carmack, C.L. Block, K.K. Howell, S.G. Anagnostaras, Methylphenidate enhances acquisition and retention of spatial memory., Neurosci. Lett. 567 (2014) 45–50.

[62] R. Morris, Developments of a water-maze procedure for studying spatial learning in the rat., J. Neurosci. Methods. 11 (1984) 47–60.

[63] R. Chen, M.R. Tilley, H. Wei, F. Zhou, F.-M. Zhou, S. Ching, N. Quan, R.L. Stephens, E.R. Hill, T. Nottoli, D.D. Han, H.H. Gu, Abolished cocaine reward in mice with a cocaine-insensitive dopamine transporter., Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 9333–8.

[64] M.R. Tilley, H.H. Gu, The Effects of Methylphenidate on Knockin Mice with a Methylphenidate-Resistant Dopamine Transporter, 327 (2008) 554–560.

[65] H. Wei, E.R. Hill, H.H. Gu, Functional mutations in mouse norepinephrine transporter reduce sensitivity to cocaine inhibition., Neuropharmacology. 56 (2009) 399–404.

[66] F. Napolitano, A. Bonito-Oliva, M. Federici, M. Carta, F. Errico, S. Magara, G. Martella, R. Nistico, D. Centonze, A. Pisani, H.H. Gu, N.B. Mercuri, A. Usiello, Role of aberrant striatal dopamine D1 receptor/cAMP/protein kinase A/DARPP32 signaling in the paradoxical calming effect of amphetamine., J. Neurosci. 30 (2010) 11043–56.

[67] F. Xu, R.R. Gainetdinov, W.C. Wetsel, S.R. Jones, L.M. Bohn, G.W. Miller, Y.M. Wang, M.G. Caron, Mice lacking the norepinephrine transporter are supersensitive to psychostimulants., Nat. Neurosci. 3 (2000) 465–71.

[68] B. Giros, M. Jaber, S.R. Jones, R.M. Wightman, M.G. Caron, Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter, Nature. 379 (1996) 606–612.

[69] B.A. Rocha, F. Fumagalli, R.R. Gainetdinov, S.R. Jones, R. Ator, B. Giros, G.W. Miller, M.G. Caron, Cocaine self-administration in dopamine-transporter knockout mice., Nat. Neurosci. 1 (1998) 132–7.

Chapter 2

Amphetamine and extinction of cued fear

Neuroscience Letters 468 (2010) 18–22

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Amphetamine and extinction of cued fear

Stephanie A. Carmack[a,*], Suzanne C. Wood[a], Stephan G. Anagnostaras[a,b]

[a] Molecular Cognition Laboratory, Department of Psychology, University of California, San Diego, CA 92093-0109, USA
[b] Program in Neurosciences, University of California, San Diego, CA 92093-0109, USA

ARTICLE INFO

Article history:
Received 17 July 2009
Received in revised form
15 September 2009
Accepted 15 October 2009

Keywords:
Amygdala
Freezing
Stimulants
Classical conditioning
Mice

ABSTRACT

Much research is focused on developing novel drugs to improve memory. In particular, psychostimulants have been shown to enhance memory and have a long history of safe use in humans. In prior work, we have shown that very low doses of amphetamine administered before training on a Pavlovian fear-conditioning task can dramatically facilitate the acquisition of cued fear. The current experiment sought to expand these findings to the extinction of cued fear, a well-known paradigm with therapeutic implications for learned phobias and post-traumatic stress disorder. If extinction reflects new learning, one might expect drugs that enhance the acquisition of cued fear to also enhance the extinction of cued fear. This experiment examined whether 0.005 or 0.05 mg/kg of D-amphetamine (therapeutic doses shown to enhance acquisition) also enhance the extinction of cued fear. Contrary to our hypothesis, amphetamine did not accelerate extinction. Thus, at doses that enhance acquisition of conditioned fear, amphetamine does not appear to enhance extinction.

© 2009 Elsevier Ireland Ltd. All rights reserved.

A large body of evidence suggests that psychostimulants can enhance learning and memory in both humans and rodents [6,21,30,31,33]. One such psychostimulant is amphetamine, a drug currently used to treat attention deficit hyperactivity disorder (ADHD; e.g. Adderall®) [1]. Our laboratory has previously found [37] that ultra-low doses (0.005 and 0.05 mg/kg) of amphetamine, similar to the therapeutic doses for ADHD, administered to mice during training, dramatically enhance cued fear memory when subjects are tested off-drug. It is clear that amphetamine can enhance the acquisition of aversive memories, but it is unclear whether amphetamine can also enhance the extinction of conditioned fear.

In Pavlovian fear conditioning, an initially neutral stimulus (the conditioned stimulus, CS, e.g. a tone) is paired with an aversive stimulus (the unconditioned stimulus, US, e.g. a footshock). Following repeated CS–US pairings, the CS alone can elicit fear in a subject. In rodents, freezing, or the absence of all movement with the exception of respiration, is often the measure of conditioned fear [2,12]. The neurobiology underlying conditioned freezing is well understood; acquisition of cued fear depends critically on the convergence of CS and US information in the basolateral amygdala [20,28]. This CS–US association is not necessarily permanent, however. Repeated presentations of the CS in the absence of the US lead to extinction of conditioned fear, evidenced by decreased freezing in response to the CS alone.

Extinction is thought to reflect new, inhibitory learning [24], whereby extinction training encodes a new memory of the CS that then competes with the original memory of the CS. Unlike acquisition of cued fear, the neural mechanisms underlying extinction are still poorly understood. For example, extinction seems to depend on the medial prefrontal cortex (which is not essential for fear acquisition) [22,25], as well as the amygdala [5,11].

Pavlovian fear conditioning can serve as a model for both the etiology and treatment of phobia because phobias, or maladaptive fear responses to conditioned stimuli [36], are frequently treated using extinction therapy [13,14]. Extinction, however, is a relatively weak and unstable form of learning, so considerable research has focused on identifying pharmacological agents, which, if given during extinction therapy would strengthen and stabilize the reduction of fear [27,35]. Therefore, if extinction reflects new, inhibitory learning, it is possible that drugs that enhance fear acquisition will also facilitate the extinction of fear memory. This study examined whether extinction could be facilitated using D-amphetamine, a psychostimulant drug previously shown to enhance acquisition of cued fear [37].

The effects of amphetamine on the extinction of conditioned freezing have only been examined in one other study. Mueller et al. [23] administered 1.0 mg/kg of amphetamine during extinction training. They found that amphetamine decreased freezing relative to saline controls during extinction training, but this effect was not seen when tested off-drug. Thus, they attributed the reduction in freezing to amphetamine-induced locomotor hyperac-

* Corresponding author at: 9500 Gilman Dr. MC 0109, La Jolla, CA 92093-0109, USA. Tel.: +1 858 822 1938; fax: +1 858 534 7190.
E-mail address: sacarmac@ucsd.edu (S.A. Carmack).
URL: http://mocolab.org (S.A. Carmack).

0304-3940/$ – see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2009.10.049

tivity rather than enhanced extinction retention. Mueller's results are not surprising in light of our recent findings, which found evidence for hyperactivity and no evidence of memory enhancement in animals administered 1 mg/kg D-amphetamine [37]. Only ultra-low doses of amphetamine (0.005–0.05 mg/kg) administered pre-training enhanced cued fear acquisition. Thus, these ultra-low doses of amphetamine are more likely than the moderate dose to enhance the extinction of Pavlovian fear-conditioning. Therefore, we administered 0.005 and 0.05 mg/kg amphetamine during extinction training and found that neither dose altered the extinction of Pavlovian fear.

Fifty-two C57B6/J inbred mice from Jackson Laboratory (West Sacramento, CA) were used in approximately equal numbers of males and females, balanced across groups. Mice were weaned at 3 weeks of age and were group housed (2–5 mice per cage) with continuous access to food and water. Mice were at least 10 weeks old before testing and subjects were handled for 5 days prior to training. The vivarium was maintained on a 14:10 light:dark schedule, and all testing was performed during the light phase of the cycle. All animal care and testing procedures were approved by the UCSD IACUC and were in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals.

Mice underwent acquisition training (tone–shock pairings) for 1 day, off-drug, followed by 6 days of extinction trials (tone-alone presentations) under saline or amphetamine conditions. One final day of extinction was conducted off-drug. Three to four mice were tested concurrently, in individual conditioning chambers housed in a windowless room. Conditioning chambers were setup as described previously [29,37]. Each conditioning chamber (32 cm × 25 cm × 25 cm) was located within a sound-attenuating chamber (63.5 cm × 35.5 cm × 76 cm) (Med-Associates Inc., St. Albans, VT) and equipped with a speaker in the sidewall. During acquisition training, the context consisted of a stainless steel grid floor (36 rods, each rod 2 mm in diameter, 8 mm center to center; Med-Associates Inc., St. Albans, VT) and a stainless steel drop pan. The sidewalls were white acrylic, and the front wall was clear to allow for viewing. Between each trial, the chambers were cleaned and scented with 7% isopropyl alcohol to provide a background odor. Ventilation fans provided background noise (65 dB). Each sound-attenuating chamber was equipped with an overhead LED light source, providing white and near-infrared light. The mice were continuously observed by a wall-mounted IEEE 1394 progressive scan video camera with a visible light filter (VID-CAM-MONO-2A; Med-Associates Inc., St. Albans, VT) connected to a computer in an adjacent room. Each chamber was connected to a solid-state scrambler, providing AC constant current shock, and an audio stimulus generator, controlled via an interface connected to a Windows computer running Video Freeze (Med-Associates, Inc., St. Albans, VT), a program designed for the automated assessment of freezing and locomotor activity. In results that will be published more fully elsewhere, computer and human scored data had a correlation of 0.971 and a fit of computer = −.007 + .974 × human (for more detail on this calculation, see [3]).

The conditioning context was altered along several dimensions for the extinction trials. White acrylic sheets were placed over the grid floors and a black plastic, triangular tent (23 cm, each side), translucent to near-infrared light, was placed inside each box. Only near-infrared light was used, creating a dark environment visible only to the video camera. Between extinction trials, the chambers were cleaned and scented with a 5% vinegar solution.

Acquisition training was conducted off-drug and consisted of a 2-min baseline activity period, followed by 9 tone–shock pairings, each separated by 20 s. During each tone–shock pairing, a 10-s tone (conditioned stimulus: 2.8 kHz, 90 dB, A scale) was presented and co-terminated with a scrambled footshock (unconditioned stimulus: 2 s, 0.75 mA, AC constant current) delivered through the floor of the cages. Freezing behavior, defined as the absence of all movement with the exception of respiration [12], was scored automatically using Video Freeze software (Med-Associates, Inc., St. Albans, VT). Mice were inside the fear-conditioning chambers for a total of 9 min before being returned to their home cages.

Twenty-four hours after training, mice began the first of 6 days of extinction trials in the alternate context described above, on-drug. Extinction consisted of a 1-min baseline, followed by 15 presentations of the training tone (10-s tone, 20-s interval between tones). Mice were removed from the chambers 30 s later and returned to their home cages. Freezing and activity were scored for the entire 9-min period during each extinction day. Drugs were administered intraperitoneally (i.p.) in a volume of 10 ml/kg. D-Amphetamine hemisulfate (Sigma–Aldrich Co., St. Louis, MO, USA) was dissolved in 0.9% sodium chloride. Amphetamine injections (salt weight: 0.005 or 0.05 mg/kg) were given i.p. 15 min prior to extinction trials. Mice were randomly assigned to one of three groups indicating the amount of amphetamine administered: 0 mg/kg (saline control, $n = 20$), 0.005 mg/kg ($n = 16$), and 0.05 mg/kg amphetamine ($n = 16$). Doses were chosen based on a previous study of cued fear acquisition [37]. A single, additional day of extinction (Day 7) was conducted off-drug, to serve as a state-dependent control.

Fig. 1 depicts each minute of acquisition training, consisting of a 2-min baseline period, followed by 9 tone–shock pairings, and a 2.5-min post-shock period. There was a main effect for minute [$F(8,392) = 66.1$, $p < 0.0001$], with freezing increasing after the onset of the tone–shock pairings. The animals were off-drug and no group differences [$F(2,49) = 0.819$, $p = 0.447$] or group by minute interactions [$F(2,49) = 0.388$, $p = 0.681$] were observed. On the first day of extinction, baseline locomotor activity (measured in arbitrary units by an automated computer scoring system) did not differ between groups [$F(2,49) = 0.156$, $p = 0.856$], suggesting that the low doses of amphetamine did not influence locomotor activity (data not depicted; see also [37]).

As we were interested in examining between-trial extinction (extinction retention [24]) and not within-trial extinction, we calculated the average freezing during the first 5 tones each day (Fig. 2A). Between-trial extinction seems more relevant to the treatment of learned fear because it is long lasting. We encountered moderately high baseline freezing during each extinction session (Fig. 2A, dashed lines), so we also measured tone freezing by subtracting baseline freezing from tone-elicited freezing (Fig. 2B). Subjects underwent 6 days of extinction trials (on-drug), and a

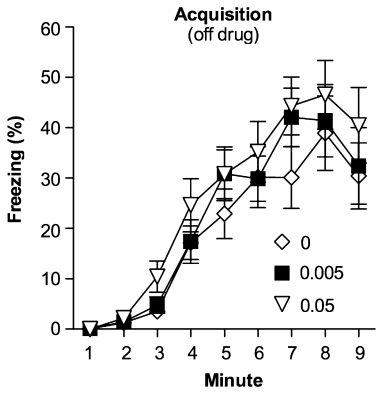

Fig. 1. Percentage of time spent freezing during training. The shocks were presented starting at 2 min. All subjects were off-drug and all groups showed the same freezing behavior. Each group represents the dose (mg/kg) of amphetamine given prior to each extinction trial (not given during acquisition). Each point represents the $M \pm SEM$.

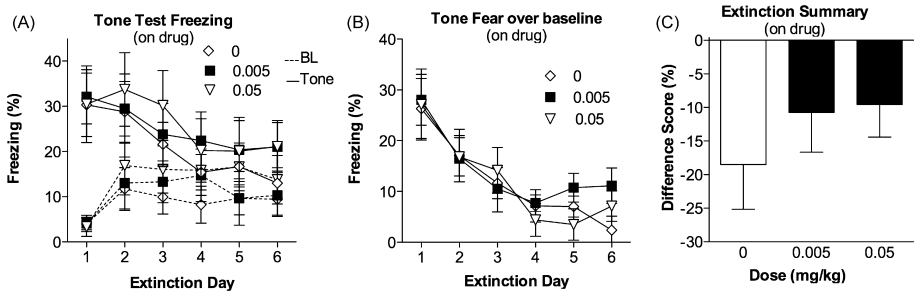

Fig. 2. (A) Percentage of time spent freezing during baseline (BL) and the average of the first 5 tone presentations (Tone) for each of the six on-drug extinction trial days. Each group represents the dose (mg/kg) of amphetamine given prior to each extinction trial. (B) Percentage of time spent freezing during the first tone block (first 5 tone presentations averaged) over baseline for each extinction day. (C) Difference between the percentages time spent freezing over baseline during the first tone block (first 5 tone presentations) on extinction Day 6 and extinction Day 1. All groups show evidence of extinction. Amphetamine did not affect between-trial or overall extinction. Each point represents the $M \pm SEM$.

main effect of day on average freezing over baseline during the first 5 tones was present [$F(5,245) = 15.890$, $p < 0.0001$] (Fig. 2B). Cued fear decreased as the number of extinction trials increased. Thus, all of the groups showed cued fear extinction; freezing decreased by at least 50% between Days 1 and 6 of the extinction trials. No group differences in between-trial extinction [$F(2,49) = 0.223$, $p = 0.801$] or group-by-day interaction [$F(10,245) = 0.498$, $p = 0.89$] were observed. To purely measure extinction, we generated a difference score by subtracting average freezing during the first 5 tones of extinction Day 1 from average freezing during the first 5 tones of extinction Day 6 (Fig. 2C). Again, all of the groups showed extinction, as demonstrated by the negative difference scores (percent freezing was greater on Day 1 than on Day 6 for all groups). No group differences were observed [$F(2,49) = 0.280$, $p = 0.757$]. Finally, although this experiment was not optimally designed to examine within-trial extinction because of the very close spacing of the tone presentations, no group differences were found in terms of short-term extinction during extinction Day 1 across the 15 tones [MANOVA, group by time interaction $F(2,49) = 0.81$, $p = 0.738$, or the difference between the average of tones 1–3 and 13–15, $F(2,49) = 0.925$, $p = 0.404$; data not depicted].

We also examined locomotor activity during the extinction trials as an alternate index of fear [3]. As in our previous analyses, we examined activity across the first 5 tone presentations to compare between-trial, rather than within-trial, changes in activity. We generated a suppression ratio to control for baseline differences in subjects' activity. The suppression ratio was defined as: (average activity during the first 5 tones)/(activity during the first 5 tones + activity during extinction trial baseline). Very low values indicate a high level of fear, 0.5 indicates no fear, and values greater than 0.5 can indicate conditioned safety [3,4]. There was a significant effect of day on the activity suppression ratios [$F(5,245) = 27.102$, $p < 0.0001$] (Fig. 3A), with suppression scores increasing (indicating decreased fear) as the number of extinction trials increased. By extinction Day 6, the suppression ratios were significantly larger (indicating less fear) than they had been on Day 1. No main effect of group [$F(2,49) = 0.337$, $p = 0.715$], or day-by-group interaction [$F(10,245) = 1.09$, $p = 0.370$] was observed.

On the last extinction day (Day 7), subjects underwent the same extinction protocol as Days 1–6, but were tested off-drug. This trial served as a state-dependent control. Regardless of treatment on prior extinction trial days, subjects displayed low levels of freezing when tested off-drug; tone-elicited freezing (average of the 5 tones) minus baseline freezing (first minute) is depicted (Fig. 3B). The extinction memory was retained and there was no evidence of state-dependent memory. No group differences in tone-elicited freezing were found [$F(2,49) = 0.007$, $p = 0.993$]. These results provide no evidence that amphetamine altered the extinction of cued fear.

We examined the effects of amphetamine on the extinction of cued fear. As has been reported with higher doses [23], we found that low (therapeutic) doses of amphetamine do not facilitate extinction of conditioned fear. We hypothesized that because cued fear extinction involves new learning, ultra-low doses of

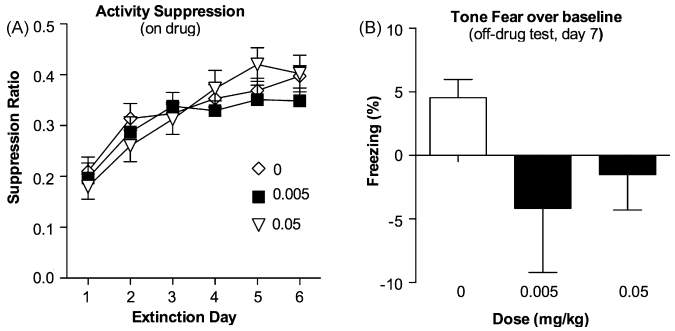

Fig. 3. (A) Activity suppression for each of the six on-drug extinction trial days. Activity suppression was computed as suppression ratio = (average activity during the first 5 tones)/(activity during the first 5 tones + activity during baseline). Values close to 0.0 reflect high levels of fear; values close to 0.5 reflect no fear [4]. Amphetamine administered before each extinction trial did not affect activity suppression. Each point represents the $M \pm SEM$. (B) Percentage time spent freezing during the state-dependent control test (extinction Day 7). All animals were off-drug and there was no evidence of state-dependent memory. Each bar represents the $M \pm SEM$.

amphetamine, previously shown to dramatically enhance cued fear acquisition [37], would also enhance extinction. Mueller et al. [23] failed to observe a facilitatory effect of amphetamine on cued fear extinction, perhaps because they used a dose (1 mg/kg) that does not affect cued fear acquisition [37]. Our results, however, are not consistent with this hypothesis.

Prior research has also found that amphetamine does not affect extinction on other behavioral paradigms. For example, a moderately high dose of amphetamine (5 mg/kg) given during extinction of fear-potentiated startle in rats failed to alter extinction [8]. Also, amphetamine (1 mg/kg) had no effect on extinction of conditioned approach [7,10]. Amphetamine (5 mg/kg) has even been found to impair extinction of passive avoidance [15,16]. As with Mueller et al. [23], however, all of these studies used moderate to high doses of amphetamine that induce locomotor hyperactivity and impair the acquisition of fear-conditioning [37]. Thus, to address this confound we used very low doses of amphetamine that do not influence activity, but can enhance memory [37]. As expected, baseline activity measurements during the first day of extinction did not differ between the amphetamine and saline groups. Thus, amphetamine's lack of effect on extinction in the current experiment cannot be attributed to amphetamine-induced alterations in locomotor activity.

One explanation for our finding is that the acquisition of aversive memories and their extinction reflect different types of new memory formation. Early evidence that N-methyl-D-aspartate (NMDA) receptors are essential for both acquisition and extinction of fear fostered enthusiasm that the mechanisms of acquisition and extinction may be similar [19,34,35]. However, more recent evidence suggests that the neural circuitry and pharmacology of fear acquisition and extinction are dissociable [for a review see [24,26]]. Li et al. [18] provide a model demonstrating how the amygdala could encode fear acquisition and extinction memories independently using discrete neural pathways. At the synaptic level, extinction, but not acquisition, depends on cannabinoid receptors [32]. At the systems level, extinction, but not acquisition, may depend on the medial prefrontal cortex [22,25]. If the neural mechanisms were different, then a drug would not necessarily be expected to enhance both acquisition and extinction. Additionally, acquisition and extinction may have different dose–response curves for pharmacological manipulation, though this seems unlikely as 1.0 mg/kg [23], and now 0.005 and 0.05 mg/kg, amphetamine has been shown to have no effect on cued fear extinction.

Several limitations in this study need to be addressed. The mice showed somewhat low levels of freezing to the tone on the first day of extinction (about 30%, after correcting for baseline, for all groups). As a result, there may have been insufficient ability to detect subtle differences in extinction. The mice were trained in a context with a bright light and underwent extinction trials in the dark. As mice are nocturnal, their activity increases in the dark and freezing behavior to the tone may have been confounded by increased activity simply due to the darker environment. Despite this, mice showed robust between-trial extinction and there was ample opportunity to observe differences between saline and amphetamine-treated mice. To address these concerns, future studies will look at the effect of different conditioning parameters (e.g. increased shock intensity and/or a different number of tone–shock pairings), and extinction training in a bright context.

To conclude, amphetamine does not appear to be a suitable candidate for facilitating fear extinction. As neural mechanisms underlying extinction learning are identified, so are potential targets for pharmacological manipulation. Exposure therapy can successfully be augmented pharmacologically [27], and it would be of significant clinical value to continue searching for those drugs that may enhance extinction. Additionally, to further investigate the dissociation between fear acquisition and extinction learning, it would be useful to concurrently examine acquisition and extinction of fear with a variety of memory-enhancing drugs [9,17,29,35,37].

Acknowledgements

We thank Jennifer Sage and Tristan Shuman for helpful comments on the manuscript. We also thank Denise Cai for excellent data analysis assistance. These studies were supported by NSF Graduate Research Fellowship (SCW), NIH NRSA DA026259 (SCW), NIH grant DA020041 (SGA) and Hellman Fellowship (SGA).

References

[1] P.A. Ahmann, F.W. Theye, R. Berg, A.J. Linquist, A.J. Van Erem, L.R. Campbell, Placebo-controlled evaluation of amphetamine mixture-dextroamphetamine salts and amphetamine salts (Adderall): efficacy rate and side effects, Pediatrics 107 (2001) E10.
[2] S.G. Anagnostaras, G.D. Gale, M.S. Fanselow, Hippocampus and contextual fear conditioning: recent controversies and advances, Hippocampus 11 (2001) 8–17.
[3] S.G. Anagnostaras, S.A. Josselyn, P.W. Frankland, A.J. Silva, Computer-assisted behavioral assessment of Pavlovian fear conditioning in mice, Learn. Mem. 7 (2000) 58–72.
[4] Z. Annau, L.J. Kamin, The conditioned emotional response as a function of intensity of the US, J. Comp. Physiol. Psychol. 54 (1961) 428–432.
[5] M. Barad, P.W. Gean, B. Lutz, The role of the amygdala in the extinction of conditioned fear, Biol. Psychiatry 60 (2006) 322–328.
[6] D.M. Barch, C.S. Carter, W. Perlstein, J. Baird, J.D. Cohen, N. Schooler, Increased stroop facilitation effects in schizophrenia are not due to increased automatic spreading activation, Schizophr. Res. 39 (1999) 51–64.
[7] C.A. Blaiss, P.H. Janak, Post-training, but not post-reactivation, administration of amphetamine and anisomycin modulates Pavlovian conditioned approach, Neurobiol. Learn. Mem. 87 (2007) 644–658.
[8] T.B Borowski, L. Kokkinidis, The effects of cocaine, amphetamine, and the dopamine D1 receptor agonist SKF 38393 on fear extinction as measured with potentiated startle: implications for psychomotor stimulant psychosis, Behav. Neurosci. 112 (1998) 952–965.
[9] J.P. Chhatwal, M. Davis, K.A. Maguschak, K.J. Ressler, Enhancing cannabinoid neurotransmission augments the extinction of conditioned fear, Neuropsychopharmacology 30 (2005) 516–524.
[10] H.J. Dudderidge, J.A. Gray, Joint effects of sodium amylobarbitone and amphetamine sulphate on resistance to extinction of a rewarded running response in the rat, Psychopharmacologia 35 (1974) 365–370.
[11] W.A. Falls, M.J. Miserendino, M. Davis, Extinction of fear-potentiated startle: blockade by infusion of an NMDA antagonist into the amygdala, J. Neurosci. 12 (1992) 854–863.
[12] M.S. Fanselow, Conditioned and unconditional components of post-shock freezing, Pavlov. J. Biol. Sci. 15 (1980) 177–182.
[13] E.B. Foa, Psychosocial treatment of posttraumatic stress disorder, J. Clin. Psychiatry 5 (Suppl. 61) (2000) 43–48, discussion 49–51.
[14] A. Garakani, S.J. Mathew, D.S. Charney, Neurobiology of anxiety disorders and implications for treatment, Mt. Sinai J. Med. 73 (2006) 941–949.
[15] L. Kokkinidis, The effects of chronic amphetamine administration on the acquisition and extinction of an active and passive avoidance response in mice, Pharmacol. Biochem. Behav. 19 (1983) 593–598.
[16] R. Kumar, Extinction of fear. I. Effects of amylobarbitone and dexamphetamine given separately and in combination on fear and exploratory behaviour in rats, Psychopharmacologia 19 (1971) 163–187.
[17] L. Ledgerwood, R. Richardson, J. Cranney, Effects of D-cycloserine on extinction of conditioned freezing, Behav. Neurosci. 117 (2003) 341–349.
[18] G. Li, S.S. Nair, G.J. Quirk, A biologically realistic network model of acquisition and extinction of conditioned fear associations in lateral amygdala neurons, J. Neurophysiol. 101 (2009) 1629–1646.
[19] K.T. Lu, D.L. Walker, M. Davis, Mitogen-activated protein kinase cascade in the basolateral nucleus of amygdala is involved in extinction of fear-potentiated startle, J. Neurosci. 21 (2001) RC162.
[20] S. Maren, G. Aharonov, D.L. Stote, M.S. Fanselow, N-methyl-D-aspartate receptors in the basolateral amygdala are required for both acquisition and expression of conditional fear in rats, Behav. Neurosci. 110 (1996) 1365–1374.
[21] J.L. Martinez Jr., R.A. Jensen, R.B. Messing, B.J. Vasquez, B. Soumireu-Mourat, D. Geddes, K.C. Liang, J.L. McGaugh, Central and peripheral actions of amphetamine on memory storage, Brain Res. 182 (1980) 157–166.
[22] M.R. Milad, G.J. Quirk, Neurons in medial prefrontal cortex signal memory for fear extinction, Nature 420 (2002) 70–74.
[23] D. Mueller, L.A. Olivera-Figueroa, D.S. Pine, G.J. Quirk, The effects of yohimbine and amphetamine on fear expression and extinction in rats, Psychopharmacology (Berl.) (2009).
[24] K.M. Myers, M. Davis, Mechanisms of fear extinction, Mol. Psychiatry 12 (2007) 120–150.

[25] G.J. Quirk, R. Garcia, F. Gonzalez-Lima, Prefrontal mechanisms in extinction of conditioned fear, Biol. Psychiatry 60 (2006) 337–343.
[26] G.J. Quirk, D. Mueller, Neural mechanisms of extinction learning and retrieval, Neuropsychopharmacology 33 (2008) 56–72.
[27] K.J. Ressler, B.O. Rothbaum, L. Tannenbaum, P. Anderson, K. Graap, E. Zimand, L. Hodges, M. Davis, Cognitive enhancers as adjuncts to psychotherapy: use of D-cycloserine in phobic individuals to facilitate extinction of fear, Arch. Gen. Psychiatry 61 (2004) 1136–1144.
[28] M.T. Rogan, U.V. Staubli, J.E. LeDoux, Fear conditioning induces associative long-term potentiation in the amygdala, Nature 390 (1997) 604–607.
[29] T. Shuman, S.C. Wood, S.G. Anagnostaras, Modafinil and memory: effects of modafinil on Morris water maze learning and Pavlovian fear conditioning, Behav. Neurosci. 123 (2009) 257–266.
[30] E. Soetens, S. Casaer, R. D'Hooge, J.E. Hueting, Effect of amphetamine on long-term retention of verbal material, Psychopharmacology (Berl.) 119 (1995) 155–162.
[31] E. Soetens, R. D'Hooge, J.E. Hueting, Amphetamine enhances human-memory consolidation, Neurosci. Lett. 161 (1993) 9–12.
[32] A. Suzuki, S.A. Josselyn, P.W. Frankland, S. Masushige, A.J. Silva, S. Kida, Memory reconsolidation and extinction have distinct temporal and biochemical signatures, J. Neurosci. 24 (2004) 4787–4795.
[33] D.C. Turner, T.W. Robbins, L. Clark, A.R. Aron, J. Dowson, B.J. Sahakian, Cognitive enhancing effects of modafinil in healthy volunteers, Psychopharmacology (Berl.) 165 (2003) 260–269.
[34] D.L. Walker, M. Davis, The role of amygdala glutamate receptors in fear learning, fear-potentiated startle, and extinction, Pharmacol. Biochem. Behav. 71 (2002) 379–392.
[35] D.L. Walker, K.J. Ressler, K.T. Lu, M. Davis, Facilitation of conditioned fear extinction by systemic administration or intra-amygdala infusions of D-cycloserine as assessed with fear-potentiated startle in rats, J. Neurosci. 22 (2002) 2343–2351.
[36] J.B. Watson, R. Rayner, Conditioned emotional reactions, 1920, Am. Psychol. 55 (2000) 313–317.
[37] S.C. Wood, S.G. Anagnostaras, Memory and psychostimulants: modulation of Pavlovian fear conditioning by amphetamine in C57BL/6 mice, Psychopharmacology (Berl.) 202 (2009) 197–206.

Chapter 2, in full, is a reprint of the material as it appears in Amphetamine and extinction of cued fear. *Neuroscience Letters*, 468, 18-22. Carmack SA, Wood SC & Anagnostaras SG (2014). The dissertation author was the primary investigator and author of this paper.

Chapter 3

Animal model of methylphenidate's long-term memory enhancing effects

Animal model of methylphenidate's long-term memory-enhancing effects

Stephanie A. Carmack, Kristin K. Howell, Kleou Rasaei, et al.

Learn. Mem. 2014 21: 82-89
Access the most recent version at doi:10.1101/lm.033613.113

Supplemental Material http://learnmem.cshlp.org/content/suppl/2014/01/10/21.2.82.DC1.html

References This article cites 57 articles, 12 of which can be accessed free at: http://learnmem.cshlp.org/content/21/2/82.full.html#ref-list-1

Creative Commons License This article is distributed exclusively by Cold Spring Harbor Laboratory Press for the first 12 months after the full-issue publication date (see http://learnmem.cshlp.org/site/misc/terms.xhtml). After 12 months, it is available under a Creative Commons License (Attribution-NonCommercial 3.0 Unported), as described at http://creativecommons.org/licenses/by-nc/3.0/.

Email Alerting Service Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or **click here.**

To subscribe to *Learning & Memory* go to:
http://learnmem.cshlp.org/subscriptions

© 2014 Carmack et al.; Published by Cold Spring Harbor Laboratory Press

Research

Animal model of methylphenidate's long-term memory-enhancing effects

Stephanie A. Carmack,[1] Kristin K. Howell,[1] Kleou Rasaei,[1] Emilie T. Reas,[2] and Stephan G. Anagnostaras[1,2,3]

[1]*Molecular Cognition Laboratory, Department of Psychology, University of California, San Diego, California 92093-0109, USA;* [2]*Program in Neurosciences, University of California, San Diego, California 92093-0109, USA*

Methylphenidate (MPH), introduced more than 60 years ago, accounts for two-thirds of current prescriptions for attention deficit hyperactivity disorder (ADHD). Although many studies have modeled MPH's effect on executive function, almost none have directly modeled its effect on long-term memory (LTM), even though improvement in LTM is a critical target of therapeutic intervention in ADHD. We examined the effects of a wide range of doses of MPH (0.01–10 mg/kg, i.p.) on Pavlovian fear learning, a leading model of memory. MPH's effects were then compared to those of atomoxetine (0.1–10 mg/kg, i.p.), bupropion (0.5–20 mg/kg, i.p.), and citalopram (0.01–10 mg/kg, i.p.). At low, clinically relevant doses, MPH enhanced fear memory; at high doses it impaired memory. MPH's memory-enhancing effects were not confounded by its effects on locomotion or anxiety. Further, MPH-induced memory enhancement seemed to require both dopamine and norepinephrine transporter inhibition. Finally, the addictive potential of MPH (1 mg/kg and 10 mg/kg) was compared to those of two other psychostimulants, amphetamine (0.005 mg/kg and 1.5 mg/kg) and cocaine (0.15 mg/kg and 15 mg/kg), using a conditioned place preference and behavioral sensitization paradigm. We found that memory-enhancing effects of psychostimulants observed at low doses are readily dissociable from their reinforcing and locomotor activating effects at high doses. Together, our data suggest that fear conditioning will be an especially fruitful platform for modeling the effects of psychostimulants on LTM in drug development.

[Supplemental material is available for this article.]

The psychostimulant methylphenidate (MPH) has been used since 1955 as a cognitive enhancer and wake-promoting agent for a variety of disorders (Challman and Lipsky 2000). Over time, it has become the mainstay of treatment for attention deficit hyperactivity disorder (ADHD) as it improves executive control, reduces impulsivity, and improves cognitive function, including learning and memory (O'Toole et al. 1997; Aron et al. 2003; Mehta et al. 2004; Arnsten 2006; Swanson et al. 2011). MPH-induced memory enhancement is often viewed as incidental to improved attention and/or cognitive control (Barkley 1997). Although many studies have modeled MPH's effect on executive function, almost none have directly modeled its effect on long-term memory (LTM) acquisition or retention, per se.

Improvement in LTM is a critical target of therapeutic intervention in ADHD, as ample evidence shows a deficit in LTM in ADHD (Rhodes et al. 2012). Psychostimulants are frequently prescribed to enhance classroom learning and are increasingly sought out by individuals without ADHD for the same reason. Indeed, stimulants also enhance learning in normal populations (Rapoport et al. 1980; Rapoport and Inoff-Germain 2002; Marshall et al. 2010). Thus, the degree to which MPH directly enhances LTM warrants further examination.

Drug development for ADHD would benefit from a simple, efficient animal model of MPH's effects on LTM. We examined the effects of a wide range of doses of MPH on Pavlovian fear learning. In this task, an initially neutral tone conditional stimulus is paired with an aversive foot-shock unconditional stimulus. As the result of this pairing, the animal comes to fear both the tone and the place of conditioning, a phenomenon known as context conditioning. Tone and context fear memory are used generally to model long-lasting memory (Anagnostaras et al. 1999; Gale et al. 2004).

Fear conditioning has become the leading model of LTM in rats and mice (Anagnostaras et al. 2000, 2001, 2010; Maren 2008). The core neuroanatomy is well studied and distinct from working memory and executive control; acquisition and retention requires the amygdala and hippocampus (Anagnostaras et al. 2001; Gale et al. 2004). The prefrontal cortex (PFC), which has an essential role in working memory and executive function, has a more limited role in fear inhibition and extinction, rather than acquisition (Morgan and LeDoux 1995; Braver et al. 2001).

MPH, a high affinity dopamine transporter (DAT) and norepinephrine transporter (NET) inhibitor (Han and Gu 2006), modulates behavior via increased monoamine neurotransmission (Kuczenski and Segal 1997, 2002; Lazzaro et al. 2010; de Oliveira et al. 2011; Johansen et al. 2011). We also tested diverse monoamine transporter inhibitors that have been used to treat ADHD, atomoxetine (ATM, NET inhibitor), bupropion (BPN, DAT inhibitor), and citalopram (CIT, SERT inhibitor), on fear learning (Fone and Nutt 2005). We further examined MPH's ability to induce locomotor hyperactivity and anxiety as they potentially confound fear conditioning.

[3]Corresponding author
E-mail stephana@ucsd.edu
Article is online at http://www.learnmem.org/cgi/doi/10.1101/lm.033613.113.

© 2014 Carmack et al. This article is distributed exclusively by Cold Spring Harbor Laboratory Press for the first 12 months after the full-issue publication date (see http://learnmem.cshlp.org/site/misc/terms.xhtml). After 12 months, it is available under a Creative Commons License (Attribution-NonCommercial 3.0 Unported), as described at http://creativecommons.org/licenses/by-nc/3.0/.

We also assessed whether MPH's procognitive and reinforcing effects are dissociable using a conditioned place preference (CPP) and behavioral sensitization paradigm. Behavioral sensitization is a progressive increase in response following repeated administration and models the transition from casual to compulsive use (Robinson and Berridge 1993, 2003). Place preference is the preference for a context previously paired with a drug and is a model of drug seeking. We compared MPH's reinforcing ability with those of amphetamine (AMPH) and cocaine (COC).

In all, we found that memory-enhancing effects of psychostimulants at low doses are readily dissociable from their reinforcing and locomotor activating effects at high doses. We further found that MPH was neither anxiogenic nor anxiolytic. We conclude that MPH's ability to enhance long-term memory appears to be due to a combination of DAT and NET inhibition. We consider whether these results support a direct effect on associativity and memory, rather than as incidental to improved executive function (Barkley 1997).

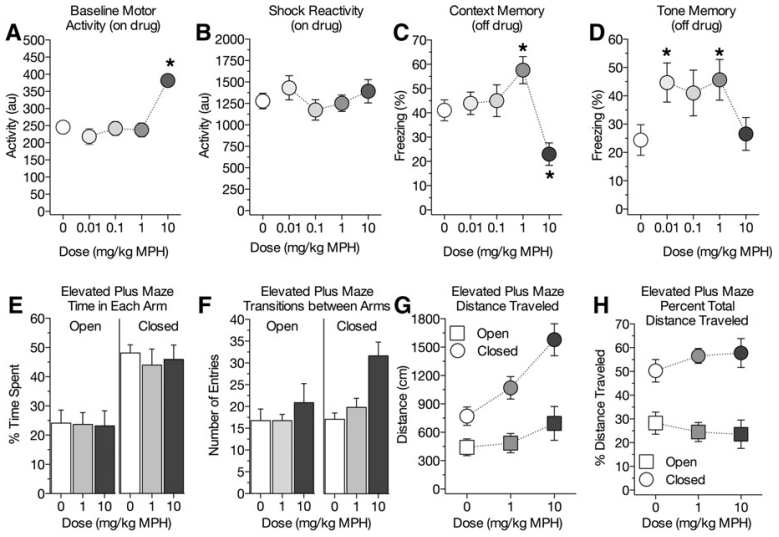

Figure 1. MPH dose-dependently modulates fear memory. (*A*) Locomotor activity during training. Mice on 10 mg/kg MPH had significantly elevated locomotor activity as compared to saline controls (0 mg/kg MPH). No other groups differed from saline controls. (*B*) Shock reactivity. The 2-sec shock presentation elicited a similar unconditioned response in all of the groups. (*C*) Context fear memory. When tested off drug 1 wk following training, the group previously given 1 mg/kg dose MPH showed enhanced contextual fear memory as compared to saline controls, while 10 mg/kg MPH impaired contextual memory. Both 0.01 and 0.1 mg/kg MPH failed to influence contextual fear memory. (*D*) Tone fear memory. MPH dose-dependently modulated tone fear memory. Both 0.01 and 1 mg/kg MPH dramatically improved tone fear memory relative to saline controls. Both 0.1 and 10 mg/kg MPH did not significantly influence tone fear learning. (*E*) Time spent in the open vs. closed arms of the elevated plus maze. Neither a high (10 mg/kg), nor a low (1 mg/kg) dose of MPH had an effect on anxiety. (*F*) Transitions into each arm of the elevated plus maze. Mice given 10 mg/kg MPH made more transitions into the enclosed arms than the saline control or 1 mg/kg groups, which did not differ. (*G*) Distance traveled in the open vs. enclosed arms. Mice given 10 mg/kg MPH traveled significantly farther in the enclosed arms than the saline control or 1 mg/kg groups, which did not differ. MPH did not affect distance traveled in the open arms. (*H*) MPH did not influence the percent of total distance traveled in the open vs. enclosed arms. Each point represents the mean ± 1 standard error. (*) Data points identify significant post-hoc comparisons against the saline control group using Fisher's protected least significant difference tests following significant omnibus comparisons.

Results

MPH dose-effect curve on fear conditioning

MPH's (0.01–10 mg/kg, i.p.) effects on long-term memory were investigated using Pavlovian fear conditioning. MPH dose-dependently increased locomotor activity during the training baseline ($F_{(4,70)} = 11.87$, $P < 0.0001$) (Fig. 1A). Only mice given 10 mg/kg MPH showed significantly more activity than the saline control group (PLSD, $P < 0.0001$; all other P values >0.3). The 2-sec shock elicited a large increase in velocity, known as the unconditioned response, which did not significantly differ between groups ($F_{(3,70)} = 0.79$, $P = 0.54$) (Fig. 1B).

MPH dose-dependently modulated freezing during the first 5 min of training (on drug data not depicted) ($F_{(4,66)} = 6.03$, $P < 0.0001$). Compared to saline controls (0 mg/kg), 0.01 mg/kg enhanced freezing ($P < 0.005$), 10 mg/kg decreased freezing ($P < 0.04$), and 0.1 and 1 mg/kg MPH produced no significant effect (P values >0.6).

There were significant overall group differences in freezing during the immediate memory test ($F_{(4,70)} = 6.74$, $P < 0.0001$) (data not graphed). Mice given 10 mg/kg froze (0.4 ± 4.2%) significantly less than saline controls (22.2 ± 3.8%, $P < 0.0001$). However, 10 mg/kg MPH's ability to stimulate activity likely influenced freezing (Fig. 1A). No other doses affected immediate memory (0.01, 28.9 ± 4.2%; 0.1, 20.1 ± 4.2%; 1, 23.4 ± 4.5%; P values >0.2).

To determine if MPH influenced long-term contextual memory, mice were returned to the conditioning context 7 d later off drug. Pretraining MPH dose-dependently modulated memory ($F_{(4,70)} = 5.46$, $P = 0.001$) (Fig. 1C). Compared to saline controls, 1 mg/kg enhanced memory ($P = 0.027$), 10 mg/kg MPH reduced memory ($P = 0.012$), and 0.01 and 0.1 mg/kg MPH failed to influence memory to the context (P values >0.5).

Tone memory was assessed 24 h later (Fig. 1D). Baseline freezing was very low and did not differ between groups (not depicted; 0 mg/kg, 4.68 ± 1.8%; 0.01 mg/kg, 8.1 ± 2.1%; 0.1 mg/kg, 14.3 ± 5.3%; 1 mg/kg, 9.2 ± 4.0%; 10 mg/kg, 3.5 ± 0.9%; $F_{(4,66)} = 1.89$, $P > 0.10$). Again, MPH dose-dependently modulated memory ($F_{(4,70)} = 2.78$, $P = 0.034$). Both 0.01 and 1 mg/kg MPH dramatically enhanced memory relative to saline controls (P values <0.05). No other doses influenced freezing to the tone (P values >0.10).

Overall, we were able to model MPH's dose-dependent memory-enhancing effects using Pavlovian fear conditioning. Clinically relevant doses of MPH given pretraining enhanced long-term contextual and tone memory. In contrast, a high dose of MPH impaired contextual memory.

MPH and elevated plus maze

MPH may have modulated anxiety rather than memory acquisition. To control for this possibility, we investigated the effect of 0, 1, and 10 mg/kg MPH on the elevated plus maze. MPH had no effect on the percent of total time spent in the open vs. enclosed

arms ($F_{(2,21)} = 0.07$, $P = 0.93$) (Fig. 1E). MPH dose-dependently modulated the number of transitions into the enclosed ($F_{(2,21)} = 10.8$, $P = 0.001$), but not open arms ($F_{(2,21)} = 0.6$, $P = 0.56$) (Fig. 1F). Mice given 10 mg/kg MPH made more transitions into the enclosed arms than the saline control or 1 mg/kg groups (P values <0.002), which did not differ (P value >0.99). MPH also dose-dependently modulated the distance traveled in the enclosed ($F_{(2,21)} = 9.7$, $P = 0.001$), but not the open arms ($F_{(2,21)} = 1.1$, $P = 0.36$) (Fig. 1G). Mice given 10 mg/kg MPH traveled significantly farther than either the saline control or 1 mg/kg groups (P values <0.02), which did not differ (P value >0.1). However, MPH had no effect on the percent of total distance traveled in the open vs. enclosed arms ($F_{(2,21)} = 0.45$, $P = 0.64$) (Fig. 1H). These findings indicate that neither 1 nor 10 mg/kg MPH altered anxiety.

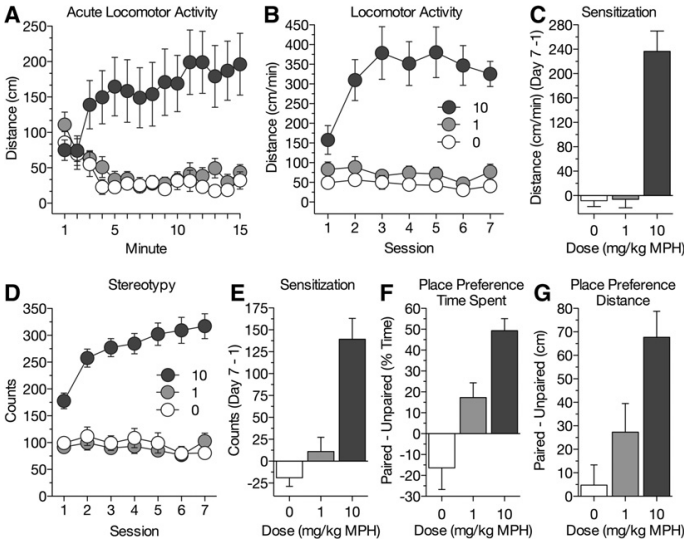

Figure 2. MPH and addiction-related behavior. (*A*) Locomotor activity on the Paired side during the first training session. Acutely, 10 mg/kg MPH greatly enhanced locomotor activity as compared to saline controls (0 mg/kg MPH), while 1 mg/kg MPH had no effect. (*B*) Locomotor activity as an average of each day across the seven training sessions on the Paired side. Mice receiving 10 mg/kg MPH showed greater locomotor activity than mice receiving saline or 1 mg/kg MPH, which did not differ from each other. (*C*) Development of locomotor sensitization. Sensitization was quantified as the difference in average locomotor response from days 1–7. Only the mice receiving 10 mg/kg MPH exhibited sensitization. Neither the saline control nor the 1 mg/kg MPH groups showed sensitization, and these groups did not differ. (*D*) Stereotyped activity as an average of each day across the seven training sessions on the Paired side. Mice receiving 10 mg/kg MPH showed greater stereotyped activity than mice receiving saline or 1 mg/kg MPH, which did not differ from one another. (*E*) Development of sensitization of stereotyped behavior. Sensitization was quantified as the difference in average stereotypic response from days 1–7. Only the mice receiving 10 mg/kg MPH sensitized. Neither the saline control nor the 1 mg/kg MPH group showed sensitization; these two groups did not differ. (*F*) Conditioned place preference. Preference was measured as the difference between the percent of time spent on the Paired side vs. the Unpaired side; positive values indicate preference for the Paired side. Mice that received 10 mg/kg MPH showed substantial place preference and greater preference for the Paired side than the other groups. Mice that received 1 mg/kg MPH showed a very small, but significant preference for the drug-paired side. The saline control group did not show any preference. (*G*) Conditioned place preference. Preference was also measured as the difference between the distance traveled on the Paired side vs. the Unpaired side. Mice that received 10 mg/kg MPH traveled farther on the Paired side than the other groups, which did not differ from one another. Each point represents the mean ± 1 standard error.

MPH-induced CPP and sensitization

We selected the two doses of MPH—1 and 10 mg/kg—that modulated memory (Fig. 1D) and investigated their addictive potential.

Figure 2A depicts locomotor activity (distance traveled) on training day 1 on the Paired side. Similar to our observations in fear conditioning (Fig. 1A), the acute response to various doses of MPH were significantly different ($F_{(2,36)} = 9.83$, $P < 0.0001$). Compared to saline controls, 10 mg/kg increased ($P < 0.0001$) and 1 mg/kg MPH had no effect on locomotor activity ($P > 0.7$).

Figure 2B shows locomotor activity across days of training on the Paired side. Significant group differences were observed ($F_{(2,36)} = 30.0$, $P < 0.0001$). Mice receiving 10 mg/kg MPH showed greater locomotor activity than mice receiving saline or 1 mg/kg (P values <0.001), which did not differ from each other ($P > 0.5$). Sensitization was quantified as the difference in average locomotor response from days 1–7 (Fig. 2C). There were significant group differences ($F_{(2,36)} = 12.54$, $P < 0.0001$). Neither the saline control nor the 1 mg/kg MPH groups showed sensitization; these groups did not differ ($P > 0.8$). Only the mice receiving 10 mg/kg MPH exhibited sensitization ($P < 0.0001$).

Figure 2D shows stereotypy during training on the Paired side. Significant group differences were observed ($F_{(2,36)} = 63.0$, $P < 0.0001$). In terms of average response, mice receiving 10 mg/kg MPH showed greater stereotyped activity than mice receiving saline or 1 mg/kg (P values <0.001), which did not differ from each other ($P > 0.7$). As with locomotor activity, there were significant group differences in sensitization ($F_{(2,36)} = 23.0$, $P < 0.0001$) (Fig. 2E). Only the mice receiving 10 mg/kg MPH sensitized ($P < 0.0001$). No other groups showed sensitization (P values <0.2).

To test CPP, mice were returned off drug with free access to both sides of the apparatus. Preference was measured as the time spent and distance traveled on the Paired vs. Unpaired sides. There were significant group differences in both time spent ($F_{(2,36)} = 17.1$, $P < 0.0001$) (Fig. 2F) and distance traveled ($F_{(2,36)} = 8.87$, $P < 0.001$) (Fig. 2G). Mice given 10 mg/kg MPH showed substantial CPP (time spent, one sample two-tailed t-test against hypothesized $\mu = 0$, $t_{(12)} = 8.49$, $P < 0.0001$; distance traveled, $t_{(12)} = 6.14$, $P < 0.0001$) and greater preference for the Paired side than the other groups (time spent, P values <0.007; distance traveled, P values <0.01). Mice given 1 mg/kg MPH showed a very small, but significant preference for the drug-paired side (time spent, $t_{(12)} = 2.46$, $P = 0.03$; distance traveled, $t_{(12)} = 2.24$, $P = 0.05$). The saline control group did not show any preference (time spent, $t_{(12)} = 1.59$, $P = 0.14$; distance traveled, $t_{(12)} = 0.56$, $P = 0.59$).

To further explore sensitization, mice were challenged with low MPH (1 mg/kg) and then high MPH (10 mg/kg) on the Paired side (Fig. 3). There were no overall group differences in locomotor activity ($P = 0.112$) (Fig. 3A, left) following the low MPH challenge, but there were significant group differences in stereotypic counts ($F_{(2,36)} = 7.95$, $P = 0.001$) (Fig. 3B, left). The group

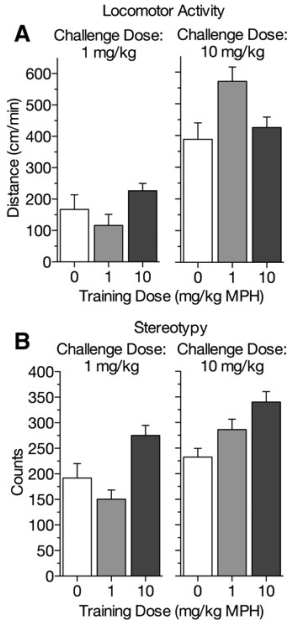

Figure 3. MPH-induced behavioral sensitization. (*A*) Sensitization of locomotor activity. No group differences in distance traveled were observed following a challenge injection of 1 mg/kg MPH (*left*). In contrast, following a challenge injection of 10 mg/kg MPH, the group trained with 1 mg/kg MPH showed significantly more locomotor activity than the groups previously given saline or 10 mg/kg MPH, which did not differ from on another (*right*). (*B*) Sensitization of stereotyped activity. The group trained with 10 mg/kg MPH showed significantly more stereotyped activity in response to a 1 mg/kg MPH challenge injection than both the saline control group and the group trained with 1 mg/kg MPH, which did not differ from one another (*left*). When challenged with a high dose of MPH (10 mg/kg), the group trained with 10 mg/kg MPH had significantly greater stereotypic counts than the other groups, which did not differ from one another (*right*). The transition to stereotyped behavior observed only in the group trained with 10 mg/kg MPH explains their lack of locomotor sensitization during the high dose MPH challenge test. Each point represents the mean ± 1 standard error.

trained with 10 mg/kg MPH showed significantly more stereotyped activity than both the saline control group and the group trained with 1 mg/kg MPH (P values <0.01), which did not differ from each other ($P > 0.2$). Only the group trained with 10 mg/kg MPH group showed evidence of a sensitized response to the low MPH challenge.

When challenged with a high dose of MPH (10 mg/kg, i.p.), there were significant group differences in both locomotor ($F_{(2,36)} = 4.82$, $P = 0.014$) (Fig. 3A) and stereotypic activity (Fig. 3B, right; $F_{(2,36)} = 7.83$, $P = 0.001$). The group trained with 1 mg/kg MPH exhibited some latent sensitization of locomotor activity and had significantly greater locomotor activity than the saline control and 10 mg/kg MPH groups (P values <0.03) (Fig. 3A, right). Surprisingly, these groups did not differ in locomotor response ($P = 0.55$). This finding appears to be driven by the 10 mg/kg MPH group's transition into stereotyped behavior. Indeed, the 10 mg/kg MPH group showed significantly greater stereotyped behavior than the other groups (P values <0.05), which did not differ ($P > 0.05$) (Fig. 3B, right). Both groups trained with MPH showed some sensitization in response to a high MPH challenge injection.

In sum, 1 mg/kg MPH had very minimal addictive potential. Repeated administration of 1 mg/kg MPH did not lead to the development of sensitization. However, challenge with a high dose (10 mg/kg MPH) injection induced some latent locomotor sensitization and there was very slight place preference. In contrast, repeated administration of 10 mg/kg MPH induced strong behavioral sensitization and CPP.

AMPH- and COC-induced CPP and sensitization

We extend these dissociable behavioral findings with MPH to two other psychostimulants, AMPH and COC. Low memory-enhancing doses of AMPH (0.005 mg/kg) (Wood and Anagnostaras 2009) and COC (0.15 mg/kg) (Wood et al. 2007) failed to induce behavioral sensitization or CPP. In contrast, high, memory-impairing doses of AMPH (1.5 mg/kg) and COC (15 mg/kg) had significant addictive potential (Fig. 4; see Supplemental Results for details).

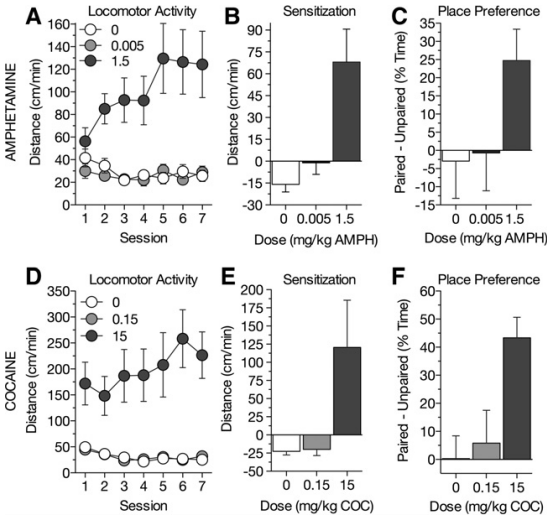

Figure 4. AMPH and COC-induced addiction-related behaviors. (*A*) Locomotor activity as an average of each day across the seven training sessions on the drug-paired side. Mice receiving 1.5 mg/kg AMPH showed greater locomotor activity than mice receiving saline or 0.005 mg/kg AMPH, which did not differ from each other. (*B*) Development of AMPH-induced locomotor sensitization. Sensitization was quantified as the difference in average locomotor response from days 1–7. Only the mice receiving 1.5 mg/kg AMPH exhibited sensitization. Neither the saline control nor the 0.005 mg/kg AMPH groups showed sensitization. (*C*) AMPH-induced conditioned place preference. Preference was measured as the difference between the percent of time spent on the Paired side vs. the Unpaired side; positive values indicate preference for the Paired side. Mice that received 1.5 mg/kg AMPH showed substantial place preference for Paired side. The saline control group and 0.005 mg/kg AMPH groups did not show any preference. (*D*) Locomotor activity as an average of each day across the seven training sessions on the drug-paired side. Mice receiving 15 mg/kg COC showed greater locomotor activity than mice receiving saline or 0.15 mg/kg COC, which did not differ from each other. (*E*) Development of COC-induced locomotor sensitization. Only the mice receiving 15 mg/kg COC sensitized. Neither the saline control nor the 0.15 mg/kg COC group sensitized. (*F*) COC-induced conditioned place preference. Mice that received 15 mg/kg COC showed substantial place preference and greater preference for the Paired side than the other groups. The saline control group and 0.15 mg/kg COC groups did not show any preference. Each point represents the mean ± 1 standard error.

Neurobiological mechanisms of MPH's dose-dependent behavioral effects

To investigate the neurobiological mechanisms that underlie MPH's dose-dependent dissociable behavioral effects, we examined the selective transporter inhibitors ATM, BUP, and CIT on fear memory (Supplemental Fig. S1A–I); for a detailed description, see Supplemental Material. Briefly, across the range of doses tested, ATM, BUP, and CIT failed to enhance LTM. In contrast, high doses of BPN and CIT impaired LTM (Supplemental Fig. S1E,H,I). Taken together with previous research in our lab demonstrating that low doses of AMPH and COC enhance LTM (Wood et al. 2007; Wood and Anagnostaras 2009), it is interesting to speculate that psychostimulants' ability to enhance LTM acquisition may be related to binding multiple transporter targets, in particular NET and DAT. In Table 1 our results are compared to published affinity studies (Wong et al. 1982; Richelson and Pfenning 1984; Forest Laboratories 2011; GlaxoSmithKline 2013). Within the realm of drugs often prescribed for ADHD, we found that drugs that are highly selective for a single transporter (ATM, CIT) failed to enhance LTM. In contrast, low doses of combined high affinity DAT and NET inhibitors AMPH, COC, and MPH enhanced memory without evidence of reinforcement. At high doses, however, many of the drugs impaired LTM (MPH, AMPH, COC, BUP, and CIT), produced locomotor hyperactivity (MPH, AMPH, COC), and showed evidence of reinforcement and addiction (AMPH, COC, MPH).

Discussion

More than 65 years after its introduction, MPH is the first-line treatment for ADHD (Spencer et al. 1996; Barkley 1998). MPH can have serious side effects, however, including growth retardation, nausea, insomnia, anxiety, tics, and cardiovascular risk (McNeil Pediatrics 2008), suggesting need for further development of psychostimulants. Though progress has been made in ADHD drug delivery, recently approved therapeutics, such as atomoxetine and guanfacine, are inferior in clinical efficacy to MPH or AMPH, despite their ability to reduce inattention and impulsivity (Wigal et al. 2005; Faraone et al. 2007; Newcorn et al. 2008).

Most clinical efficacy studies only report inattention/hyperactivity–impulsivity measures (ADHD IV) (Dittmann et al. 2013) and clinical global impression (CGI) (Setyawan et al. 2013). These studies do not assess efficacy in improving LTM. Rather, they focus on improvements in problem classroom behaviors even though a growing body of evidence shows an impairment of LTM in ADHD (Rhodes et al. 2012).

MPH's clinical efficacy is generally modeled using attention or cognitive control tasks, such as attentional set-shift, stop-signal, and five-choice serial reaction time (Puumala et al. 1996; Robbins 2002; Arnsten and Dudley 2005; Eagle et al. 2007; Berridge et al. 2012; Humby et al. 2013). However, these models do not assess LTM and are difficult to implement in high throughput drug development as they are complex, sometimes require extensive training, and often are in monkeys. Drug development will benefit from the addition of this simple, efficient mouse model of MPH's effects on LTM because of cost, the widespread use of mice preclinically, and the widely available genetic tools in mice.

At 1 mg/kg, MPH enhanced the acquisition of both contextual and tone memory. Even lower doses (0.01–0.1 mg/kg) dramatically enhanced tone memory. This finding is consistent with previous research showing that low doses of AMPH, modafinil, and COC enhance fear memory (Wood et al. 2007; Shuman et al. 2009; Wood and Anagnostaras 2009). Further, MPH modulates fear memory independent of its effects on locomotor activity or anxiety.

Pavlovian fear conditioning has become especially useful as an experimental model in psychiatric research because of its simplicity (LeDoux 1998; Maren 2008; Mahan and Ressler 2012):

Table 1. Behavioral effects and binding affinities of methylphenidate, amphetamine, cocaine, atomoxetine, bupropion, and citalopram

		Behavior			Binding affinity (K_i)[a]		
Drug	Dose	Locomotion[b]	Reinforcement[c]	Memory[d]	DAT (nM)	NET (nM)	SERT (nM)
Methylphenidate[e]	Low	–	–	↑	160	40	22,000
	High	↑	↑	↓			
D-Amphetamine[f]	Low	–	–	↑	82	50	1840
	High	↑	↑	↓			
Cocaine[g]	Low	↑	–	↑	270	155	180
	High	↑	↑	↓			
Atomoxetine[h]	Low	–	–	–	1800	1.9	750
	High	↓	–	–			
Bupropion[i]	Low	–	–	–	630	2300	15,600
	High	↑	?	↓			
Citalopram[j]	Low	–	–	–	28,000	4000	1.3
	High	–	?	↓			

[a]Published K_i values are shown for methylphenidate, amphetamine, cocaine, bupropion, citalopram (Richelson and Pfenning 1984), and atomoxetine (Wong et al. 1982) in the rat brain. Please note low K_i values indicate high affinity.

[b](↑) The drug elevates locomotor activity at the specified dose; (↓) the drug decreases locomotor activity; (–) no effect.

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

[d](↑) The drug enhances memory at the specified dose; (↓) the drug impairs memory; (–) no effect.

[e]Methylphenidate's locomotor and reinforcing effects are depicted in Figures 1A, 2, and 3; its effects on memory are shown in Figure 1, C and D.

[f]D-Amphetamine's locomotor and reinforcing effects are shown in Figure 4A–C; its effect on memory is previously published (Fig. 3 in Wood and Anagnostaras 2009).

[g]Cocaine's locomotor and reinforcing effects are depicted in Figure 4D–F; its effect on memory is previously published (Fig. 3 in Wood et al. 2007).

[h]Atomoxetine's locomotor and reinforcing effects are shown in Supplemental Figures S1A and S2; its effects on memory are shown in Supplemental Figure S1, B and C.

[i]Bupropion's locomotor and reinforcing effects are reported in Wellbutrin's FDA approved labeling (GlaxoSmithKline 2013); its effects on memory are shown in Supplemental Figure S1, E and F.

[j]Citalopram's locomotor and reinforcing effects are reported in Celexa's FDA approved labeling (Forest Laboratories 2011); its effects on memory are shown in Supplemental Figure S1, H and I.

a single tone–shock pairing can result in a long-lasting memory (Fig. 1). Additionally, its established neural circuitry is similar between rodents and humans (LeDoux 1998; Delgado et al. 2006). MPH, a high affinity DAT and NET inhibitor (Han and Gu 2006), likely enhances memory acquisition by increasing monoamine neurotransmission (Kuczenski and Segal 1997, 2002; Lazzaro et al. 2010; de Oliveira et al. 2011; Johansen et al. 2011). We tested CIT, ATM, and BPN on fear learning to investigate the consequences of selectively blocking SERT, NET, and DAT. Reviewing these very generally, one is left with the impression that considerable affinity for both NET and DAT may be required for the cognitive enhancing effects of psychostimulants (Table 1; Wong et al. 1982; Richelson and Pfenning 1984; Forest Laboratories 2011; GlaxoSmithKline 2013).

MPH's effects on memory acquisition are often construed to be the exclusive result of improved attention or executive control (Barkley 1997). This interpretation is difficult to reconcile with our observation that MPH dramatically enhances long-term tone memory (Fig. 1D). The attentional demands in tone fear conditioning are modest at best; a very loud tone is followed by an even more attention-grabbing, inescapable foot shock. Furthermore, although working memory is heavily conflated with executive control, decades of evidence suggest that the core neurobiology of LTM is distinct from that of executive control (Morgan and LeDoux 1995; Braver et al. 2001). This suggests that MPH may also directly influence core associative mechanisms such as long-term potentiation (LTP).

Substantial evidence does exist that MPH acts on cellular substrates implicated in LTM; for example, MPH enhances long-term potentiation and depression (Dommett et al. 2008; Tye et al. 2010). Recently, acute administration of MPH in rats has been shown to facilitate plasticity in the amygdala via an increase in AMPA receptor-mediated currents following a cue–reward learning task (Tye et al. 2010). MPH also increases hippocampal norepinephrine in vivo (Kuczenski and Segal 2002) and such changes are known to influence synaptic plasticity (Akirav and Richter-Levin 2002). Thus, the potential that MPH directly improves learning or associability directly warrants further investigation. Ultimately, improved classroom learning will be demonstrated by improvements in LTM, such as on exams.

We further demonstrate that MPH's memory-enhancing effect at low doses is dissociable from its reinforcing effects induced by high doses. Most animal studies have used doses 2–40 times higher than the clinically relevant dose in an effort to model addiction (Gainetdinov et al. 1999; Kuczenski and Segal 2002; Abraham et al. 2012). We have advocated using a one-to-one dosing scheme unless specific evidence warrants using a different dose in mice (Wood et al. 2007; Shuman et al. 2009; Wood and Anagnostaras 2009). No evidence suggests that appropriate rodent dosing should be 40 times higher than human dosing. MPH is available in a variety of time-released preparations, but is typically prescribed around 0.5–1 mg/kg, and is not meant to exceed 2 mg/kg/day (McNeil Pediatrics 2008). The memory-enhancing doses that we observed (0.01–1 mg/kg) accord well with and are on the same order of magnitude as prescribed doses.

The memory-enhancing dose (1 mg/kg MPH) showed little evidence of reinforcement. In contrast, 10 mg/kg MPH not only produced sensitization, place preference, and a marked stimulating effect, but it also impaired memory. This dissociation is supported by our observation that memory-enhancing doses of AMPH (0.005 mg/kg) and COC (1.5 mg/kg) also showed little evidence of reinforcement, while high, addictive, doses impaired memory (Fig. 4). Together, these results substantiate the view that psychostimulant dosage explains the "paradox" of cognitive enhancements in patient populations and cognitive deficits in addicts (Rapoport et al. 1980; Ellinwood et al. 1998; Rapoport and Inoff-Germain 2002; Berridge and Devilbiss 2011; Wood et al. 2013). As dosage dramatically dissociates psychostimulants' procognitive and reinforcing effects, it is likely that one can develop an MPH-like drug, which retains all of MPH's procognitive effects, but lacks any reinforcing effects. Though, to date, such efforts have been limited.

Overall, we found a clear long-term enhancement of memory by MPH at doses similar to those prescribed for ADHD; these memory-enhancing effects were not confounded by effects on locomotion or anxiety and were readily dissociable from the reinforcing effects seen at high doses. Together, our data suggest that fear conditioning will be an especially fruitful platform for modeling the effects of psychostimulants on LTM in drug development.

Materials and Methods

Subjects

We used 380 hybrid C57BL/6Jx129S1/SvImJ (Jackson Labs) group-housed mice, at least 10 wk old before testing. The vivarium was on a 14:10-h light–dark schedule and testing occurred during the light phase. All procedures were approved by the UCSD IACUC and compliant with the NRC Guide.

Drugs

Dosing was by salt weight and the vehicle was always 0.9% saline. Methylphenidate HCl (Sigma-Aldrich) was given in 0.01, 0.1, 1, or 10 mg/kg. Atomoxetine HCl (Tata) was given in 0.01, 0.5, 1, or 10 mg/kg. Bupropion HCl (Biomol) was given in 0.5, 5, 10, or 20 mg/kg. Citalopram HBr (Enzo) was given in 0.01, 0.1, 1, or 10 mg/kg. D-Amphetamine hemisulfate (Sigma) was given in 0.005 or 1.5 mg/kg. Cocaine HCl (Sigma) was given in 0.15 or 15 mg/kg. All injections were given intraperitoneally (i.p.), 10 mL/kg.

Fear conditioning

Eight mice were tested concurrently in individual conditioning chambers. The VideoFreeze system (Med Associates) was used as described previously (Anagnostaras et al. 2010; Carmack et al. 2010, 2013); see Supplemental Methods for details of all drugs tested. For MPH experiments mice were injected 30 min before training. Mice were randomly assigned to groups by dose of MPH administered: 0 (saline control, $n = 17$), 0.01 ($n = 14$), 0.1 ($n = 14$), 1 ($n = 12$), or 10 mg/kg ($n = 14$).

Training began with a 3-min baseline, followed by one tone–shock pairing, consisting of a 30-sec tone (2.8 kHz, 85 dBA) that co-terminated with a 2-sec scrambled, AC foot shock (0.75 mA, RMS). Mice were in the chambers for a total of 10 min (Wood and Anagnostaras 2011). Freezing behavior and locomotor activity were recorded (Anagnostaras et al. 2000; Carmack et al. 2010).

Mice were returned to the training context, without drug, 7 d later. Freezing was scored for 5 min to measure context fear. Mice were placed in an alternate context 24 h later, also off drug, to measure tone fear. The training context was altered for tone testing trials: white acrylic sheets were placed over the grid floors and a black plastic, triangular teepee was placed inside each box. Only near-infrared light was used, creating a dark environment. The chambers were cleaned and scented with a 5% vinegar solution. Tone testing consisted of a 2-min baseline, followed by a 3-min tone (2.8 kHz, 85 dBA).

Elevated plus maze

The plus maze (MED Associates) had two open and two enclosed arms (6.5 cm × 36 cm each) joined at a center hub (6.5 cm × 6.5 cm) elevated 74 cm from the ground. Testing lasted 5 min in dim light. The floor of the maze had near infrared backlighting invisible to the mice to provide video contrast. Mice were tracked using a camera and video tracking software (Panlab Smart 3.0, Harvard Apparatus). Mice were given 0 (saline control, $n = 8$),

1 ($n = 8$), or 10 mg/kg MPH ($n = 8$) 30 min prior to testing. Time spent, distance traveled, and transitions (head and shoulder entries) between each section were recorded.

Conditioned place preference (CPP) and behavioral sensitization

Eight mice were tested concurrently in individual CPP chambers ($43 \times 43 \times 31$ cm, Med Associates) as previously described (Carmack et al. 2013). Each chamber consisted of two distinct (visual, tactile, and odor cues) sides bisected by an opaque wall with a removable insert. Activity Monitor software (Med Associates) used infrared beams to detect mouse position and to derive locomotor activity (distance) and stereotypy (counts). Mice were habituated to the apparatus for 30 min per side per day for 2 d prior to training.

On each of seven daily CPP training sessions, mice were placed into each side of the apparatus for 15 min per side per day. All mice were first given saline prior to placement into the first side (Unpaired). Then, all mice were given drug prior to placement into the second side (Paired). The compartments were counterbalanced. For MPH experiments, mice were assigned to one of three drug groups ($n = 13$/group): 0 (saline control), 1 (low dose), or 10 (high dose) mg/kg MPH. These doses of MPH maximally enhanced and impaired fear memory in the fear conditioning experiment (Fig. 1C).

Twenty-four hours after the final training session, mice were tested off drug for CPP. The insert was removed and subjects were allowed access to both sides of the chamber for 15 min.

To measure the development of sensitization, distance traveled and stereotyped activity were recorded during training on the Paired side. Development of sensitization was calculated as the difference between day 1 (acute) and day 7 (sensitized) response. Additionally, all mice received two challenge tests: one with a low dose (1 mg/kg MPH) 48 h after training, and one with a high dose (10 mg/kg MPH) 72 h after training. For both tests, all mice were injected with drug and immediately placed into the Paired side for 45 min.

AMPH's and COC's ability to induce CPP and sensitization at low (0.005 and 0.15 mg/kg) and high doses (1.5 and 15 mg/kg) were also investigated using the above protocol. These doses maximally enhanced or impaired memory in previously published work (Wood et al. 2007; Wood and Anagnostaras 2009); see Supplemental Methods for more details.

Statistical analyses

Data were analyzed using multivariate or univariate analyses of variance (ANOVAs). Post-hoc comparisons were performed following significant omnibus comparisons using Fisher's protected least significant difference tests. The level of significance was $P \leq 0.05$. We found no evidence of sex-related differences in any measures (P values >0.2), so male and female data were collapsed.

Acknowledgments

We gratefully acknowledge Leila Davis, Alexandra Cifelli, Monette Leyva, Vanessa Wu, Sarah D'Angelo, Carina Block, and Erin McNalley for technical assistance. We also thank Antonio Mora, Roy Jungay and Gilberto Sanchez for excellent animal care. We thank Jordan Comins and Liz Harrison for helpful comments on an earlier version of this manuscript. These studies were supported by NIH grant DA020041 (S.G.A.), Hellman Fellowship (S.G.A.), NSF Graduate Research Fellowship (K.K.H.), and UCSD Chancellor's Interdisciplinary Collaboratories Grant (S.A.C., E.T.R.).

References

Abraham AD, Cunningham CL, Lattal KM. 2012. Methylphenidate enhances extinction of contextual fear. *Learn Mem* **19:** 67–72.

Akirav I, Richter-Levin G. 2002. Mechanisms of amygdala modulation of hippocampal plasticity. *J Neurosci* **22:** 9912–9921.

Anagnostaras SG, Maren S, Fanselow MS. 1999. Temporally graded retrograde amnesia of contextual fear after hippocampal damage in rats: Within-subjects examination. *J Neurosci* **19:** 1106–1114.

Anagnostaras SG, Josselyn SA, Frankland PW, Silva AJ. 2000. Computer-assisted behavioral assessment of Pavlovian fear conditioning in mice. *Learn Mem* **7:** 58–72.

Anagnostaras SG, Gale GD, Fanselow MS. 2001. Hippocampus and contextual fear conditioning: Recent controversies and advances. *Hippocampus* **11:** 8–17.

Anagnostaras SG, Wood SC, Shuman T, Cai DJ, Leduc AD, Zurn KR, Zurn JB, Sage JR, Herrera GM. 2010. Automated assessment of pavlovian conditioned freezing and shock reactivity in mice using the video freeze system. *Front Behav Neurosci* **4:** 1–11.

Arnsten AF. 2006. Stimulants: Therapeutic actions in ADHD. *Neuropsychopharmacology* **31:** 2376–2383.

Arnsten AF, Dudley AG. 2005. Methylphenidate improves prefrontal cortical cognitive function through α2 adrenoceptor and dopamine D1 receptor actions: Relevance to therapeutic effects in attention deficit hyperactivity disorder. *Behav Brain Funct* **1:** 2–10.

Aron AR, Dowson JH, Sahakian BJ, Robbins TW. 2003. Methylphenidate improves response inhibition in adults with attention-deficit/hyperactivity disorder. *Biol Psychiatry* **54:** 1465–1468.

Barkley RA. 1997. Behavioral inhibition, sustained attention, and executive functions: Constructing a unifying theory of ADHD. *Psychol Bull* **121:** 65–94.

Barkley RA. 1998. *Attention deficit hyperactivity disorder: A handbook for diagnosis and treatment*. Guilford Press, New York.

Berridge CW, Devilbiss DM. 2011. Psychostimulants as cognitive enhancers: The prefrontal cortex, catecholamines, and attention-deficit/hyperactivity disorder. *Biol Psychiatry* **69:** e101–e111.

Berridge CW, Shumsky JS, Andrzejewski ME, McGaughy JA, Spencer RC, Devilbiss DM, Waterhouse BD. 2012. Differential sensitivity to psychostimulants across prefrontal cognitive tasks: Differential involvement of noradrenergic α1- and α2-receptors. *Biol Psychiatry* **71:** 467–473.

Braver TS, Barch DM, Kelley WM, Buckner RL, Cohen NJ, Miezin FM, Snyder AZ, Ollinger JM, Akbudak E, Conturo TE, et al. 2001. Direct comparison of prefrontal cortex regions engaged by working and long-term memory tasks. *NeuroImage* **14:** 48–59.

Carmack SA, Wood SC, Anagnostaras SG. 2010. Amphetamine and extinction of cued fear. *Neurosci Lett* **468:** 18–22.

Carmack SA, Kim JS, Sage JR, Thomas AW, Skillicorn KN, Anagnostaras SG. 2013. The competitive NMDA receptor antagonist CPP disrupts cocaine-induced conditioned place preference, but spares behavioral sensitization. *Behav Brain Res* **239:** 155–163.

Challman TD, Lipsky JJ. 2000. Methylphenidate: Its pharmacology and uses. *Mayo Clin Proc* **75:** 711–721.

Delgado MR, Olsson A, Phelps EA. 2006. Extending animal models of fear conditioning to humans. *Biol Psychol* **73:** 39–48.

de Oliveira AR, Reimer AE, de Macedo CEA, de Carvalho MC, Silva MA, Brandão ML. 2011. Conditioned fear is modulated by D2 receptor pathway connecting the ventral tegmental area and basolateral amygdala. *Neurobiol Learn Mem* **95:** 37–45.

Dittmann RW, Cardo E, Nagy P, Anderson CS, Bloomfield R, Caballero B, Higgins N, Hodgkins P, Lyne A, Civil R, et al. 2013. Efficacy and safety of lisdexamfetamine dimesylate and atomoxetine in the treatment of attention-deficit/hyperactivity disorder: a head-to-head, randomized, double-blind, phase IIIb study. *CNS Drugs* doi: 10.1007/s40263-013-0104-8.

Dommett EJ, Henderson EL, Westwell MS, Greenfield SA. 2008. Methylphenidate amplifies long-term plasticity in the hippocampus via noradrenergic mechanisms. *Learn Mem* **15:** 580–586.

Eagle DM, Tufft MR, Goodchild HL, Robbins TW. 2007. Differential effects of modafinil and methylphenidate on stop-signal reaction time task performance in the rat, and interactions with the dopamine receptor antagonist cis-flupenthixol. *Psychopharmacology* **192:** 193–206.

Ellinwood EH, King G, Lee T. 1998. Chronic amphetamine use and abuse. In *Psychopharmacology: The fourth generation of progress* (ed. Watson SJ), http://www.acnp.org/g4/gn401000166/ch162.htm. Lippincott, Williams & Wilkins, Philadelphia, PA.

Faraone SV, Wigal SB, Hodgkins P. 2007. Forecasting three-month outcomes in a laboratory school comparison of mixed amphetamine salts extended release (Adderall XR) and atomoxetine (Strattera) in school-aged children with ADHD. *J Atten Disord* **11:** 74–82.

Fone KCF, Nutt DJ. 2005. Stimulants: Use and abuse in the treatment of attention deficit hyperactivity disorder. *Curr Opin Pharmacol* **5:** 87–93.

Forest Laboratories. 2011. Celexa: Full prescribing information. http://www.accessdata.fda.gov/drugsatfda_docs/label/2011/020822s038s040,021046s016s017lbl.pdf.

Gainetdinov RR, Wetsel WC, Jones SR, Levin ED, Jaber M, Caron MG. 1999. Role of serotonin in the paradoxical calming effect of psychostimulants on hyperactivity. *Science* **283:** 397–401.

Gale GD, Anagnostaras SG, Godsil BP, Mitchell S, Nozawa T, Sage JR, Wiltgen B, Fanselow MS. 2004. Role of the basolateral amygdala in the storage of fear memories across the adult lifetime of rats. *J Neurosci* **24:** 3810–3815.

GlaxoSmithKline. 2013. Wellbutrin: Full prescribing information. http://www.accessdata.fda.gov/drugsatfda_docs/label/2011/018644s043lbl.pdf.

Han DD, Gu HH. 2006. Comparison of the monoamine transporters from human and mouse in their sensitivities to psychostimulant drugs. *BMC Pharmacol* **6:** 6.

Humby T, Eddy JB, Good MA, Reichelt AC, Wilkinson LS. 2013. A novel translational assay of response inhibition and impulsivity: Effects of prefrontal cortex lesions, drugs used in ADHD, and serotonin 2C receptor antagonism. *Neuropsychopharmacology* **38:** 2150–2159.

Johansen JP, Cain CK, Ostroff LE, LeDoux JE. 2011. Molecular mechanisms of fear learning and memory. *Cell* **147:** 509–524.

Kuczenski R, Segal DS. 1997. Effects of methylphenidate on extracellular dopamine, serotonin, and norepinephrine: Comparison with amphetamine. *J Neurochem* **68:** 2032–2037.

Kuczenski R, Segal DS. 2002. Exposure of adolescent rats to oral methylphenidate: Preferential effects on extracellular norepinephrine and absence of sensitization and cross-sensitization to methamphetamine. *J Neurosci* **22:** 7264–7271.

Lazzaro SC, Hou M, Cunha C, Ledoux JE, Cain CK. 2010. Antagonism of lateral amygdala α1-adrenergic receptors facilitates fear conditioning and long-term potentiation. *Learn Mem* **17:** 489–493.

LeDoux J. 1998. Fear and the brain: Where have we been, and where are we going? *Biol Psychiatry* **44:** 1229–1238.

Mahan AL, Ressler KJ. 2012. Fear conditioning, synaptic plasticity and the amygdala: Implications for posttraumatic stress disorder. *Trends Neurosci* **35:** 24–35.

Maren S. 2008. Pavlovian fear conditioning as a behavioral assay for hippocampus and amygdala function: Cautions and caveats. *Eur J Neurosci* **28:** 1661–1666.

Marshall P, Schroeder R, O'Brien J, Fischer R, Ries A, Blesi B, Barker J. 2010. Effectiveness of symptom validity measures in identifying cognitive and behavioral symptom exaggeration in adult attention deficit hyperactivity disorder. *Clin Neuropsychol* **24:** 1204–1237.

McNeil Pediatrics. 2008. Concerta: Full prescribing information. http://www.accessdata.fda.gov/drugsatfda_docs/label/2008/021121s015s017lbl.pdf.

Mehta MA, Goodyer IM, Sahakian BJ. 2004. Methylphenidate improves working memory and set-shifting in AD/HD: Relationships to baseline memory capacity. *J Child Psychol Psychiatry* **45:** 293–305.

Morgan MA, LeDoux JE. 1995. Differential contribution of dorsal and ventral medial prefrontal cortex to the acquisition and extinction of conditioned fear in rats. *Behav Neurosci* **109:** 681–688.

Newcorn JH, Kratochvil CJ, Allen AJ, Casat CD, Ruff DD, Michelson D. 2008. Atomoxetine and osmotically released methylphenidate for the treatment of attention deficit hyperactivity disorder: acute comparison and differential response. *Am J Psychiatry* **165:** 721–730.

O'Toole K, Abramowitz A, Morris R, Dulcan M. 1997. Effects of methylphenidate on attention and nonverbal learning in children with attention-deficit hyperactivity disorder. *J Am Acad Child Adolesc Psychiatry* **36:** 531–538.

Puumala T, Ruotsalainen S, Jäkälä P, Koivisto E, Riekkinen P, Sirviö J. 1996. Behavioral and pharmacological studies on the validation of a new animal model for attention deficit hyperactivity disorder. *Neurobiol Learn Mem* **66:** 198–211.

Rapoport JL, Inoff-Germain G. 2002. Responses to methylphenidate in attention-deficit/hyperactivity disorder and normal children: Update 2002. *J Atten Disord* **6:** S57–S60.

Rapoport JL, Buchsbaum MS, Weingartner H, Zahn TP, Ludlow C, Mikkelsen EJ. 1980. Dextroamphetamine: It's cognitive and behavioral effects in normal and hyperactive boys and normal men. *Arch Gen Psychiatry* **37:** 933–943.

Rhodes SM, Park J, Seth S, Coghill DR. 2012. A comprehensive investigation of memory impairment in attention deficit hyperactivity disorder and oppositional defiant disorder. *J Child Psychol Psychiatry* **53:** 128–137.

Richelson E, Pfenning M. 1984. Blockade by antidepressants and related compounds of biogenic amine uptake into rat brain synaptosomes: Most antidepressants selectively block norepinephrine uptake. *Eur J Pharmacol* **104:** 277–286.

Robbins TW. 2002. The 5-choice serial reaction time task: Behavioural pharmacology and functional neurochemistry. *Psychopharmacology* **163:** 362–380.

Robinson TE, Berridge KC. 1993. The neural basis of drug craving: An incentive-sensitization theory of addiction. *Brain Res Rev* **18:** 247–291.

Robinson TE, Berridge KC. 2003. Addiction. *Ann Rev Psychol* **54:** 25–53.

Setyawan J, Guerin A, Hodgkins P, Gauthier G, Cloutier M, Wu E, Erder MH. 2013. Treatment persistence in attention deficit hyperactivity disorder: A retrospective analysis of patients initiated on lisdexamfetamine vs other medications. *J Med Econ* **16:** 1275–1289.

Shuman T, Wood SC, Anagnostaras SG. 2009. Modafinil and memory: Effects of modafinil on Morris water maze learning and Pavlovian fear conditioning. *Behav Neurosci* **123:** 257–266.

Spencer T, Biederman J, Wilens T, Harding M, O'Donnell D, Griffin S. 1996. Pharmacotherapy of attention-deficit hyperactivity disorder across the life cycle. *J Am Acad Child Adolesc Psychiatry* **35:** 409–432.

Swanson J, Baler RD, Volkow ND. 2011. Understanding the effects of stimulant medications on cognition in individuals with attention-deficit hyperactivity disorder: A decade of progress. *Neuropsychopharmacology* **36:** 207–226.

Tye KM, Tye LD, Cone JJ, Hekkelman EF, Janak PH, Bonci A. 2010. Methylphenidate facilitates learning-induced amygdala plasticity. *Nat Neurosci* **13:** 475–481.

Wigal SB, McGough JJ, McCracken JT, Biederman J, Spencer TJ, Posner KL, Wigal TL, Kollins SH, Clark TM, Mays DA, et al. 2005. A laboratory school comparison of mixed amphetamine salts extended release (Adderall XR) and atomoxetine (Strattera) in school-aged children with attention deficit/hyperactivity disorder. *J Atten Disord* **9:** 275–289.

Wong DT, Threlkeld PG, Best KL, Bymaster FP. 1982. A new inhibitor of norepinephrine uptake devoid of affinity for receptors in rat brain. *J Pharmacol Exp Ther* **222:** 61–65.

Wood SC, Anagnostaras SG. 2009. Memory and psychostimulants: Modulation of Pavlovian fear conditioning by amphetamine in C57BL/6 mice. *Psychopharmacology* **202:** 197–206.

Wood SC, Anagnostaras SG. 2011. Interdependence of measures in pavlovian conditioned freezing. *Neurosci Lett* **505:** 134–139.

Wood SC, Fay J, Sage JR, Anagnostaras SG. 2007. Cocaine and Pavlovian fear conditioning: Dose–effect analysis. *Behav Brain Res* **176:** 244–250.

Wood SC, Sage JR, Shuman T, Anagnostaras SG. 2013. Psychostimulants and cognition: A continuum of behavioral and cognitive activation. *Pharmacol Rev* (in press).

Received October 15, 2013; accepted in revised form October 28, 2013.

Animal model of methylphenidate's long-term memory enhancing effects

Supplementary Information

Supplemental Methods

Fear conditioning with atomoxetine, bupropion, and citalopram

For fear conditioning experiments with ATM, BUP, and CIT, mice were randomly assigned to groups by dose of drug administered. The protocol was identical to that described for experiments with MPH with the following exceptions: for atomoxetine (ATM) experiments, mice were injected with saline or ATM 30 min prior training in a dose of 0 (saline control, n = 20), 0.1 (n = 13), 0.5 (n = 13), 1 (n = 18), or 10 mg/kg (n = 20). For bupropion (BUP) experiments, mice were injected with saline or BUP 30 min prior to training. The following doses of BUP were given: 0 (saline control, n = 14), 0.5 (n = 11), 5 (n = 11), 10 (n = 10), or 20 mg/kg (n = 10). For citalopram (CIT) experiments, mice were injected with saline or CIT 30 min prior to training. CIT was administered in a dose of: 0 (saline control, n = 12), 0.01 (n = 8), 0.1 (n = 8), 1 (n = 10), or 10 mg/kg (n = 8). Tone testing consisted of a 2 min baseline period, followed by a 3, 30-sec tone presentations identical to the training tone (2.8 kHz, 85 dBA).

Addiction-related behaviours with amphetamine, cocaine, and atomoxetine

For AMPH experiments, mice were randomly assigned to one of three drug groups: 0 (saline control, n = 12), 0.005 mg/kg (low dose, n = 16), or 1.5 mg/kg (high dose, n = 12) mg/kg MPH. In previously published work, these doses of AMPH enhanced and impaired fear memory, respectively, as compared to saline controls (Wood and Anagnostaras, 2009). For COC experiments, mice were randomly assigned to one of three drug groups (n = 8/group): 0 (saline control), 0.15 mg/kg (low dose), or 15 mg/kg (high dose). These doses of COC were selected because they enhanced and impaired fear memory, respectively (Wood et al., 2007). For ATM experiments, mice were randomly assigned to one of three drug groups: 0 (saline control, n = 12), 1 mg/kg (low dose, n = 8), or 10 mg/kg (high dose, n = 8).

Supplemental Results

Fear conditioning with atomoxetine, bupropion, and citalopram

For brevity, only significant post-hoc comparisons using Fisher's PLSD, following significant omnibus comparisons, are reported (starred data points in Figure S1). At 10 mg/kg, the selective norepinephrine transporter inhibitor atomoxetine (ATM; e.g. Straterra) reduced activity during the 3 min training baseline period (Figure S1A) ($p = 0.04$). No other doses had an effect on locomotor activity (p values > 0.3). Pre-training administration of ATM had no effect on freezing to the context [$F(4,78) = 0.59$, $p = 0.67$] (Figure S1B) or tone [$F(4,78) = 1.23$, $p = 0.31$] (Figure S1C) when subjects were tested off drug 7 days later.

The dopamine and norepinephrine transporter inhibitor bupropion (BUP; e.g. Wellbutrin) had no effect on locomotor activity during the 3 min training baseline period (Figure S1D) [$F(4,51) = 0.58$, $p = 0.68$]. When tested off drug 7 days later, only pre-training administration of 20 mg/kg BUP trended toward significantly reducing contextual fear memory ($p = 0.06$, all other p values > 0.3) (Figure S1E). Pre-training administration of BUP had no effect tone fear memory [$F(4,51) = 0.18$, $p = 0.95$] (Figure S1F).

The highly selective serotonin transporter inhibitor, citalopram (CIT; e.g. Celexa) did not alter locomotor activity during the 3 min training baseline period [ANOVA; $F(4,39) = 0.88$, $p = 0.48$] (Figure S1G). Pre-training administration of a high dose of CIT (10 mg/kg) significantly impaired context ($p = 0.026$) (Figure SIH) and tone fear memory ($p = 0.035$) (Figure S1I) when

subjects were tested off drug 7 days later. No other doses of CIT had an effect on fear memory (p values > 0.2).

Addiction-related behaviours with amphetamine, cocaine, or atomoxetine

AMPH

Following repeated administration of saline (control), a pro-cognitive low dose (0.005 mg/kg), or a memory-impairing high dose (1.5 mg/kg) of AMPH, only the group given 1.5 mg/kg AMPH showed evidence of locomotor sensitization [$F(2,37) = 13.7$, $p < 0.0001$]. Locomotor activity (horizontal distance traveled) as an average of each day across the 7 days of training on the Paired side is shown in Figure 4A. In terms of average response, mice receiving 1.5 mg/kg AMPH showed greater locomotor activity than mice receiving saline or 0.005 mg/kg AMPH (p values < 0.001). Sensitization was quantified as the difference in average locomotor response from day 1 to 7 (Figure 4B). There were significant group differences [$F(2,37) = 10.5$, $p < 0.0001$]. The group receiving 1.5 mg/kg was significantly different from zero and was significantly different from the other two groups (p values < 0.001). Neither the saline control nor the 0.005 mg/kg AMPH groups showed sensitization (p values > 0.5), and these two groups did not differ ($p > 0.4$).

To test place preference, mice were returned, off drug, to the CPP apparatus with access to both sides of the chamber for 15 min, 24 hours after training. Preference (Paired % time – Unpaired % time) is depicted in Figure 4C. There were significant group differences [$F(2,37) = 3.75$, $p < 0.04$]. Mice that received 1.5 mg/kg AMPH showed significant place preference (one sample two-tailed t-test against hypothesized $\mu = 0$, $t(11) = 2.69$, $p < 0.03$). Neither the saline control group, nor the group administered 0.005 mg/kg AMPH during training show place preference (p values > 0.2).

COC

Locomotor activity (horizontal distance traveled) as an average of each day across the 7 days of training on the Paired side is shown in Figure 4D. Visual inspection of Figure 4D suggests that across training only the group receiving 15 mg/kg COC sensitized, while the other groups did not. Significant group differences were observed [$F(2,21) = 15.4$, $p < 0.0001$]. In terms of average response, mice receiving 15 mg/kg COC showed greater locomotor activity than mice receiving saline or 0.15 mg/kg COC (p values < 0.001). Sensitization was quantified as the difference in average locomotor response from day 1 to 7 (Figure 4E). The group receiving 15 mg/kg was significantly different from zero ($p < 0.05$) and was nearly significantly different from the other two groups ($p = 0.056$). Neither the saline control nor the 0.15 mg/kg groups showed sensitization (p values > 0.5).

Preference (Paired % time – Unpaired % time) for the drug-paired side is depicted in Figure 4F. There were significant group differences [$F(2,21) = 6.41$, $p = 0.007$]. Mice that received 15 mg/kg COC showed significant place preference (one sample two-tailed t-test against hypothesized $\mu = 0$, $t(7) = 5.92$, $p = 0.001$) and greater preference for the Paired side than the other groups (p values < 0.01). Neither the saline control group, nor the group administered 0.15 mg/kg COC during training show place preference (p values > 0.6).

ATM

Following repeated administration of saline (control), a low dose (1 mg/kg) or a high dose (10 mg/kg) of ATX, no groups showed evidence of locomotor sensitization. Locomotor activity (horizontal distance traveled) as an average of each day across the 7 days of training on the Paired side is shown in Figure S2A. Sensitization was quantified as the difference in average locomotor response from day 1 to 7 (Figure S2B). There were no significant group differences and no group was significantly greater than 0 (p values > 0.9). Preference for the drug-paired side (Paired % time – Unpaired % time) is depicted in Figure S2C. There were significant group

differences [$F(2,37) = 3.75$, $p < 0.04$]. Mice that received 10 mg/kg ATM showed significant place preference (one sample two-tailed t-test against hypothesized $\mu = 0$, $t(11) = 2.69$, $p < 0.03$) and greater preference for the Paired side than the other groups (p values < 0.01). Neither the saline control group, nor the group administered 1 mg/kg ATM during training show place preference (p values > 0.4).

Supplemental Figures

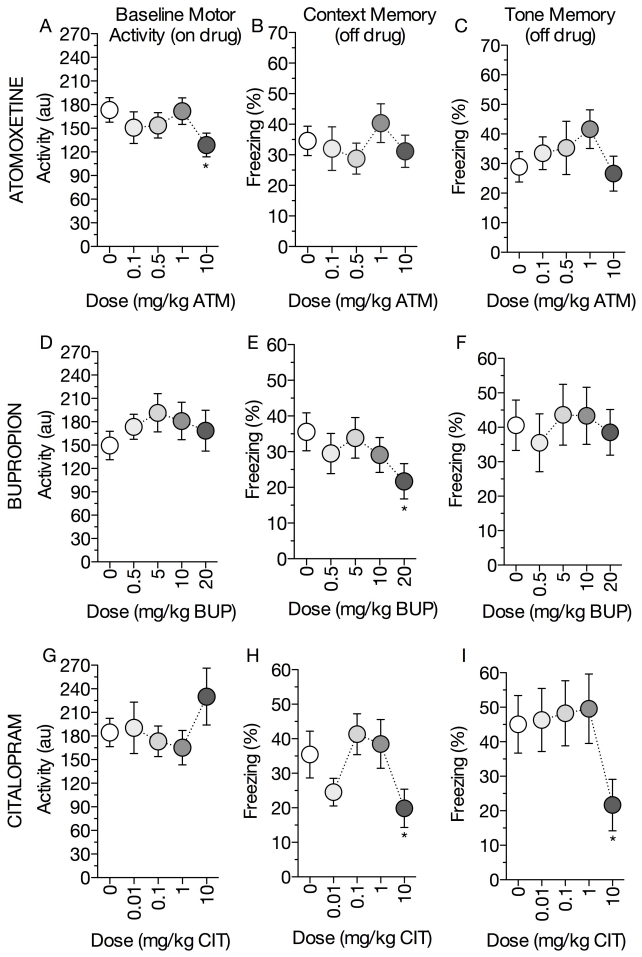

Figure S1. Atomoxetine, bupropion, and citalopram on fear learning. **(A)** ATM and baseline locomotor activity during training. 10 mg/kg ATM significantly decreased locomotor activity as compared to saline controls. No other groups differed from saline controls. (**B**) ATM and context fear memory. Pre-training administration of ATM failed to significantly influence freezing to the context. (**C**) ATM and tone fear memory. Pre-training administration of ATM also had no significant effects freezing to the tone. **(D)** BPN and baseline locomotor activity during training. BPN had no effect on locomotor activity. (**E**) BPN and context fear memory. Pre-training administration of a high dose (20 mg/kg) of BPN reduced freezing to the context as compared to saline controls. No other groups differed from saline controls. (**F**) BPN and tone fear memory. Pre-training administration of BPN failed to influence freezing to the tone. **(G)** CIT and baseline locomotor activity during training. CIT had no effect on locomotor activity. (**H**) CIT and context fear memory. Pre-training administration of a high dose (10 mg/kg) CIT impaired context fear memory as compared to saline controls. No other doses differed from saline controls. (**I**) CIT and tone fear memory. 10 mg/kg CIT also reduced freezing to the tone as compared to saline controls. No other doses had significant effects on tone fear memory. Each point represents the mean ± 1 standard error. Starred (*) data points identify significant post-hoc comparisons against the saline control group.

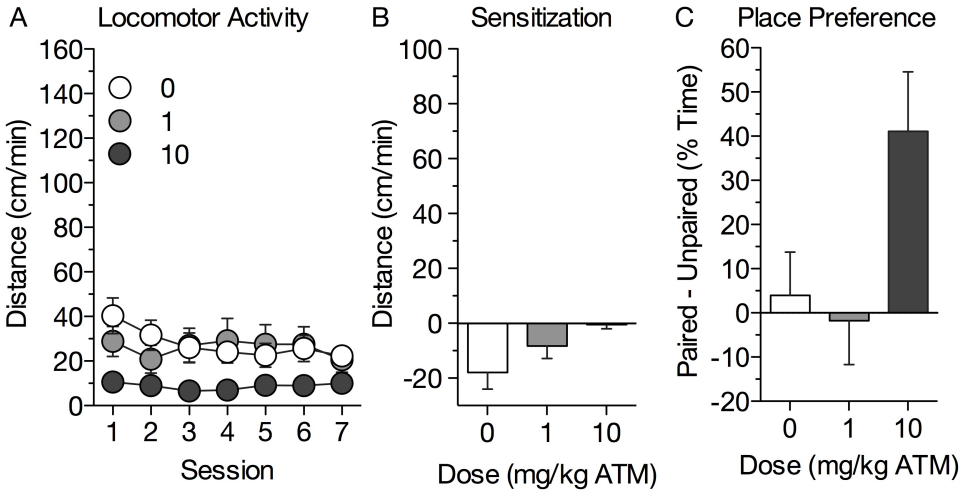

Figure S2. Atomoxetine and addiction-related behaviours. (**A**) Locomotor activity for each of the 7 training sessions on the drug-paired side. (**B**) Development of sensitization. Sensitization was quantified as the difference in average locomotor response from day 1 to 7. No groups showed evidence of sensitization. (**C**) ATM-induced conditioned place preference. Preference was measured as the difference between the percent of time spent on the paired side versus the unpaired side; positive values indicate preference for the paired side. Mice that received 10 mg/kg ATM showed significant place preference. The saline control group and 1 mg/kg ATM groups did not show any preference. Each point represents the mean ± 1 standard error.

Chapter 3, in full, is a reprint of the material as it appears in Animal model of methylphenidate's long-term memory-enhancing effects. *Learning & Memory*, 21, 82-89. Carmack SA, Howell KK, Rasaei K, Reas ET & Anagnostaras SG (2014). The dissertation author was the primary investigator and author of this paper.

Chapter 4

Methylphenidate enhances acquisition and retention of spatial memory

Neuroscience Letters 567 (2014) 45–50

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Methylphenidate enhances acquisition and retention of spatial memory

Stephanie A. Carmack[a], Carina L. Block[a], Kristin K. Howell[a], Stephan G. Anagnostaras[a,b,*]

[a] Molecular Cognition Laboratory, Department of Psychology, University of California, San Diego 92093-0109, United States
[b] Program in Neurosciences, University of California, San Diego 92093-0109, United States

HIGHLIGHTS

- 10 mg/kg MPH given pre-training enhances learning on the hidden platform version of the Morris water maze.
- 1 or 10 mg/kg MPH given pre-training enhances retention of spatial memory in the water maze.
- 10 mg/kg MPH given chronically before Pavlovian fear conditioning dramatically impairs long-term fear memory.

ARTICLE INFO

Article history:
Received 27 November 2013
Received in revised form 7 March 2014
Accepted 18 March 2014

Keywords:
Psychostimulant
Fear conditioning
Hippocampus
Mouse
Cognitive enhancement
Water maze

ABSTRACT

Psychostimulants containing methylphenidate (MPH) are increasingly being used both on and off-label to enhance learning and memory. Still, almost no studies have investigated MPH's ability to specifically improve spatial or long-term memory. Here we examined the effect of training with 1 or 10 mg/kg MPH on hidden platform learning in the Morris water maze. 10 mg/kg MPH improved memory acquisition and retention, while 1 mg/kg MPH improved memory retention. Taken together with prior evidence that low, clinically relevant, doses of MPH (0.01–1 mg/kg MPH) enhance fear memory we conclude that MPH broadly enhances memory.

© 2014 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Psychostimulants containing methylphenidate (MPH) are used therapeutically to enhance cognition, improve executive function, promote wakefulness, and reduce impulsivity (for a review see [1]). Increasingly, MPH is being used both on and off-label to specifically improve long-term memory (LTM) [2–4]. Few studies, however, have examined MPH's ability to modulate spatial or long-term memory [5–7]. Rather, most research has focused on MPH-induced improvements in working memory, attention, and cognitive control [8–10].

Prior research in our laboratory has shown that low, clinically relevant doses of MPH (0.01–1 mg/kg) enhance LTM in Pavlovian fear conditioning, a leading model of memory in rats and mice [11–13]. In this paradigm animals learn to fear previously neutral tone and contextual stimuli following their pairing with an aversive foot-shock [12]. Both tone and contextual conditioning require the amygdala; contextual conditioning additionally requires the hippocampus [14,15]. While lower MPH doses enhanced fear memory, a relatively high dose (10 mg/kg) dramatically impaired fear memory [11]. Importantly, these memory-modulating effects were independent of any effects on locomotion, anxiety, or reinforcement [11].

Here we selected the doses of MPH that maximally enhanced (1 mg/kg) or impaired (10 mg/kg) fear memory acquisition [11] and assessed their effect on spatial memory using the well-established hidden platform version of the Morris water maze [16–18]. This hippocampal-dependent task requires subjects to use distal spatial cues to locate a fixed hidden platform in order to escape from a pool of opaque water [19–21]. In earlier work, we found that a much higher dose of the atypical psychostimulant modafinil [1] was necessary to enhance water maze acquisition (75 mg/kg) as compared to fear conditioning (0.75 mg/kg) [22].

* Corresponding author at: 9500 Gilman Drive MC 0109, University of California, San Diego, La Jolla, CA 92093-0109, United States. Tel.: +858 224 2531; fax: +858 534 7190.
E-mail addresses: stephana@ucsd.edu, sanagnostaras@ucsd.edu (S.G. Anagnostaras).
URL: http://www.mocolab.org (S.G. Anagnostaras).

http://dx.doi.org/10.1016/j.neulet.2014.03.029
0304-3940/© 2014 Elsevier Ireland Ltd. All rights reserved.

One possible explanation for the difference in dosing across fear conditioning and water maze is tolerance [23]. Unlike our earlier fear conditioning experiments where MPH or modafinil was given acutely [11,22], water maze training involves repeated stimulant injections. We examined this possibility by chronically administering 10 mg/kg MPH and then testing its effect on fear learning. Tolerance proved to be an unlikely explanation. We instead consider whether the difference in dosing is better explained by a difference in the level of arousal required for optimal performance on each task.

2. Materials and methods

2.1. Subjects

51 hybrid C57BL/6Jx129S1/SvlmJ mice (129B6; stock mice from the Jackson Laboratory, West Sacramento, CA) were used in approximately equal numbers of females ($n = 24$) and males ($n = 27$); treatment groups were balanced across sexes. Mice were 12 weeks old before testing and group housed (4–5 mice per cage) with continuous access to food and water. Mice were handled for 5 days (1 min/day) prior to experiments. The vivarium was maintained on a 14:10 h light:dark schedule and all testing was performed during the light phase of the cycle. Animal care and testing procedures were approved by the UCSD IACUC and were compliant with the NRC Guide.

2.2. Drugs

Methylphenidate HCl (MPH; Sigma–Aldrich) was dissolved in physiological 0.9% saline (vehicle) and administered in a dose of 1 or 10 mg/kg (salt weight). All saline and drug injections were administered intraperitoneally (i.p.) in a volume of 10 ml/kg.

2.3. Apparatus

2.3.1. Water maze

The water maze was 114 cm in diameter and 74 cm high. The water was made opaque with white tempera paint and heated to 23.5 °C using a built-in heater and thermostat. The maze was divided into four quadrants (Target Quadrant, TQ; Target Left, TL; Target Right, TR; Target Opposite, OP). Although the maze itself appeared isotropic, distal cues were placed around the room and included a door, a computer, and several posters. The white acrylic escape platform was an electromagnetically controlled Atlantis platform, 10 cm in diameter, covered with plastic mesh to provide a textured surface for the mice to grip. In the raised position the top of the platform was 1 cm below the surface of the water, available to the mouse. Location was tracked and scored using a computerized video tracking system connected to an overhead video camera (Water Maze, Med Associates).

2.3.2. Fear conditioning

Three to four mice were trained concurrently in individual conditioning chambers. Locomotor activity and freezing behaviour were recorded during conditioning and testing trials using the VideoFreeze system (Med Associates) as described previously [12,24].

2.4. Experimental procedures

2.4.1. Water maze

2.4.1.1. Acquisition. Mice were injected 30 min prior to each of 15 training days and were randomly assigned to groups by dose of MPH administered: 0 (saline control, $n = 10$), 1 ($n = 12$), or 10 mg/kg ($n = 10$). Each training day had 3 standard platform training trials and 1 variable interval (VI) platform probe trial.

For platform training trials the mouse was lowered into the pool facing the wall from one of four randomly assigned start locations. The trial lasted until the mouse found the hidden platform where it remained for 5 s. If the mouse did not find the platform in 60 s it was placed onto the platform for 5 s to provide reinforcement and exposure to the platform's location. Latency to the platform was measured as the time between the mouse leaving the starting location and climbing onto the platform. Swim speed was calculated as the average centimetres swam per second for the duration of the trial. Data were averaged for each day.

A single VI probe trial immediately followed the platform training trials each training day. The platform was unavailable for 10, 20, 30 or 40 s, after which it was raised. The intervals for the 15 training sessions were as follows: 10, 30, 20, 40, 40, 20, 30, 10, 40, 10, 30, 20, 40, 10, and 20 s. VI probe trials provide a more sensitive measure of spatial memory than no platform probe trials as they lead to more accurate and persistent searching at the platform location [17]. Additionally, VI trials can be used repeatedly because they are reinforcing and do not produce extinction [17,21]. Time spent in each quadrant was recorded.

No platform (NP) probe trials followed the training and VI probe trials on training days 5, 10, and 15 as a traditional measure of spatial learning. Mice were placed in the OP quadrant and the platform was unavailable for the entire 60 s trial. Time spent in each quadrant and platform crossings were recorded. Platform crossings were defined as the number of times a mouse swam across the exact location of the platform (10-cm diameter).

2.4.1.2. Retention. Mice were given off drug NP probe trials both one day (Day 16) and one week (Day 23) following training. Mice were placed in the OP quadrant and the trial lasted for 60 s with the platform unavailable for the entire trial. Time spent in each quadrant and platform crossings were recorded.

2.4.2. Fear conditioning

Mice were randomly assigned to groups by dose of MPH administered. Mice were injected with either 0 (saline control, $n = 9$) or 10 mg/kg MPH ($n = 7$) daily for 12 days before conditioning. On Day 13 mice were injected 30 min prior to the 10 min conditioning session. Drug treatment and sex were counterbalanced across conditioning chambers. Following a 3 min baseline period, mice received one tone-shock pairing in which a 30 s tone (2.8 kHz, 85 dBA) co-terminated with a 2 s scrambled, AC foot shook (0.75 mA, RMS) [12,24].

Seven days later mice were returned to the conditioning chambers without drug to assess context memory. Freezing was measured for 5 min. Twenty-four hours later mice were placed in an alternate context (modified along several dimensions [11,24]), also off drug, to assess tone fear. Tone testing consisted of a 2 min baseline followed by 3–30 s tone presentations (2.8 kHz, 85 dBA). Freezing behaviour was again recorded.

2.5. Statistical analyses

Data were entered into a multivariate analysis of variance (MANOVA) and the level of significance was set at $p \leq 0.05$. Post hoc comparisons were done with Fisher's protected least significant difference (unpaired tests) or paired two-tailed t-tests (paired tests). Three mice, one from each drug group, were excluded early in training for failing to perform the task (floating). Data from male and female mice were collapsed because there were no differences between the sexes on any measures (p values >0.3).

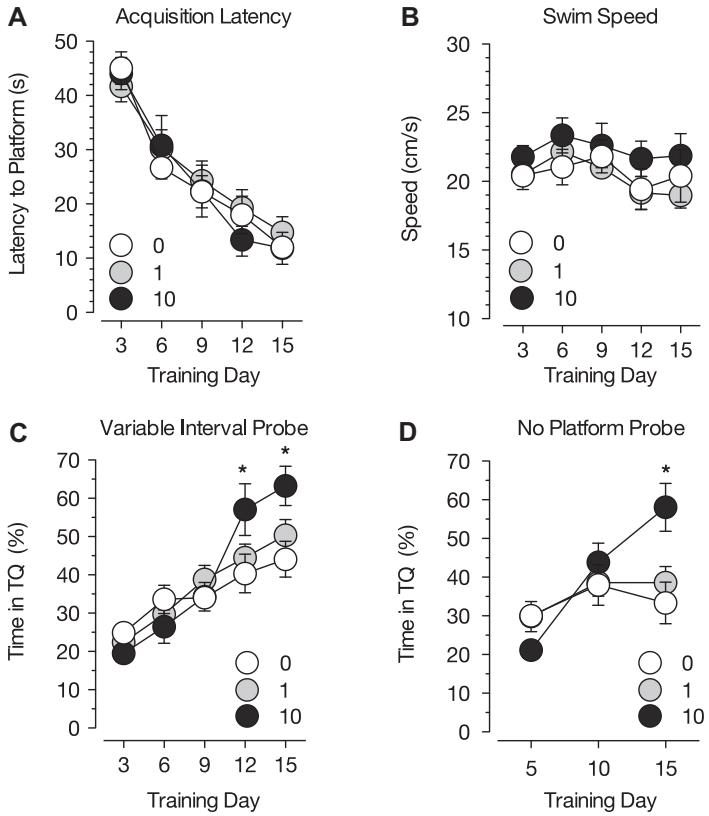

Fig. 1. Water maze acquisition. (A) Latency to find the platform across the 15 days of training presented in blocks of 3 days. Mice were given 0 (saline control, white circles), 1 (grey circles) or 10 mg/kg (black circles) MPH prior to each session. No group differences were found. (B) Average swim speed during the platform training trials did not differ between groups. (C) Time spent in the target quadrant (TQ) during variable interval probe trials. Mice trained with 10 mg/kg MPH spent more time in the TQ on Days 11–15 than saline controls. (D) Time spent in the TQ during no platform probe trials given on Days 5, 10, and 15. Mice trained with 10 mg/kg MPH spent more time in the TQ during the Day 15 trial than saline controls. Each point represents the mean ± 1 SEM. Starred (*) data points identify significant post hoc comparisons against the saline control group using Fisher's protected least significant difference tests following significant omnibus comparisons.

3. Results

3.1. Water maze

3.1.1. Water maze acquisition

3.1.1.1. Platform training trials. All groups learned the task over the 15 days of training (Days 1–15). For clarity, data are depicted in blocks of three training days (Fig. 1). Subjects took less time to find the platform on Day 15 versus Day 1 [0 mg/kg: $t(9) = 4.69$, $p = 0.001$; 1 mg/kg: $t(11) = 4.08$, $p = 0.002$; 10 mg/kg: $t(9) = 4.82$, $p = 0.001$] (Fig. 1A). Pre-training MPH had no effect on performance; no group differences were found in the latency to reach the platform [$F(2,29) = 0.15$, $p = 0.86$, *ns*] (Fig. 1A) or average swim speed [$F(2,29) = 1.04$, $p = 0.37$, *ns*] during platform training trials (Fig. 1B).

3.1.1.2. Variable interval probe trials. Each day subjects were given one VI probe trial, on drug, following the 3 platform training trials. Subjects spent significantly more time in the TQ during the VI probe trial on Day 15 versus Day 1 [0 mg/kg: $t(9) = 2.78$, $p = 0.021$; 1 mg/kg: $t(11) = 6.16$, $p < 0.001$; 10 mg/kg: $t(9) = 7.27$, $p < 0.001$] (Fig. 1C). There was a significant day by group interaction for time spent in the TQ [$F(28,406) = 2.32$, $p < 0.001$] (Fig. 1C). The 10 mg/kg MPH group spent more time in the TQ on Days 11–15 than saline controls (p values <0.03). The saline control and 1 mg/kg MPH groups did not differ from one other (p value >0.3).

3.1.1.3. No platform probe trials. On Days 5, 10, and 15 subjects were given standard NP probe trials, on drug. Subjects spent significantly more time in the TQ on Day 15 versus Day 5 [0 mg/kg: $t(9) = 2.62$, $p = 0.03$; 1 mg/kg: $t(11) = 3.8$, $p = 0.003$; 10 mg/kg: $t(9) = 5.23$, $p = 0.001$] (Fig. 1D). There was a significant NP probe test day by group interaction for time spent in the TQ [$F(4,58) = 4.36$, $p = 0.004$] (Fig. 1D). Mice given 10 mg/kg MPH spent more time in the TQ during the Day 15 NP probe trial than the saline and 1 mg/kg MPH groups (p values <0.01), which did not differ from one another ($p > 0.4$).

3.1.2. Water maze retention

One day after training (Day 16) all mice were given a NP probe test, off drug. Fig. 2A depicts the percent time spent in each quadrant. To assess learning in each group paired two tailed t-tests between the time spent in the TQ versus the mean time spent in the other three quadrants were used. Mice trained with 1 mg/kg or 10 mg/kg MPH spent more time in the TQ than the other quadrants averaged, but saline controls did not: saline [$t(9) = 1.71$, $p = 0.12$], 1 mg/kg MPH [$t(11) = 4.30$, $p = 0.001$], 10 mg/kg MPH [$t(9) = 6.68$,

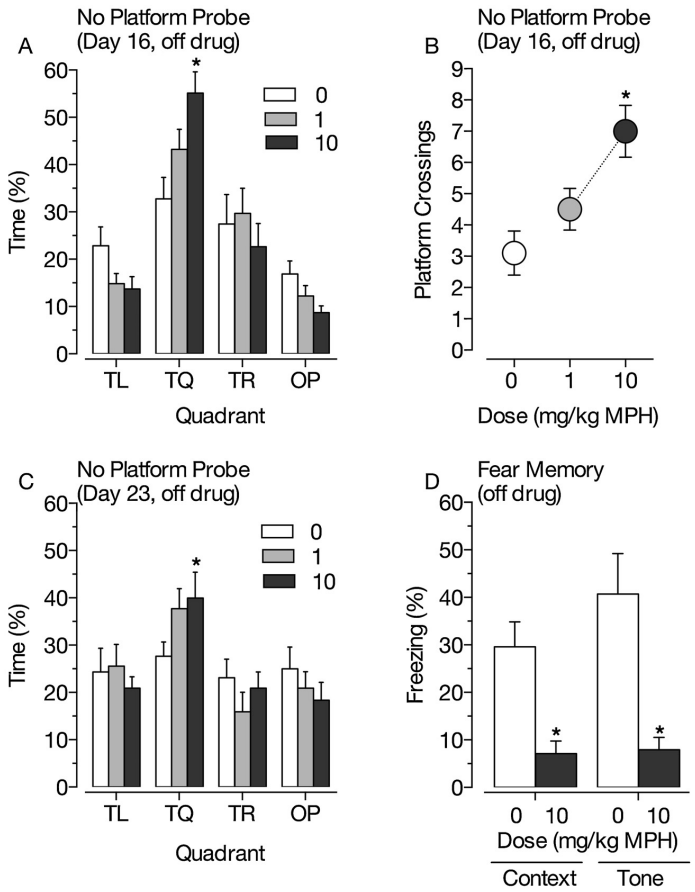

Fig. 2. Water maze retention. (A) Time spent in each quadrant during the off drug no platform probe trial conducted one-day post-training. Mice trained with 1 (grey bars) or 10 mg/kg (black bars) MPH, but not saline (0 mg/kg MPH, white bars), spent more time in the TQ than the other quadrants (TL: target left; TR: target right; OP: opposite). (B) Crossings over the exact platform location during the Day 16 NP probe trial. Mice trained with 10 mg/kg MPH crossed the platform location significantly more than saline controls. (C) Time spent in each quadrant during the off drug NP probe trial conducted one week after training. Mice trained with 1 or 10 mg/kg MPH, but not saline (0 mg/kg MPH), spent more time in the TQ than the other quadrants. Each point or bar represents the mean ± 1 SEM. (D) Pavlovian fear conditioning. Chronic dosing with 10 mg/kg MPH (black bars) prior to conditioning dramatically impaired long-term context (left) and tone fear memory (right) as compared to saline controls (0 mg/kg MPH, white bars). Each bar represents the mean ± 1 SEM average percent time freezing for the entire 5 min context test or the three 30-s tone presentations during the tone test. Starred (*) data points identify significant post hoc comparisons against the saline control group using Fisher's protected least significant difference tests following significant omnibus comparisons.

$p < 0.0001$]. This indicates that mice trained with MPH retained the location of the platform, while the saline control group had relatively weak memory. To determine whether the groups learned differently, we performed a MANOVA of time spent in the TQ and platform crossings. Significant group differences were found [Time in TQ: $F(2,29) = 5.98$, $p = 0.007$, Platform Crossings: $F(2,29) = 6.85$, $p = 0.004$]. Mice trained with 10 mg/kg MPH spent significantly more time in the TQ ($p = 0.002$; Fig. 2A) and crossed the platform location more times than the saline control group (p value <0.02; Fig. 2B). The saline control and 1 mg/kg MPH groups did not differ in terms of time spent in the TQ ($p = 0.1$) or platform crossings ($p = 0.18$).

One week after training (Day 23) mice were given a second off-drug NP probe test. Mice trained with 1 mg/kg or 10 mg/kg MPH spent more time in the TQ than the other quadrants averaged, though saline control mice did not: saline [$t(9) = 0.87$, $p = 0.45$], 1 mg/kg MPH [$t(11) = 2.98$, $p = 0.01$], 10 mg/kg MPH [$t(9) = 2.73$, $p = 0.02$] (Fig. 2C). Mice trained with 10 mg/kg MPH spent significantly more time in the TQ ($p < 0.01$; Fig. 2C) and crossed the platform location significantly more than the saline control group ($p < 0.03$; data not graphed). Thus, mice trained with MPH retained the location of the platform one-week post-training, while the saline control group did not.

3.2. Fear conditioning

10 mg/kg MPH enhanced spatial memory in the water maze. In previous work we found that acute administration of 1 mg/kg MPH enhanced and 10 mg/kg MPH impaired long-term fear memory [11]. It is possible that chronic administration of 10 mg/kg MPH produces tolerance [23], which might explain the different dose–response curves across the two tasks. To examine this possibility we gave 0 (saline control) or 10 mg/kg MPH once for each of

12 days prior to training and during fear conditioning, mimicking the water maze drug administration protocol.

3.2.1. Training

After 12 days of chronic dosing mice were given 0 or 10 mg/kg MPH 30 min prior to training in the conditioning chambers (Day 13). 10 mg/kg MPH significantly increased locomotor activity during the baseline period relative to saline controls (0 mg/kg: 177 ± 35.9, 10 mg/kg: 306.5 ± 40.7 arbitrary units) [$F(1,14) = 5.67$, $p = 0.03$] (data not graphed). The 2-s shock elicited a large increase in velocity, the unconditioned response, which did not differ between groups [$F(1,14) = 0.84$, $p = 0.38$] (data not graphed).

3.2.2. Testing

One week after training mice were returned to the conditioning context, off drug, to assess contextual memory. As compared to saline controls, chronic dosing with 10 mg/kg MPH prior to training dramatically impaired contextual memory [$F(1,14) = 10.20$, $p = 0.01$] (Fig. 2D, left). Twenty-four hours later tone memory was assessed, also off drug. Baseline locomotor activity in the alternate context did not differ between groups ($p > 0.2$). Mice chronically given 10 mg/kg MPH during training had significantly less tone memory than saline controls [$F(1,14) = 11.03$, $p = 0.005$] (Fig. 2D, right). Overall, these data suggest that the difference in dosing with regards to enhancing fear conditioning versus water maze memory cannot simply be explained by tolerance.

4. Discussion/conclusions

Here we demonstrate that mice given 10 mg/kg MPH pre-training learned the location of a fixed hidden platform faster than mice trained on saline or 1 mg/kg MPH (Fig. 1). Further, mice trained with either 1 or 10 mg/kg MPH retained the location of the platform both one day and one week post-training, while saline control mice did not (Fig. 2A–C). Together, these findings indicate that MPH dose-dependently enhances spatial learning and memory and that these effects persist when animals are tested off drug.

Interestingly, we observed different dose–response curves on fear conditioning and the water maze [11]. In the current study 10 mg/kg MPH optimally enhanced water maze learning; we previously found that 10 mg/kg MPH impaired fear memory [11]. Additionally, the 1 mg/kg dose of MPH that optimally enhanced fear learning [11] only modestly enhanced the retention of spatial memory in the water maze (Fig. 2C). It is important to note that this 1 mg/kg dose is the same as that typically prescribed therapeutically to humans (0.5–1 mg/kg) [13]. It is unclear how a dose of MPH in a mouse translates to a human dose [25] and unless specific evidence warrants otherwise, we have advocated using one-to-one dosing between humans and mice (see [1] and [11] for an extensive discussion).

Tolerance is one possible explanation for the different dose–response curves we observed across the two tasks [23]. In earlier work, a single injection of 10 mg/kg MPH was administered prior to fear conditioning [11], whereas water maze training involved injections of 10 mg/kg MPH for 15 days. Given that an enhancement in water maze learning was not seen until the 11th day of training (Fig. 1C), it is possible that subjects grew tolerant to the adverse behavioural effects seen with acute administration [23]. If this was the case then one might predict that mice given 10 mg/kg MPH chronically before fear conditioning would not have impaired fear memory. However, chronic dosing with 10 mg/kg MPH led to dramatically impaired fear memory when subjects were tested off drug (Fig. 2D). This decrement was independent of any effects on locomotion (see [11] for a discussion).

Instead, these dosing differences likely reflect different levels of arousal required for each task or differential action on the neural substrates for each task. It has been widely hypothesized that cognitive tasks have different optimal levels of arousal [26–28]. Often, high levels of arousal/activation are associated with impaired performance, while moderate arousal/activation is associated with the best performance [1]. Consistent with the present study, we previously found that a much higher dose of modafinil, an atypical psychostimulant [1], was required to enhance water maze learning (75 mg/kg) in comparison to the dose required to enhance fear learning (0.75 mg/kg) [22]. Thus, our results suggest that fear conditioning and the water maze themselves may produce different levels of arousal/activation or may require different amounts of monoamine activation for optimal learning [29–31]. We have argued that psychostimulant dose can be viewed as a proxy for the level of arousal/activation in animal models [1]. One may speculate that the water maze requires a greater level of activation than fear conditioning for optimal performance, which shifts the MPH dose–response curve to the right [22]. Still, we would expect that very high doses of MPH would impair water maze performance.

Nonetheless, 10 mg/kg MPH produced a compelling long-term enhancement of spatial learning that persisted when subjects were tested off-drug. Taken together with evidence that MPH (0.01–1 mg/kg) can enhance fear memory [11], it is clear that MPH produces a broad improvement in associative memory. We suggest that psychostimulant-induced memory enhancement should be the standard with which novel nootropics are compared. Indeed, although many novel cognitive enhancers are being developed, it remains to be seen if they will be definitively more effective and/or safe than the classical psychostimulants.

Acknowledgements

We thank Antonio Mora, Roy Jungay and Gilberto Sanchez for excellent animal care and Monette Leyva, Vanessa Wu, Erin McNalley, and Leila Oueini for invaluable technical assistance. We thank Jordan Comins for thoughtful comments on the manuscript. These studies were supported by NIH grant DA020041 (SGA), Hellman Fellowship (SGA), UCSD Chancellor's Interdisciplinary Collaboratories Grant (SAC), and NSF Graduate Research Fellowship (KKH).

References

[1] S.C. Wood, J.R. Sage, T. Shuman, S.G. Anagnostaras, Psychostimulants and cognition: a continuum of behavioral and cognitive activation, Pharmacol. Rev. 66 (2014) 193–221.
[2] P. Marshall, R. Schroeder, J. O'Brien, R. Fischer, A. Ries, B. Blesi, J. Barker, Effectiveness of symptom validity measures in identifying cognitive and behavioral symptom exaggeration in adult attention deficit hyperactivity disorder, Clin. Neuropsychol. 24 (2010) 1204–1237.
[3] S.M. Rhodes, J. Park, S. Seth, D.R. Coghill, A comprehensive investigation of memory impairment in attention deficit hyperactivity disorder and oppositional defiant disorder, J. Child Psychol. Psychiatry 53 (2012) 128–137.
[4] C.J. Teter, S.E. McCabe, K. LaGrange, J.A. Cranford, C.J. Boyd, Illicit use of specific prescription stimulants among college students: prevalence, motives, and routes of administration, Pharmacotherapy 26 (2006) 1501–1510.
[5] L. Kinney, C.V. Vorhees, A comparison of methylphenidate induced active avoidance and water Mmze performance facilitation, Pharmacol. Biochem. Behav. 10 (1979) 437–439.
[6] M.L. Zeise, S. Espinoza, A. González, F.S. Cerda, J. Nacarate, C.G. Yáñez, B. Morales, Methylphenidate improves cue navigation in the Morris water maze in rats, Neuroreport 18 (2007) 1059–1062.
[7] Y. Tian, Y. Wang, Y. Deng, K. Maeda, Methylphenidate improves spatial memory of spontaneously hypertensive rats: evidence in behavioral and ultrastructural changes, Neurosci. Lett. 461 (2009) 106–109.
[8] M.A. Mehta, I.M. Goodyer, B.J. Sahakian, Methylphenidate improves working memory and set-shifting in AD/HD: relationships to baseline memory capacity, J. Child Psychol. Psychiatry 45 (2004) 293–305.
[9] C.W. Berridge, J.S. Shumsky, M.E. Andrzejewski, J.A. McGaughy, R.C. Spencer, D.M. Devilbiss, B.D. Waterhouse, Differential sensitivity to psychostimulants across prefrontal cognitive tasks: differential involvement of noradrenergic α_1- and α_2-receptors, Biol. Psychiatry 71 (2012) 467–473.
[10] D.M. Eagle, M.R.A. Tufft, H.L. Goodchild, T.W. Robbins, Differential effects of modafinil and methylphenidate on stop-signal reaction time task performance

in the rat, and interactions with the dopamine receptor antagonist cis-flupenthixol, Psychopharmacology (Berl.) 192 (2007) 193–206.
[11] S.A. Carmack, K.K. Howell, K. Rasaei, E.T. Reas, S.G. Anagnostaras, Animal model of methylphenidate's long-term memory-enhancing effects, Learn. Mem. 21 (2014) 82–89.
[12] S.G. Anagnostaras, S.C. Wood, T. Shuman, D.J. Cai, A.D. Leduc, K.R. Zurn, J.R. Sage, G.M. Herrera, Automated assessment of Pavlovian conditioned freezing and shock reactivity in mice using the video freeze system, Front. Behav. Neurosci. 4 (2010) 1–11.
[13] McNeil Pediatrics, Concerta: Full Prescribing Information, 2008, pp. 1–30.
[14] G.D. Gale, S.G. Anagnostaras, B.P. Godsil, S. Mitchell, T. Nozawa, J.R. Sage, B. Wiltgen, M.S. Fanselow, Role of the basolateral amygdala in the storage of fear memories across the adult lifetime of rats, J. Neurosci. 24 (2004) 3810–3815.
[15] S.G. Anagnostaras, G. Gale, M.S. Fanselow, Hippocampus and contextual fear conditioning: recent controversies and advances, Hippocampus 11 (2001) 8–17.
[16] R. Morris, Developments of a water-maze procedure for studying spatial learning in the rat, J. Neurosci. Methods 11 (1984) 47–60.
[17] A.L. Markowsa, J.M. Long, C.T. Johnson, D.S. Olton, Variable-interval probe test as a tool for repeated measurements of spatial memory in the water maze, Behav. Neurosci. 107 (1993) 627–632.
[18] C.V. Vorhees, M.T. Williams, Morris water maze: procedures for assessing spatial and related forms of learning and memory, Nat. Protoc. 1 (2006) 848–858.
[19] N.J. Broadbent, L.R. Squire, R.E. Clark, Reversible hippocampal lesions disrupt water maze performance during both recent and remote memory tests, Learn. Mem. 13 (2006) 187–191.
[20] R.E. Clark, N.J. Broadbent, L.R. Squire, Hippocampus and remote spatial memory in rats, Hippocampus 15 (2005) 260–272.
[21] R.E. Clark, S.J. Martin, Interrogating rodents regarding their object and spatial memory, Curr. Opin. Neurobiol. 15 (2005) 593–598.
[22] T. Shuman, S.C. Wood, S.G. Anagnostaras, Modafinil and memory: effects of modafinil on Morris water maze learning and Pavlovian fear conditioning, Behav. Neurosci. 123 (2009) 257–266.
[23] J. Swanson, S. Gupta, D. Guinta, D. Flynn, D. Agler, M. Lerner, L. Williams, I. Shoulson, S. Wigal, Acute tolerance to methylphenidate in the treatment of attention deficit hyperactivity disorder in children, Clin. Pharmacol. Ther. 66 (1999) 295–305.
[24] S.A. Carmack, S.C. Wood, S.G. Anagnostaras, Amphetamine and extinction of cued fear, Neurosci. Lett. 468 (2010) 18–22.
[25] R. Kuczenski, D.S. Segal, Stimulant actions in rodents: implications for attention-deficit/hyperactivity disorder treatment and potential substance abuse, Biol. Psychiatry 57 (2005) 1391–1396.
[26] R.M. Yerkes, J.D. Dodson, The relation of strength of stimulus to rapidity of habit-formation, J. Comp. Neurol. Psychol. (1908) 459–482.
[27] D.O. Hebb, Drives and the C.N.S. (conceptual nervous system), Psychol. Rev. 62 (1955) 243–254.
[28] H. Schlosberg, Three dimensions of emotion, Psychol. Rev. 61 (1954) 81–88.
[29] P.L. Clatworthy, S.J.G. Lewis, L. Brichard, Y.T. Hong, D. Izquierdo, L. Clark, R. Cools, F.I. Aigbirhio, J. Baron, T.D. Fryer, T.W. Robbins, Dopamine release in dissociable striatal subregions predicts the different effects of oral methylphenidate on reversal learning and spatial working memory, J. Neurosci. 29 (2009) 4690–4696.
[30] N.D. Volkow, G.J. Wang, J.S. Fowler, J. Logan, S.J. Gatley, C. Wong, R. Hitzemann, N.R. Pappas, Reinforcing effects of psychostimulants in humans are associated with increases in brain dopamine and occupancy of D(2) receptors, J. Pharmacol. Exp. Ther. 291 (1999) 409–415.
[31] S. Vijayraghavan, M. Wang, S.G. Birnbaum, G.V. Williams, A.F.T. Arnsten, Inverted-U dopamine D1 receptor actions on prefrontal neurons engaged in working memory, Nat. Neurosci. 10 (2007) 376–384.

Chapter 4, in full, is a reprint of the material as it appears in Methylphenidate enhances acquisition and retention of spatial memory. *Neuroscience Letters*, 567, 45-50. Carmack SA, Block CL, Howell KK & Anagnostaras SG (2014). The dissertation author was the primary investigator and author of this paper.

Chapter 5

Obligatory role for the dopamine transporter in learning and memory

Summary

Dopaminergic neurotransmission has traditionally been thought to play a modulatory, rather than obligatory, role in learning and memory [1]. Psychostimulant drugs, such methylphenidate and amphetamine, enhance neurotransmission of dopamine, norepinephrine, and serotonin by acting on reuptake transporters [2]. These drugs form the cornerstone of treatment for attention-deficit hyperactivity disorder, the most common psychiatric disorder in children, because of their ability to improve learning [2]. Here we examine triple point mutant knockin mice of the gene coding the dopamine transporter (DAT) [3]. This knockin mutation was engineered to hinder the ability of drugs such as methylphenidate and cocaine to act at DAT and also leads to reduced efficiency of the transporter, without affecting total transporter expression [3]. Although we expected a small facilitatory role for DAT in learning and memory, the mutations produced severe learning and memory defects across multiple domains. The dramatic memory impairments were not confounded by effects on locomotor activity, anxiety, nociception, and motivation, and were without any apparent effect on two forms of hippocampal synaptic plasticity. Together these findings strongly challenge the notion that DAT plays a nonessential or modulatory role in learning and memory. Rather, our results demonstrate an obligatory role for DAT, perhaps by acting as a critical salience control mechanism for opening the temporal window of learning. As DAT dysfunction has been associated with many debilitating disorders, including ADHD [4], renewed focus should be placed on this transporter as a pharmacological target.

Psychostimulant drugs that act on DAT and the norepinephrine transporter (NET) are cognitive enhancers known to increase monoaminergic neurotransmission [2]. Monoamine transmitters are generally considered to have memory-modulating effects as a result of their ability to facilitate synaptic plasticity [1,5,6]. We recently reported that low doses of the psychostimulants methylphenidate, cocaine, amphetamine, and modafinil enhance the acquisition of Pavlovian fear conditioning [2,7]. It remains an open question, however, whether DAT and/or NET binding mediate psychostimulant-induced memory enhancement.

In order to determine if DAT is essential for the enhancement of memory by psychostimulants, we pursued a strategy of using knockin mice that markedly reduce the binding of methylphenidate and cocaine (DAT^{CI} mutants) [3,8]. Because the binding sites for dopamine (DA), methylphenidate, and cocaine partially overlap [9], the knockin mutation also reduces transporter efficiency [3]. DAT^{CI} mice are unique in that the mutation does not lead to dramatic compensation of other neurotransmitter systems[3,10]. We were then quite surprised to find a stunning effect of the DAT^{CI} knockin on learning and memory, even in the absence of methylphenidate or cocaine (Figure 1).

We first recognized severe memory impairments in DAT^{CI} mutants' on Pavlovian fear conditioning, the leading model of learning and memory in rodents. In fear conditioning, an animal is brought to a conditioning chamber and presented with a tone that is immediately followed by an aversive but mild foot shock [11]. As the result of this pairing, the animal exhibits a learned fear response (freezing) in response to presentation of the tone or the conditioning context alone. Both tone context fear memory depend on the amygdala, while context fear memory additionally requires the hippocampus[12]. In striking contrast to the wild type controls, DAT^{CI} mutants showed almost no contextual fear memory acquisition when tested immediately [$F_{1,20} = 33.5$, $p <$

0.0001] (Fig. 1a), one hour [$F_{1,28}$ = 23.2, p < 0.0001] (Fig. 1b), or one week after conditioning [$F_{1,20}$ = 46.1, p < 0.0001] (Fig. 1c). The DAT^{Cl} knockin also abolished tone fear memory [$F_{1,20}$ = 19.1, p < 0.0001] (Fig. 1d). Further, the dramatic fear learning impairment could not be rescued by methylphenidate (Extended Data 1).

Prior studies with a complete DAT knockout were difficult to interpret due to extreme hyperactivity and dramatic compensation by other transmitter systems [13,14]. DAT^{Cl} mutants are only slightly hyperactive as compared to wild type controls during the pre-conditioning baseline period [$F_{1,69}$ = 8.1, p = 0.006] (Fig. 1e,) and on standard measures of activity in the open field [$F_{1,20}$ = 19.6, p < 0.0001] (Extended Data 2). To rule out the possibility that freezing deficits observed freezing deficits may reflect a performance failure (i.e., a disruption of freezing behaviour) rather than a deficit in memory, we split the sample of DAT^{Cl} mice on locomotor activity during the conditioning baseline. This median split created a group of DAT^{Cl} mutants ($LoDAT^{Cl}$) with activity comparable to the wild-type controls (p = 0.44) and a group (Hi DAT^{Cl}) that was about twice as active as wild-type controls (p < 0.0001) [group $F_{2,19}$ = 15.0, p < 0.0001] (Fig. 1f). In terms of severity of memory deficits, however, both $LoDAT^{Cl}$ and $HiDAT^{Cl}$ mice froze significantly less than wild type controls (p values < 0.005), and did not differ from each other (p values > 0.8) (Fig. 1g-i). Thus, profound amnesia is evident even in a sample of DAT^{Cl} mice that is not hyperactive.

We then tested whether the DAT^{Cl} knockin altered nociception because the mutants showed a slight, but significant, increased reactivity to the shock as compared wild type controls [$F_{1,69}$ = 6.3, p = 0.014] (Fig. 1e, right). This was already unlikely since higher shock reactivity would not explain reduced freezing. Moreover, using footshock threshold testing, we found no evidence of altered nociception (p values > 0.3) (Fig. 1j). We also found that the DAT^{Cl} knockin had no effect on anxiety behavior, suggesting the effects were on memory, rather than fear per se (p values > 0.2) (Extended Data 3).

Next, we assessed whether the DAT^{Cl} mutants were capable of showing more freezing behavior by using intensive training. Mice received four unsignaled shocks per day for 14 consecutive days (Fig. 1k). By the 14th day of training, despite still having a deficit relative to WT mice [genotype $F_{1,11} = 4.8$, $p = 0.05$], DAT^{Cl} mice show evidence of good freezing behavior (49.9±6.4%, Fig 1k). This suggests that deficits seen in fear memory (Fig. 1b-d) are not due simply to an inability to display adequate levels of freezing. This finding provides further support that the DAT^{Cl} mutants have a deficit in the *memory acquisition*.

We then asked whether the learning and memory deficit evident in the DAT^{Cl} mutants would generalize beyond fear learning by testing the mice on two other hippocampus-dependent tasks [15,16]: place learning in the Morris water maze and novel object recognition (Fig. 2). In the Morris maze animals must learn to use distal spatial cues to locate a fixed, hidden platform in order to escape from a pool of opaque water. During acquisition, short-term memory was assessed in probe trials every 5 days that immediately followed daily training trials. DAT^{Cl} mice show some evidence of short-term retention of memory for the platform location by the 20th day of training (Fig. 2a,b). Long-term memory for the platform location was assessed one week after the last day of training (Day 27). While the wild-type controls exhibited memory comparable to performance on the last day of training, the DAT^{Cl} showed severe amnesia [genotype $F_{1,24} = 9.6$, $p = 0.005$] (Fig. 2c). Because performance of DAT^{Cl} mice and wild-type controls was nearly identical during the early stages of training (p values > 0.9) (Fig. 2d,e) differences in motivation, perception, or swim speed do not account for this rapid forgetting.

Because there were differences in swim speed between the two genotypes [genotype x probe trial $F_{3,72} = 6.051$, $p = 0.001$] (Fig. 2e) we split (median) the sample of DAT^{Cl} mice on speed during the Day 20 probe trial (Fig. 2f). This produced a group of mutants ($LoDAT^{Cl}$) whose swim speed was equivalent to that of wild-type mice ($p > 0.9$)

(Fig. 2f), but whose memory impairment was just as dramatic as that of the DAT^{Cl} mice who swam significantly faster ($HiDAT^{Cl}$, p = 0.83) relative to wild type controls (p values < 0.03) [Speed: $F_{2,23}$ = 7.7, p = 0.003; Time in target quadrant: group $F_{2,23}$ = 4.6, p = 0.021] (Fig. 2g). Overall, these data indicate that the DAT^{Cl} knockin also produces a severe deficit in place memory.

We further investigated hippocampus-dependent memory formation using a non-spatial, non-aversive, novel object recognition task [15]. This task takes advantage of the natural investigatory behaviour of mice as they tend to investigate familiar objects less than novel objects following re-exposure. While wild-type controls showed preference for the novel object at all time points tested (p values < 0.05), DAT^{Cl} mutants failed to show a preference for the novel object starting 2 hours after initial exposure to the familiar objects (p values > 0.07) (Fig. 2h, left). This intriguing finding suggests that DAT^{Cl} mutants forgot that they had previously been exposed to the familiar object or were unable to detect novelty[17]. It is interesting to consider this notion in light of the recent proposal that the dopamine system, activated by novelty, controls hippocampus-dependent memory processing by regulating proteins necessary for temporal persistence of synaptic plasticity and long-term memory [6,17] .

Based on this hypothesis and the observation of severe hippocampus-dependent memory deficits in DAT^{Cl} mice, we suspected that the mutants would have impaired hippocampal long-term potentiation (LTP) [1,18]. Thus, we assessed LTP *in vitro* using two stimulation protocols: high-frequency stimulation known to induce persistent LTP (Fig. 3a) and two-theta burst stimulation, a minimal protocol thought to mimic endogenous physiological activity[19] (Fig. 3b). Surprisingly, we found that both forms of LTP were remarkably intact in the DAT^{Cl} mutants as compared to wild-type controls (p values > 0.6) (Fig. 3).

The finding that a highly selective disruption of the DAT produces severe memory defects across multiple domains, independent of any apparent effects on hippocampal

LTP, is difficult to integrate into the traditional understanding of how dopamine works to modulate memory at the synaptic level [6,20,21]. Is DAT disruption acting just by increasing post-synaptic dopamine signaling? Beyond its well-known ability to modulate synaptic dopamine levels, DAT itself has channel-like [22] properties and can significantly modify the pre-synaptic current [23]. Recent work suggests this is a putative therapeutic mechanism of action of psychostimulants [23]. Investigating this possibility will require a different approach to the physiology, away from glutamate signaling [1], and with focus on the channel action of DAT.

The mechanism by which reduced efficiency of the DAT leads to memory failure remains intriguing and may not directly involve association formation [24–26]. Aside from contingency detection, one necessary function in associative learning is the opening of an "associative window" that enables the onset of memory acquisition [27–32]; this mechanism is argued to limit learning to biologically important events in order to avoid exceeding memory capacity [25]. The DAT^{Cl} knockin may produce a deficit in saliency detection and therefore, animals fail to orient and attend to important events in the environment [33,34]. As a result, they fail to open the "window of learning." In this sense, the role of DAT could be to enable low-level assignment of salience by novelty detection to drive attention toward biologically significant events [25], thus opening the associative window so that events may be encoded in memory. Inappropriate salience and attention, as well as DAT dysfunction, have been implicated in disorders like schizophrenia [35] and ADHD [36], suggesting that DAT^{Cl} mutants provide a promising model for therapeutic development.

Methods Summary

All procedures were conducted in accordance with the animal care standards set forth by the National Institutes of Health (8th Guide) and were approved by the UCSD

IACUC. Adult mice (at least 10 weeks of age) on a C57Bl/6J background were used for all studies.

Methods

Animals. Generation of **DAT^{CI}** knockin mice by homologous recombination has been previously described [3]. **DAT^{CI}** mice have a triple point mutation (L104V/F105C/ A109V) in transmembrane domain 2 within the DAT protein that renders it ~90-fold more insensitive to cocaine inhibition [3] and ~18-fold more insensitive to methylphenidate inhibition as compared to wildtype DAT [8]. Mice were originally generated from 129SvJ embryonic (ES) cells and crossed with C57Bl/6J mice at The Ohio State University (Columbus, Ohio). Mutant mice have been backcrossed with C57Bl/6J mice for at least 10 generations and are considered to be in the C57Bl/6J background. Heterozygous mice were shipped to UCSD (La Jolla, California) where they were crossed to generate the littermate wild type and mutant mice used in the present studies. Transnetyx (Cordova, TN) performed genotyping. Animals were maintained on a 14:10 light/dark cycle and testing occurred during the light phase. All procedures were done blind with respect to genotype.

Drugs. Methylphenidate HCl (MPH; Sigma-Aldrich) was dissolved in physiological 0.9% saline (vehicle) and given in a dose of 1, 10, 18, or 50 mg/kg (salt weight). All saline and drug injections were administered intraperitoneally (i.p.) in a volume of 10 ml/kg.

Fear conditioning: tone conditioning. Mice were placed into a novel conditioning chamber and after a 2 min baseline were given three tone-shock pairings (tone: 30 s, 2.8 kHz, 85 dBA, shock: 2 s, AC, 0.75 mA, RMS) at min 2, 3, 4. After an additional 5 min (immediate memory test), they were returned to their home cages. One

hour later, one group was returned to the conditioning chambers for a 5 min context memory test. Seven days later a second group was returned to the conditioning chambers for a 5 min context memory test. The next day the second group was brought to an alternate environment and after a 2-min baseline period, the training tone was presented three times at 30 s intervals (tone test). For experiments with methylphenidate, drug was administered 30 min prior to conditioning. Memory was assessed during the last 5 minutes of conditioning (immediate memory, *on drug*), 7 days later (context test, *off drug*), and 8 days later (tone test, *off drug*). Locomotor activity and freezing behaviour were recorded and scored during conditioning and testing trials using the VideoFreeze system (MedAssociates). The basic protocol and apparatus have been described previously [7,37].

Fear conditioning: context acquisition. Mice were placed into a novel conditioning chamber and after a 4-min baseline period, received 4 unsignaled shocks (2 s, 0.75 mA, RMS) separated by 1 min each. After an additional 30 s, they were returned to their home cages. This was repeated for 14 days. Locomotor activity and freezing behaviour were recorded during the 4-min baseline period before the shock each day to form an acquisition curve.

Shock reactivity thresholds. We measured the sensitivity of wild type and DAT^{Cl} mutants to foot shocks of increasing intensity. Mice were individually placed in conditioning chambers and given 1-s foot shocks, starting at 0.05 mA and increasing in 0.05 mA intervals every 10-s. The test was terminated when flinching, running, vocalization, and jumping behaviors had been elicited or when the shocks reached 0.5 mA. The level of current required to elicit each behaviour was recorded.

Elevated Plus Maze. The plus maze (MedAssociates) had two open and two enclosed arms joined at a center hub, elevated 74 cm from the ground. Testing lasted 5 min in dim light in a windowless room. Mice were tracked using a camera and video tracking software (Panlab Smart 3.0, Harvard Apparatus). Time spent and distance traveled in each section of the maze were recorded.

Open Field. Four mice were tested concurrently in dim light in individual activity chambers (MedAssociates) housed in a windowless room. Each chamber had clear polycarbonate walls and white acrylic floors. The chambers were cleaned and scented with 10% ZEP. Mice were tracked using Activity Monitor software (MedAssociates). Locomotor activity (distance traveled) and rearing (vertical counts) were measured for 30 min.

Morris water maze. The basic protocol for the water maze experiment has been described elsewhere [38]. The water maze (114 cm in diameter) had a 10 cm diameter acrylic escape platform hidden 1 cm below the surface of the water. The water was heated to 23.5°C using a built-in heater and thermostat and made opaque using white tempera paint. Distal cues were arranged around the room (e.g. posters), but the maze itself appeared isotropic. Location was tracked using a computerized video tracking system (WaterMaze, MedAssociates). Mice were given four training trials per day for 20 d. For each trial, mice started from one of four randomly assigned start locations and had a maximum of 60 s to find the hidden platform in a fixed location. If 60 s elapsed, the mouse was manually placed onto the platform. Mice were allowed to rest for 5 s on the platform to provide reinforcement and exposure to the platform's location. Following training trials on days 5, 10, 15, and 20, mice were given a 60 s probe trials in which the platform had been removed. One week after training (Day 27), mice were given a final probe trial to assess retention of the hidden platform's location.

Object Recognition. Mice were habituated to the testing chambers 10 min per day for 7 d. The testing room had white noise and was dimly lit with red light. To habituate to object presentation mice were given two identical objects in the testing chambers for 2, 10-min sessions (1-h interval). These objects were not used again. Object recognition training and testing occurred over two days. Each session was 5 min long. On Day 1 the animals were trained with two identical to-be-familiar objects. Tests 1-5 each had one familiar object and one novel object and were conducted at 5 min (Test 1), 1 hr (Test 2), 2 hr (Test 3), 3 hr (Test 4), or 24 hr (Test 5) after the training session. Seven objects were used: a weight, toy horn, plastic food cup, spiky rubber ball, binder clip, plastic block, and a jack. The objects used were counterbalanced across genotype, location, and novelty. Behavior was video-recorded and later scored for the duration of olfactory investigation of objects (defined as the subjects nose oriented toward and in close contact with the object). A novel preference score was generated to indicate learning: (novel time) / (novel + familiar time) for each testing trial.

Hippocampal LTP. The basic protocol and apparatus have been described elsewhere[19]. Recordings were made using transverse hippocampal slices in a submerged recording chamber perfused with artificial cerebrospinal fluid. Extracellular excitatory postsynaptic field potentials (EPSPs) were recorded in CA1. Long-term potentiation (LTP) was induced after a 15 min baseline according to a high-frequency stimulation (HFS) protocol (4 x 100 Hz for 1s) or two-theta burst stimulation (TBS) protocol (two bursts, each burst 4 pulses at 100 Hz, 200 ms inter-burst interval). Slices in which there was significant drift were excluded. Data reported reflect individual animals, rather than slices; when multiple slices were used from a single animal, data were averaged into a single data point.

Statistical Analyses. Data were entered into a multivariate analysis of variance (MANOVA) and the level of significance was set at $p \leq 0.05$. Post-hoc comparisons were done with Fisher's protected least significant difference (unpaired tests) or paired two-tailed t-tests (paired tests) following significant omnibus comparisons. Results from male and female mice were combined because there were no significant differences between the sexes on any measures.

Figures

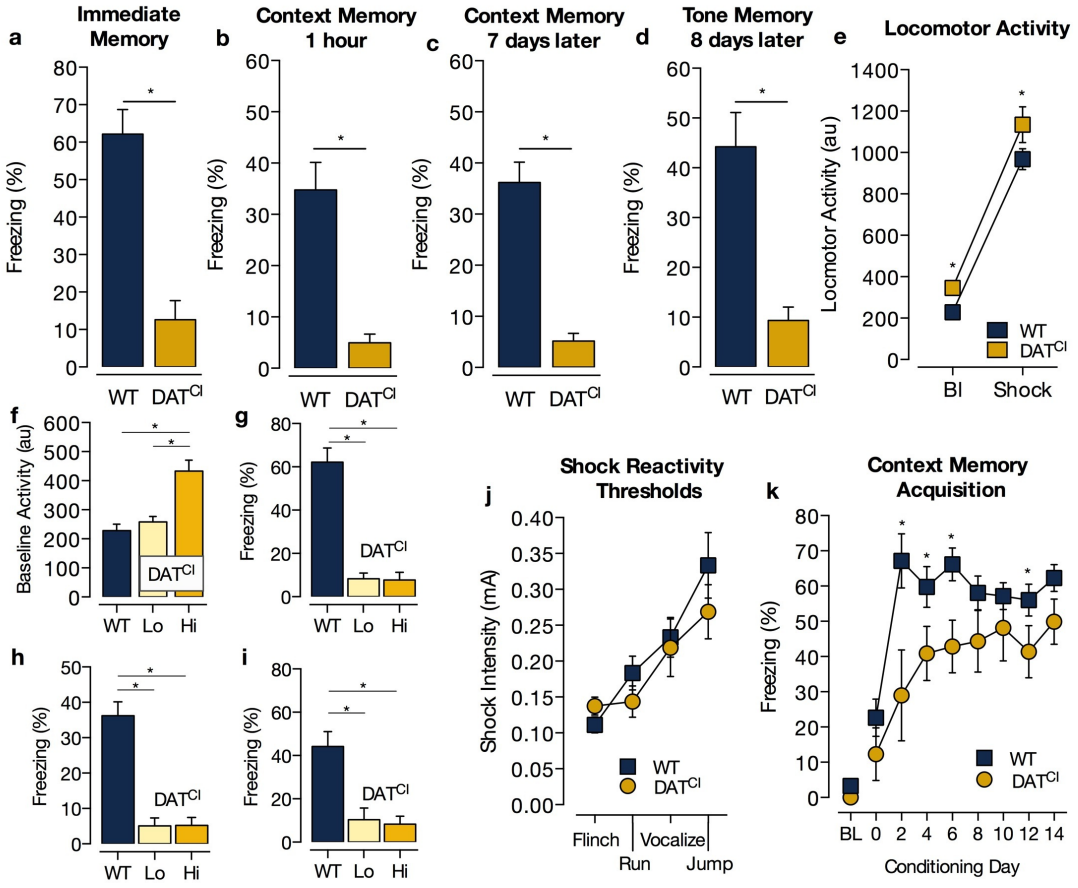

Figure 5.1. DAT^{Cl} mutants have profound defects in fear learning and memory. a-c, Context memory, measured as percentage of trial freezing, during the last 5 minutes of conditioning (**a**), one hour (**b**), or seven days later (**c**). (**d**) Tone fear memory. Mutants were impaired on all memory tests compared to wildtype (WT) controls (n≥10/group). (**e**) Activity during conditioning baseline (Bl) and in response to foot shock (Shock). Mutants are slightly hyperactive, but can be split to produce a group with activity comparable to WT (**f**). Baseline activity does not predict memory deficits in the last 5 minutes of conditioning (**g**), context test (**h**), or tone test (**i**). (**j**) Foot-shock threshold testing (n≥8/group) (**k**) Freezing during the 4 min baseline at the start of each context conditioning day (n≥5/group). Error bars, standard error of the mean (s.e.m.). * $p < 0.05$.

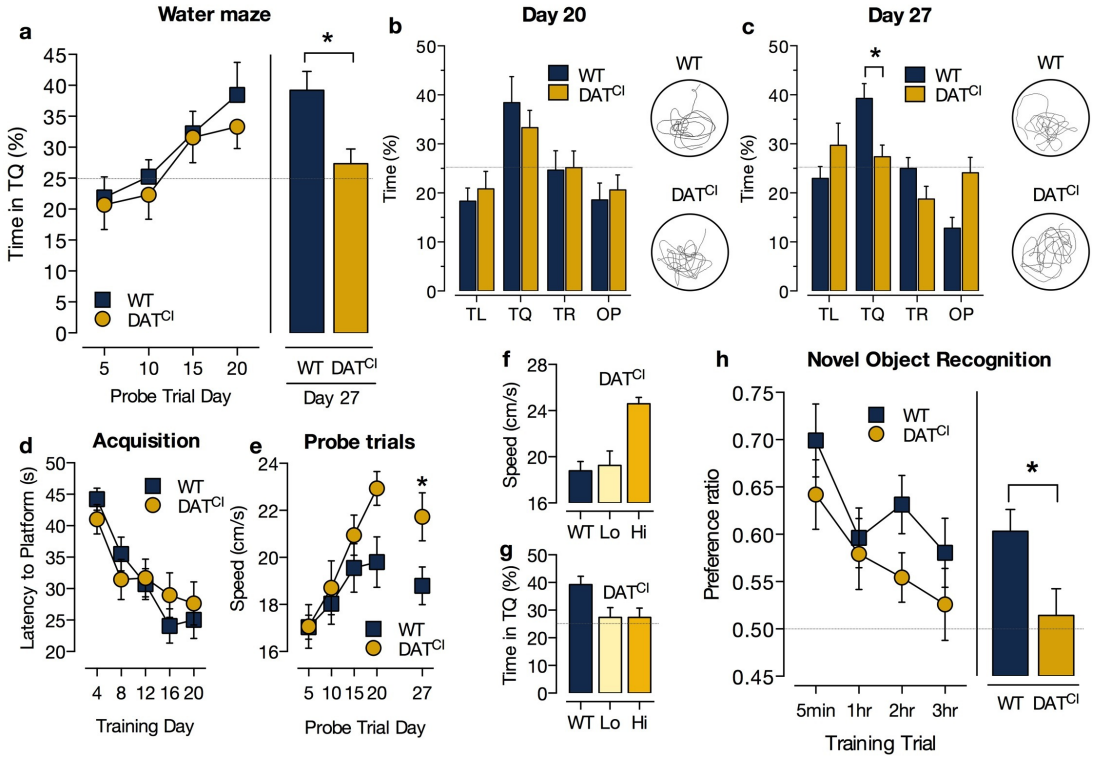

Figure 5.2. DAT^{Cl} mutants have impaired hippocampal-dependent memory. a-g, Morris water maze training and testing. Short-term memory probes on Days 5-20 (**a,b**) and long-term memory (LTM) probe on Day 27 (**a,c**) with representative swim paths. Wildtype (WT) controls have LTM for the platform location; mutants do not (*n*=13/group). (**d**) Latency to reach the platform during training. (**e**) DAT^{Cl} mutants swim faster than WT mice (Day 20 probe), but can be split to produce a group with swim speeds comparable to WT (**f**). (**g**) Swim speed during Day 20 probe does not predict LTM deficits. (**h**) Novel object recognition. Training trials (*left*) and testing 24 hours later (*right*). Preference score is calculated as (novel object time)/(novel + familiar time). Mutants perform worse than WT controls (*n*=9/group). TQ, target quadrant (platform location); TR, target right; TL, target left; OP, opposite. Dashed lines, chance. Error bars, s.e.m. * $p < 0.05$.

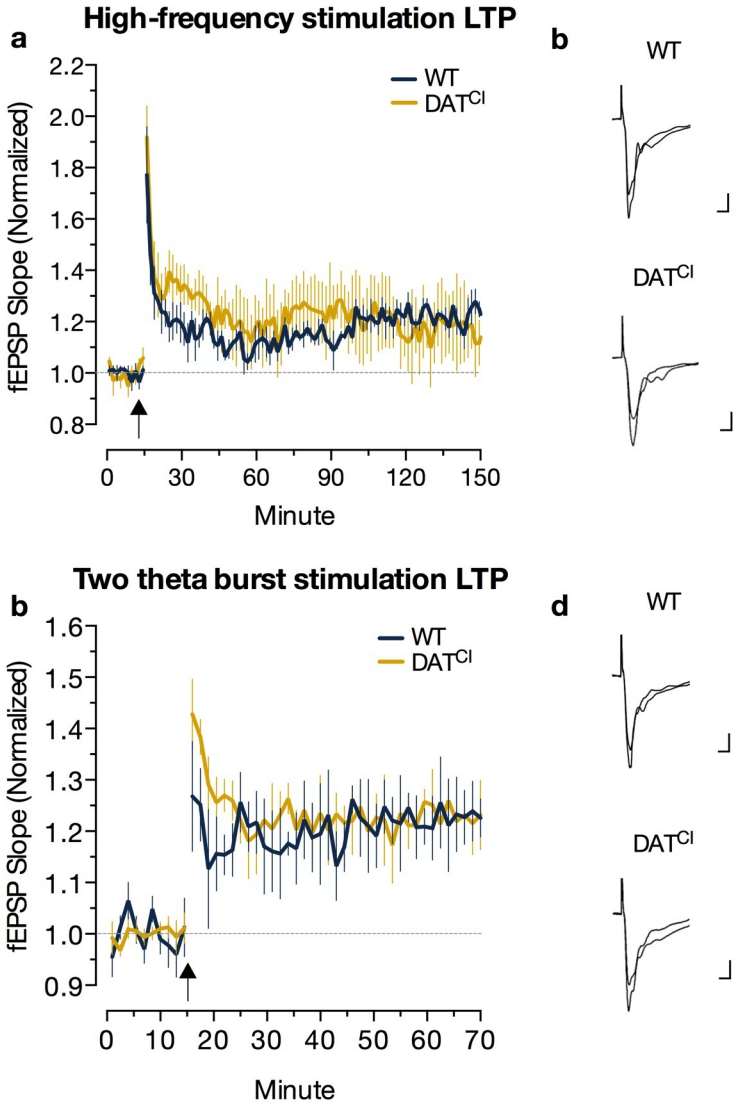

Figure 5.3. DAT^{Cl} mutants have spared hippocampal CA1 long-term potentiation (LTP) *in vitro*. (**a**) LTP after high-frequency stimulation (HFS, 4 x 100 Hz for 1s). LTP was similar in mutant and wildtype (WT) mice (*n*=3/group). (**b**) Representative recordings during baseline and after induction of LTP by HFS. (**c**) LTP after two theta-burst stimulation (TBS, two bursts separated by 200 ms, each burst 4 pulses at 100 Hz). LTP was similar in mutant and WT mice (*n*=4/group). (**d**) Representative recordings during baseline and after induction of LTP by TBS. Arrow, tetanus. Error bars, s.e.m. Scale bars, 0.5 mV and 10 ms.

Supplemental Figures

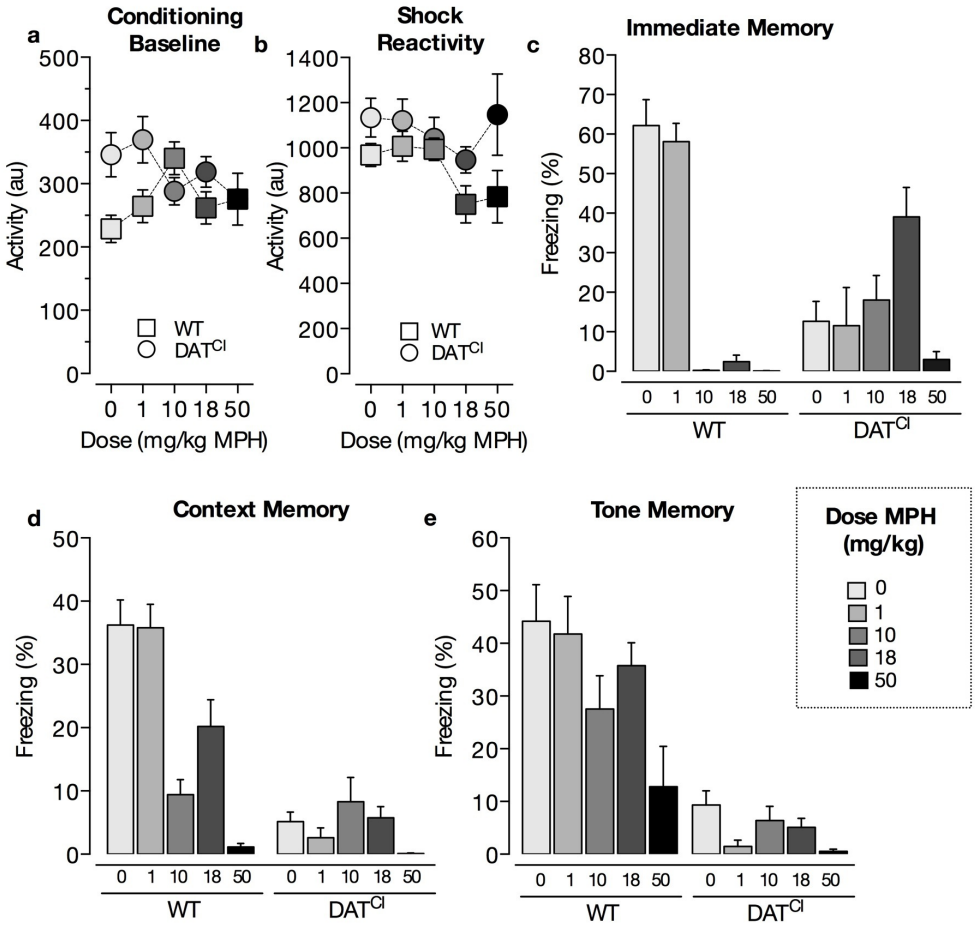

Figure 5.S1. Methylphenidate (MPH) does not rescue the DAT^{Cl} fear memory deficit. (**a**) Baseline activity. MPH was given 30 min prior to conditioning. DAT^{Cl} mutants are more active than wildtype (WT) controls. MPH dose-dependently increases activity in WT and decreases activity in DAT^{Cl} mice [genotype x dose $F_{4,73}$ = 3.3, p = 0.01; genotype $F_{1,73}$ = 6.1, p = 0.016]. (**b**) Reactivity to the footshock. DAT^{Cl} mutants have greater shock reactivity than WT controls [genotype $F_{1,73}$ = 14.4, p < 0.001]. MPH dose-dependently modulates shock reactivity [dose $F_{4,73}$ = 3.7, p = 0.009]. (**c**) Immediate memory measured as percentage of time spent freezing during the last 5 minutes of conditioning. DAT^{Cl} mutants freeze less than WT controls [genotype $F_{1,73}$ = 5.7, p = 0.02]. MPH modulates immediate fear memory based on dose and genotype [genotype x dose $F_{4,73}$ = 22.7, p < 0.001; dose $F_{4,73}$ = 14.9, p = 0.02]. (**d**) Context fear memory tested 7 days after conditioning. DAT^{Cl} mutants freeze less than WT controls [genotype $F_{1,73}$ = 63.3, p < 0.001]. MPH dose-dependently decreases context memory in WT, but not DAT^{Cl} mice [genotype x dose $F_{4,73}$ = 12.2, p < 0.001; dose $F_{4,73}$ = 12.4, p < 0.001]. (**e**) Tone fear memory tested 8 days after conditioning. DAT^{Cl} mutants freeze less than WT mice [genotype $F_{1,73}$ = 60.4, p < 0.001]. MPH dose-dependently decreases tone memory in WT, but does not affect DAT^{Cl} mutants [dose $F_{4,73}$ = 3.4, p = 0.01]. (*n* > 6/ group). Error bars, s.e.m. * p < 0.05.

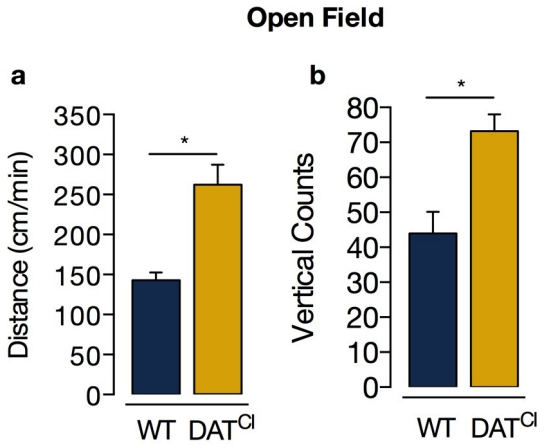

Figure 5.S2. DAT^{Cl} mutants are hyperactive in the open field. (**a**) DAT^{Cl} mutants have greater locomotor activity during the 30 min test than wild type (WT) controls [genotype $F_{1,20}$ = 19.6, p < 0.0001]. (**b**) DAT^{Cl} mutants exhibit greater rearing behaviour than WT mice, measured as number of vertical counts [genotype $F_{1,20}$ = 13.8, p = 0.001] (*n*=11/group). Error bars, s.e.m. * p < 0.05.

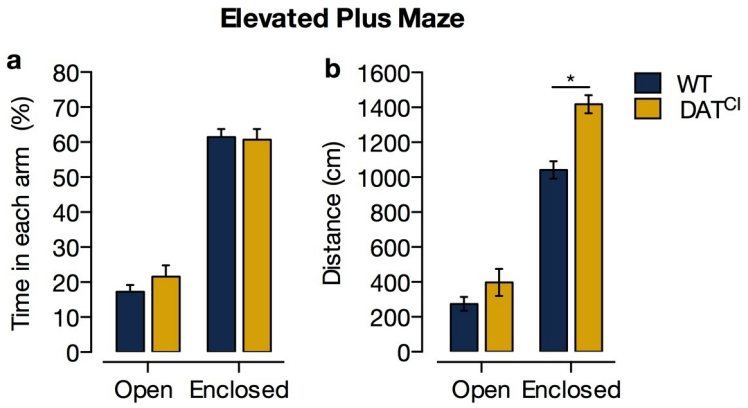

Figure 5.S3. DAT^{Cl} mutants show wild-type levels of anxiety. (**a**) Results are expressed as the percentage of time spent in the open (anxiety provoking) as compared to the enclosed arms of the maze. DAT^{Cl} and wildtype (WT) mice spend comparable amounts of time in each arm (p values > 0.2). (**b**) DAT^{Cl} mice are hyperactive as compared to WT controls in the maze [genotype $F_{1,20}$ = 27.1, p < 0.001] (*n*=11/group). Error bars, s.e.m.

References

[1] E.R. Kandel, Y. Dudai, M.R. Mayford, The molecular and systems biology of memory., Cell. 157 (2014) 163–86.

[2] S.C. Wood, J.R. Sage, T. Shuman, S.G. Anagnostaras, Psychostimulants and Cognition: A Continuum of Behavioral and Cognitive Activation, Pharmacol. Rev. 66 (2014) 193–221.

[3] R. Chen, M.R. Tilley, H. Wei, F. Zhou, F.-M. Zhou, S. Ching, N. Quan, R.L. Stephens, E.R. Hill, T. Nottoli, D.D. Han, H.H. Gu, Abolished cocaine reward in mice with a cocaine-insensitive dopamine transporter., Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 9333–8.

[4] J. Swanson, R.D. Baler, N.D. Volkow, Understanding the effects of stimulant medications on cognition in individuals with attention-deficit hyperactivity disorder: a decade of progress., Neuropsychopharmacology. 36 (2011) 207–26.

[5] S.J. Sara, The locus coeruleus and noradrenergic modulation of cognition., Nat. Rev. Neurosci. 10 (2009) 211–23.

[6] J.I. Rossato, L.R.M. Bevilaqua, I. Izquierdo, J.H. Medina, M. Cammarota, Dopamine controls persistence of long-term memory storage., Science. 325 (2009) 1017–20.

[7] S.A. Carmack, K.K. Howell, K. Rasaei, E.T. Reas, S.G. Anagnostaras, Animal model of methylphenidate's long-term memory-enhancing effects, Learn. Mem. 21 (2014) 82–89.

[8] M.R. Tilley, H.H. Gu, The Effects of Methylphenidate on Knockin Mice with a Methylphenidate-Resistant Dopamine Transporter, 327 (2008) 554–560.

[9] T. Beuming, J. Kniazeff, M.L. Bergmann, L. Shi, L. Gracia, K. Raniszewska, A.H. Newman, J.A. Javitch, H. Weinstein, U. Gether, C.J. Loland, The binding sites for cocaine and dopamine in the dopamine transporter overlap., Nat. Neurosci. 11 (2008) 780–9.

[10] F. Napolitano, A. Bonito-Oliva, M. Federici, M. Carta, F. Errico, S. Magara, G. Martella, R. Nistico, D. Centonze, A. Pisani, H.H. Gu, N.B. Mercuri, A. Usiello, Role of aberrant striatal dopamine D1 receptor/cAMP/protein kinase A/DARPP32 signaling in the paradoxical calming effect of amphetamine., J. Neurosci. 30 (2010) 11043–56.

[11] S.G. Anagnostaras, S.C. Wood, T. Shuman, D.J. Cai, A.D. Leduc, K.R. Zurn, J.R. Sage, G.M. Herrera, Automated assessment of pavlovian conditioned freezing and shock reactivity in mice using the video freeze system., Front. Behav. Neurosci. 4 (2010) 1–11.

[12] S.G. Anagnostaras, S. Maren, M.S. Fanselow, Temporally graded retrograde amnesia of contextual fear after hippocampal damage in rats: within-subjects examination., J. Neurosci. 19 (1999) 1106–14.

[13] B. Giros, M. Jaber, S.R. Jones, R.M. Wightman, M.G. Caron, Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter, Nature. 379 (1996) 606–612.

[14] B.A. Rocha, F. Fumagalli, R.R. Gainetdinov, S.R. Jones, R. Ator, B. Giros, G.W. Miller, M.G. Caron, Cocaine self-administration in dopamine-transporter knockout mice., Nat. Neurosci. 1 (1998) 132–7.

[15] R.E. Clark, S.J. Martin, Interrogating rodents regarding their object and spatial memory., Curr. Opin. Neurobiol. 15 (2005) 593–8.

[16] N.J. Broadbent, L.R. Squire, R.E. Clark, Reversible hippocampal lesions disrupt water maze performance during both recent and remote memory tests., Learn. Mem. 13 (2006) 187–91.

[17] J.E. Lisman, A.A. Grace, The hippocampal-VTA loop: controlling the entry of information into long-term memory., Neuron. 46 (2005) 703–13.

[18] J. Lisman, A.A. Grace, E. Duzel, A neoHebbian framework for episodic memory; role of dopamine-dependent late LTP., Trends Neurosci. 34 (2011) 536–47.

[19] S.G. Anagnostaras, G.G. Murphy, S.E. Hamilton, S.L. Mitchell, N.P. Rahnama, N.M. Nathanson, A.J. Silva, Selective cognitive dysfunction in acetylcholine M1 muscarinic receptor mutant mice., Nat. Neurosci. 6 (2003) 51–8.

[20] R.L. Redondo, R.G.M. Morris, Making memories last: the synaptic tagging and capture hypothesis., Nat. Rev. Neurosci. 12 (2011) 17–30.

[21] U. Frey, H. Matthies, K.G. Reymann, H. Matthies, The effect of dopaminergic D1 receptor blockade during tetanization on the expression of long-term potentiation in the rat CA1 region in vitro., Neurosci. Lett. 129 (1991) 111–4.

[22] M.S. Sonders, S.G. Amara, Channels in transporters., Curr. Opin. Neurobiol. 6 (1996) 294–302.

[23] S.L. Ingram, B.M. Prasad, S.G. Amara, Dopamine transporter-mediated conductances increase excitability of midbrain dopamine neurons., Nat. Neurosci. 5 (2002) 971–8.

[24] A.M.J. Young, R.G. Ahier, R.L. Upton, M.H. Joseph, J.A. Gray, Increased extracellular dopamine in the nucleus accumbens of the rat during associative learning of neutral stimuli, Neuroscience. 83 (1998) 1175–1183.

[25] P. Redgrave, T.J. Prescott, K. Gurney, Is the short-latency dopamine response too short to signal reward error?, Trends Neurosci. 22 (1999) 146–51.

[26] R. Spanagel, F. Weiss, The dopamine hypothesis of reward: past and current status., Trends Neurosci. 22 (1999) 521–7.

[27] N.J. Mackintosh, A theory of attention: Variations in the associability of stimuli with reinforcement., Psychol. Rev. 82 (1975) 276–298.

[28] J.M. Pearce, G. Hall, A model for Pavlovian learning: variations in the effectiveness of conditioned but not of unconditioned stimuli., Psychol. Rev. 87 (1980) 532–52.

[29] M.R. Roesch, D.J. Calu, G.R. Esber, G. Schoenbaum, Neural correlates of variations in event processing during learning in basolateral amygdala., J. Neurosci. 30 (2010) 2464–71.

[30] M.A. Gluck, R.F. Thompson, Modeling the neural substrates of associative learning and memory: a computational approach., Psychol. Rev. 94 (1987) 176–91.

[31] R. Rescorla, A. Wagner, A Theory of Pavlovian Conditioning : Variations in the Effectiveness of Reinforcement and Nonreinforcement II, in: A.H. Black, W.F. Prokasy

(Eds.), Class. Cond. II Curr. Res. Theory, Appleton-Century-Crofts, New York, 1972: pp. 64–99.

[32] C.R. Gallistel, P.D. Balsam, Time to rethink the neural mechanisms of learning and memory., Neurobiol. Learn. Mem. 108 (2014) 136–44.

[33] C.L. Hull, The problem of stimulus equivalence in behavior theory, Psychol. Rev. 46 (1939) 9.

[34] T.W. Robbins, Arousal systems and attentional, Biol. Psychol. 45 (1997) 57–71.

[35] S. Kapur, Psychosis as a state of aberrant salience: a framework linking biology, phenomenology, and pharmacology in schizophrenia, Am. J. Psychiatry. 160 (2003) 13–23.

[36] N.D. Volkow, G. Wang, J. Newcorn, J.S. Fowler, F. Telang, M. V Solanto, J. Logan, C. Wong, Y. Ma, J. Swanson, K. Schulz, K. Pradhan, Brain dopamine transporter levels in treatment and drug naïve adults with ADHD, Neuroimage. 34 (2007) 1182–1190.

[37] S.A. Carmack, S.C. Wood, S.G. Anagnostaras, Amphetamine and extinction of cued fear., Neurosci. Lett. 468 (2010) 18–22.

[38] S.A. Carmack, C.L. Block, K.K. Howell, S.G. Anagnostaras, Methylphenidate enhances acquisition and retention of spatial memory., Neurosci. Lett. 567 (2014) 45–50.

Chapter 5, in full, is currently being prepared for submission for publication of the material. Carmack SA, Scudder SL, Howell KK, Harrison EM, Patrick GN, Gu HH & Anagnostaras SG (2014). The dissertation author was the primary investigator and author of this paper.

We thank A. Mora for husbandry support. We thank members of the Anagnostaras lab for excellent technical assistance: D. Ma, C. Block, S. D'Angelo, V. Wu, L. Oueini, and M. Kullar. We thank J. Comins for thoughtful discussion. This work was supported by funding from NIH grant DA020041 (S.G.A), Hellman Fellowship (S.G.A), UCSD Chancellor's Interdisciplinary Collaboratory Grant (S.A.C.), and NSF Graduate Research Fellowships (K.K.H, S.L.S.).

Chapter 6

General Conclusions

1. Methylphenidate dose-dependently modulates long-term memory.
2. Methylphenidate's enhancement of memory is independent of its effects on anxiety, movement, and reinforcement.
3. Psychostimulants have differential effects on hippocampus-dependent versus -independent memories.
4. DAT and possibly NET mediate psychostimulant-induced memory enhancement.
5. DAT has an obligatory, rather than modulatory, role in learning and memory.
6. DAT is likely involved in a low-level, basic process such as association formation or salience attribution.

1. Methylphenidate dose-dependently modulates long-term memory.

Psychostimulant-induced memory enhancement is often interpreted as the result of reduced impulsivity and improved executive control [1]. Recently, our lab has shown that low doses of the stimulants cocaine [2], amphetamine [3], and modafinil [4] enhance the acquisition of Pavlovian fear conditioning, a leading rodent model of long-term memory with modest attentional demands [5,6], while high doses impair it. In Chapter 3 we extended this work to include the stimulant methylphenidate [7]. Acute, low doses (0.01-1 mg/kg, ip) of methylphenidate given prior to training enhanced fear memory when animals were tested off drug one week later. In contrast, high doses (10 mg/kg, ip) impaired fear memory. Fear acquisition requires brain structures distinct from those required for working memory and executive control [6,8,9]; thus, our results indicate that stimulants can enhance learning and memory directly.

In Chapter 4 we were able to generalize these findings to spatial learning in the Morris water maze [10]. 10 mg/kg methylphenidate given daily before training enhanced acquisition of the location of a hidden platform, as well as retention of the platform's location one week later (off drug). Interestingly, the 10 mg/kg dose that optimally enhanced water maze learning, impaired fear memory [7]; and the 1 mg/kg dose that

optimally enhanced fear learning only modestly enhanced retention of spatial memory. We previously observed a similar shift in the stimulant dose-response curve for modafinil, an atypical psychostimulant [11]. A much higher dose of modafinil (75 mg/kg) was required for enhancement of water maze learning compared to that required for enhancement of fear learning (0.75 mg/kg) [4]. Though water maze training involves repeated stimulant injections and fear conditioning involves a single, acute injection, additional experiments indicated that tolerance was not a likely explanation for the difference in dosing.

These findings are consistent with a large literature demonstrating that the stimulant dose optimal for enhancing memory consolidation varies by task [12]. One may speculate that the fear conditioning and water maze tasks produce different levels of activation/arousal or require different levels of catecholamine transmission for optimal learning [11,13–15]. If the water maze is itself a less arousing task than fear conditioning, this would shift the stimulant dose-response curve to the right, consistent with our findings (see [11] for a discussion of stimulant dose as a proxy for level of activation). Further, these findings highlight the need to construct dose-response curves when assessing a stimulant's effect on a behavior [11]. Stimulant actions at the synapse are dose-dependent [16–18], and the vast majority of animal studies have used doses that far exceed those that are clinically relevant [19,20]. Taken together, these experiments demonstrate that methylphenidate dose-dependently modulates associative memory, in addition to its already well-established effects on attention, working memory, and executive function [15,21–23].

2. Methylphenidate enhances memory independently of effects on anxiety, movement, and reinforcement.

As described above, stimulant effects on memory are generally considered secondary to effects on other systems [24]. For instance, one could argue that

methylphenidate enhances fear memory at low doses because it increases anxiety, or that it impairs fear memory at high doses because it's anxiolytic [25]. This hypothesis is reasonable because increased anxiety is one of the listed side effects of Concerta, a formulation of methylphenidate [26]. In Chapter 3, however, we demonstrate that neither a memory enhancing (1 mg/kg, ip) nor a memory impairing dose of methylphenidate (10 mg/kg, ip) alters anxiety on the elevated plus maze [7].

Additionally, we have consistently observed that stimulants' effects on locomotor activity are not directly related to their effects on memory (Table 1). First, we conducted our memory tests one week after training off drug to ensure that residual effects on activity did not confound our results. Second, there is no reliable pattern between memory enhancements/impairments, off drug, and locomotor hyperactivity/hypoactivity, on drug. 1 mg/kg (ip) methylphenidate enhanced both tone and context fear even though it was slightly behaviorally activating. 10 mg/kg (ip) methylphenidate greatly elevated locomotor activity and produced profound context memory deficits, but did not affect tone memory. Earlier work with cocaine clearly illustrate this point as well [2]; though 0.1 mg/kg cocaine and 15 mg/kg cocaine have opposite effects on fear memory, they stimulate locomotor activity to a similar degree (see [2] their Fig. 1a and Fig. 3).

Importantly, we were also able to dissociate methylphenidate, cocaine, and amphetamine's memory-enhancing effects from their reinforcing effects by dose. High (10 mg/kg), but not low (0.01-1 mg/kg) doses of methylphenidate induced sensitization and place preference, and impaired memory. Low, memory enhancing, doses of amphetamine and cocaine also showed little evidence of reinforcement, while high, addictive, doses impaired memory. Together, these results corroborate the notion that psychostimulant dosage explains the apparent "paradox" of cognitive enhancements in patient populations and cognitive deficits in addicts [27–31].

In the United States, stimulants are scheduled under the Controlled Substances Act because of their potential for abuse [11]. As we have shown in Chapter 3 [7], dosage

dramatically dissociates psychostimulants' pro-cognitive and reinforcing effects. Psychostimulants have dose-dependent actions at the molecular [16,32], synaptic [33,34] and neuroanatomical [35] levels. Thus, we propose that it is likely that one can develop a stimulant-like drug, which retains all of the stimulants' pro-cognitive effects, but lacks any reinforcing effects.

3. Psychostimulants have differential effects on hippocampus-dependent versus -independent memories.

While cocaine [2], amphetamine [3], modafinil [4], and methylphenidate (Chapter 3 [7]) dose-dependently enhance or impair the acquisition of fear memory, they differ with respect to which aspects of fear memory they modulate (Table 1). Both context and tone fear acquisition require the amygdala; context fear additionally requires the hippocampus [36,37]. Most work with stimulants has focused on the striatum in an effort to model addiction [19,38–40] or on the prefrontal cortex to investigate working memory and executive function [15,41,42]; relatively little work has examined stimulants and their effects in the hippocampus and amygdala [12,39,43–45].

With the exception of amphetamine, stimulants enhanced context fear at low doses. In contrast, all of the stimulants impaired context memory at high doses. It is possible that low doses of amphetamine may also enhance context fear and that Wood and colleagues [3] failed to observe an enhancement as a result of ceiling effects. The conditioning protocol used in that study elicited robust context fear; saline control animals froze approximately 70 percent of the test duration even when tested one week after conditioning (their Fig. 3a [3]); the task itself appears to have already produced optimal levels of activation [10,11]. It would be interesting to see if low doses of amphetamine enhance context memory acquisition using a minimal protocol. One theory of stimulant action posits that stimulants only have effects when the task initially produces low levels of performance or the subject has compromised catecholamine

levels [11].

From these results, it appears that stimulants reliably dose-dependently modulate hippocampus-dependent memory. Our findings that modafinil [4] and methylphenidate (Chapter 4 [10]) enhance another hippocampus-dependent task, spatial learning in the Morris water maze, corroborate our fear conditioning results. Additionally, amphetamine infused directly into the hippocampus or amygdala enhances consolidation of water maze learning [45]; and stimulants clearly act in the hippocampus at therapeutic doses [38,39,46]. For example, *in vivo* microdialysis studies in rats revealed that amphetamine and methylphenidate dose-dependently modulate norepinephrine levels in the hippocampus [38,39] and such changes are known to influence synaptic plasticity [47]. Indeed, Dommett and colleagues [44] recently demonstrated that methylphenidate alters hippocampal synaptic plasticity *in vitro*.

In contrast to context fear, low doses of methylphenidate, amphetamine, and cocaine, all classic stimulants, enhanced hippocampus-independent tone fear memory (Table 1). Consistent with this, it has been suggested that dopamine can modulate the formation of aversive associations because it modulates excitatory synaptic plasticity through local inhibitory circuits within the amygdala [43,48–50]; and stimulants enhance dopamine neurotransmission in the amygdala [51]. Recently, methylphenidate was shown to enhance learning on an amygdala-dependent cue-reward learning paradigm and to facilitate dopamine-dependent synaptic plasticity *in vitro* [43]. This does not exclude the possibility, however, that stimulants affect amygdala-dependent memory via a noradrenergic-dependent mechanism.

High doses of amphetamine and cocaine impaired tone fear memory, but surprisingly, high doses of methylphenidate and modafinil did not. One may speculate that these differences are the result of differing transporter binding affinities between the stimulants. As compared to methylphenidate and modafinil [11,33], amphetamine and cocaine have higher affinities for the serotonin transporter (SERT) [11] and increased

serotonergic neurotransmission in the amygdala is associated with impaired fear memory [52]. This would not explain, though, why modafinil and methylphenidate impair context memory, which also requires the amygdala. We would predict, however, that even higher doses of methylphenidate and modafinil would impair tone fear memory. It remains an open question as to why stimulant effects diverge when it comes to amygdala-dependent tone fear memory.

As a final point, it is important to again highlight the fact that long-term memory in fear conditioning's core neuroanatomy is distinct from that of working memory and executive control, which require the PFC [6,8,9]. In Chapter 2 we demonstrate that amphetamine (0.005 and 0.05 mg/kg, ip) does not enhance PFC-dependent fear extinction at doses capable of enhancing PFC-independent fear acquisition [53]. Research on stimulants and memory have been dominated by studies on PFC-dependent tasks; this finding points to the need for studying stimulant effects in other regions of catecholaminergic circuits.

4. DAT and possibly NET mediate psychostimulant-induced memory enhancement.

The observation that methylphenidate's memory-enhancing and reinforcing effects are dissociable based on dose led us to hypothesize that these effects may also be dissociable based on requirement for NET and/or DAT inhibition (Chapters 3 and 5). NET and DAT ratios vary across discrete brain regions and stimulants have varying affinities for the separate transporters [33–35]. Current neurobiological models of stimulants' pro-cognitive effects emphasize the critical role of enhancing dopaminergic and noradrenergic transmission through NET [35], particularly in the PFC [34,42,54,55], and strong evidence implicates affinity for DAT in stimulants' reinforcing effects [14,56].

To address whether DAT, NET, or DAT and NET were required for psychostimulant-induced memory enhancement, we first took a pharmacological

approach and assessed the effects of diverse monamine transporter inhibitors that have been used to treat ADHD on fear learning. We predicted that if stimulants exert their memory-enhancing effects via inhibition of a particular transporter, then one of the selective inhibitors should mimic the effects of the stimulants on fear learning. Atomoxetine (NET inhibitor), bupropion (low affinity DAT and NET inhibitor with additional post-synaptic effects [57]), and citalopram (SERT inhibitor) [58] all failed to enhance long-term memory across a range of doses [7]. This led us to speculate that psychostimulant-induced enhancements in long-term memory acquisition are either related to: (1) selectively binding to multiple transporter targets (see [7], Table 1), or (2) binding to DAT specifically, as we did not test a selective DAT inhibitor.

Selective action exclusively at NET does not seem to be critical for stimulants' long-term memory enhancing effects; atomextine, a potent NET inhibitor, did not mimic the memory-modulating effects characteristic of any of the doses tested. Though atomoxetine (brand name Strattera) enhances catecholamine levels in the PFC and reduces inattention and impulsivity, it is clinically inferior to amphetamine and methylphenidate in treating ADHD [57,59,60]. Further, another selective NET inhibitor, edivoxetine, recently failed clinical trials for failing to meet therapeutic efficacy endpoints [61]. It is interesting to consider whether this has something to do with selective NET inhibitors' ability to improve long-term memory.

As a final note, ADHD drug developers have avoided drugs with affinity for DAT (e.g. GBR12935, GBR12909 [43]) because of their significant abuse potential [14,56]. Increasingly, though, evidence points to action at DAT in stimulants' therapeutic effects [43,61–63]. For example, certain alleles of the DAT gene correlate with memory performance [64], as well as hyperactivity and impulsivity scores in ADHD [65]. The evidence we present here suggests that one could develop a drug that is a weak or partial agonist at DAT that retains the memory-enhancing effects typical of stimulants, with reduced abuse potential.

5. DAT has an obligatory, rather than modulatory, role in learning and memory

In order to determine if DAT is essential for the enhancement of memory by psychostimulants, we examined the effects of methylphenidate in a triple point mutant knockin mice of the gene coding DAT (DAT^{Cl} mutants) (Chapter 5) [66,67]. This mutation hinders the ability of both cocaine and methylphenidate to bind to DAT. As a result, DAT is 89-fold less sensitive to cocaine inhibition and 50-fold less sensitive to methylphenidate inhibition [66,67].

Previous behavioral work with complete DAT knockout mice was largely uninterpretable because the knockout produced dramatic locomotor hyperactivity, skeletal abnormalities, and altered body weight [64,68–71]. The knockout mice had substantial adaptive changes in dopamine homeostasis and dramatic up-regulation of the remaining monoamine transporters [64]; surprisingly, they also still self-administered and showed place preference to cocaine [71]. By contrast, DAT^{Cl} mutants do not have dramatic compensation [68,72,73]. They have wild type levels of total transporter and receptor expression and norepinephrine and serotonin levels [66].

Based on our findings in Chapter 3 [7], we predicted that DAT would have a minor, facilitatory role in learning and memory. Unexpectedly, we found that the DAT^{Cl} knockin produced severe learning and memory defects in fear conditioning, water maze, and novel object recognition. The deficits were not confounded by effects on locomotor activity, anxiety, nociception, and motivation, and were without any obvious effect on two forms of hippocampal synaptic plasticity: (1) high-frequency stimulation known to induce persistent long-term potentiation (LTP), and (2) two-theta burst stimulation, a minimal protocol thought to mimic endogenous physiological activity [75]. This result was particularly surprising because dopamine has recently been implicated in controlling both the persistence of LTP [76–78] and the persistence of memory [79].

It is difficult to reconcile our finding that a highly selective mutation of the DAT produces a profound memory deficit, without any obvious effects on hippocampal LTP,

with the traditional understanding of how dopamine works to modulate memory at the synaptic level. The DAT^{Cl} knockin reduces transporter efficiency [66], most likely because the binding sites for dopamine (DA), methylphenidate, and cocaine partially overlap [74]. As a result, the mutants have increased synaptic levels of dopamine. It is possible that the DAT^{Cl} knockin produced abnormal post-synaptic dopaminergic signaling; recent evidence suggests that DAT^{Cl} mutants have abnormal dopamine D1 receptor responses following phasic dopamine signaling [80]. D1 receptors directly interact with N-methyl-D-aspartate glutamate (NMDA) receptors [81] and NMDA receptors are the cornerstones of synaptic models of memory [82].

It could also be the case that DAT serves a critical pre-synaptic function in learning and memory [70,83]. DAT itself can function like a channel and elicit a significant pre-synaptic current [83,84]. Intriguingly, amphetamine, a DAT substrate, can induce a current through DAT and increase dopamine cell excitability; further, cocaine can block this effect [84]. Both stimulant actions at DAT and the DAT^{Cl} knockin could affect memory by modifying DAT's channel-like properties. Further work needs to be done investigating the possibility that the channel action at DAT is critical for memory and will require a different approach to physiology.

In sum, monoaminergic transmitters are traditionally considered modulatory, rather than necessary, for learning and memory [85,86]; one view posits that monoamine projections are too diffuse and sparse, compared to glutamate synapses, to represent a critical learning and memory mechanism [24]. Our findings suggest that DAT plays more than just a modulatory role. The memory impairments we observed are as striking those produced by deletion of CaMKII, a molecule now known to be obligatory for memory [87,88].

6. DAT is likely involved in a low-level, basic process like association formation or salience attribution.

A necessary function in associative learning is deciding which information to encode into memory in order to avoid exceeding memory capacity [89]. Formal learning theories posit that there is an “associative window” that must first be opened to enable memory acquisition [90–95]. It is argued that this mechanism limits learning to biologically important events [96,97], and that without it, an animal would exceed memory capacity. Processes like salience detection and arousal may drive attention toward biologically significant events [98] and trigger the opening of the “associative window” so that events may be encoded.

The role of DAT, then, may be to enable a low-level assignment of salience by novelty detection [51,99]. Indeed, dopamine neurons respond maximally to novel stimuli [51,97,100]. The DAT^{CI} mutants may have failed to learn because the knockin interfered with salience detection, rendering them incapable of orienting and attending to important events. In other words, consistent with optimal arousal theory, DAT^{CI} mutants may not have been aroused enough to learn [11].

Conversely, low doses of stimulants may enhance memory by binding to DAT and artificially opening the window [78]. This idea is supported by the observation that stimulants disrupt latent inhibition [96] and facilitate learning in sub-optimal learning conditions [101]. One could also speculate that high doses of stimulants impair memory by preventing the closure of the window [11]. Current theories on the biological bases of memory posit that the classic neuromodulators, dopamine and norepinephrine, exert top-down control over memory based on factors like motivation and reward [102–104]. The data we present here suggest that DAT may additionally have a low-level, basic role in memory.

Table 6.1 Psychostimulants and their effects on fear memory acquisition and activity. Generally, low doses of stimulants are associated with enhancements in fear memory, while high doses are associated with impairments. ↑, enhanced memory or increased activity. ↓, impaired memory or decreased activity. -, no effect.

			Memory	
Psychostimulant	**Dose**	**Activity**	**Context**	**Tone**
Methylphenidate[7]	Low	-	↑	↑
	High	↑	↓	-
d-Amphetamine[3]	Low	-	-	↑
	High	↑	↓	↓
Cocaine[2]	Low	↑	↑	↑
	High	↑	↓	↓
Modafinil[4]	Low	↓	↑	-
	High	-	↓	-

References

[1] R.A. Barkley, Behavioral inhibition, sustained attention, and executive functions: constructing a unifying theory of ADHD., Psychol. Bull. 121 (1997) 65–94.

[2] S.C. Wood, J. Fay, J.R. Sage, S.G. Anagnostaras, Cocaine and Pavlovian fear conditioning: dose-effect analysis., Behav. Brain Res. 176 (2007) 244–50.

[3] S.C. Wood, S.G. Anagnostaras, Memory and psychostimulants: modulation of Pavlovian fear conditioning by amphetamine in C57BL/6 mice., Psychopharmacology (Berl). 202 (2009) 197–206.

[4] T. Shuman, S.C. Wood, S.G. Anagnostaras, Modafinil and Memory: Effects of Modafinil on Morris Water Maze Learning and Pavlovian Fear Conditioning, Behav. Neurosci. 123 (2009) 257–266.

[5] S.G. Anagnostaras, S.C. Wood, T. Shuman, D.J. Cai, A.D. Leduc, K.R. Zurn, J.R. Sage, G.M. Herrera, Automated assessment of pavlovian conditioned freezing and shock reactivity in mice using the video freeze system., Front. Behav. Neurosci. 4 (2010) 1–11.

[6] S. Maren, Pavlovian fear conditioning as a behavioral assay for hippocampus and amygdala function: cautions and caveats., Eur. J. Neurosci. 28 (2008) 1661–6.

[7] S.A. Carmack, K.K. Howell, K. Rasaei, E.T. Reas, S.G. Anagnostaras, Animal model of methylphenidate's long-term memory-enhancing effects, Learn. Mem. 21 (2014) 82–89.

[8] T.S. Braver, D.M. Barch, W.M. Kelley, R.L. Buckner, N.J. Cohen, F.M. Miezin, A.Z. Snyder, J.M. Ollinger, E. Akbudak, T.E. Conturo, S.E. Petersen, Direct comparison of prefrontal cortex regions engaged by working and long-term memory tasks., Neuroimage. 14 (2001) 48–59.

[9] M.A. Morgan, J.E. LeDoux, Differential contribution of dorsal and ventral medial prefrontal cortex to the acquisition and extinction of conditioned fear in rats., Behav. Neurosci. 109 (1995) 681–8.

[10] S.A. Carmack, C.L. Block, K.K. Howell, S.G. Anagnostaras, Methylphenidate enhances acquisition and retention of spatial memory., Neurosci. Lett. 567 (2014) 45–50.

[11] S.C. Wood, J.R. Sage, T. Shuman, S.G. Anagnostaras, Psychostimulants and Cognition: A Continuum of Behavioral and Cognitive Activation, Pharmacol. Rev. 66 (2014) 193–221.

[12] J.L. McGaugh, B. Roozendaal, Drug enhancement of memory consolidation: historical perspective and neurobiological implications., Psychopharmacology (Berl). 202 (2009) 3–14.

[13] P.L. Clatworthy, S.J.G. Lewis, L. Brichard, Y.T. Hong, D. Izquierdo, L. Clark, R. Cools, F.I. Aigbirhio, J. Baron, T.D. Fryer, T.W. Robbins, Dopamine release in dissociable striatal subregions predicts the different effects of oral methylphenidate on reversal learning and spatial working memory., J. Neurosci. 29 (2009) 4690–6.

[14] N.D. Volkow, G.J. Wang, J.S. Fowler, J. Logan, S.J. Gatley, C. Wong, R. Hitzemann, N.R. Pappas, Reinforcing effects of psychostimulants in humans are associated with increases in brain dopamine and occupancy of D(2) receptors., J. Pharmacol. Exp. Ther. 291 (1999) 409–15.

[15] S. Vijayraghavan, M. Wang, S.G. Birnbaum, G. V Williams, A.F.T. Arnsten, Inverted-U dopamine D1 receptor actions on prefrontal neurons engaged in working memory., Nat. Neurosci. 10 (2007) 376–84.

[16] E.S. Calipari, M.J. Ferris, Amphetamine mechanisms and actions at the dopamine terminal revisited., J. Neurosci. 33 (2013) 8923–5.

[17] P. Seeman, B.K. Madras, Anti-hyperactivity medication: methylphenidate and amphetamine, Mol. Psychiatry. 3 (1998) 386–396.

[18] R. Kuczenski, D.S. Segal, Stimulant actions in rodents: implications for attention-deficit/ hyperactivity disorder treatment and potential substance abuse., Biol. Psychiatry. 57 (2005) 1391–6.

[19] R. Kuczenski, D.S. Segal, Locomotor effects of acute and repeated threshold doses of amphetamine and methylphenidate: relative roles of dopamine and norepinephrine., J. Pharmacol. Exp. Ther. 296 (2001) 876–83.

[20] R.R. Gainetdinov, W.C. Wetsel, S.R. Jones, E.D. Levin, M. Jaber, M.G. Caron, Role of Serotonin in the Paradoxical Calming Effect of Psychostimulants on Hyperactivity, Science (80-.). 283 (1999) 397–401.

[21] C.W. Berridge, D.M. Devilbiss, M.E. Andrzejewski, A.F.T. Arnsten, A.E. Kelley, B. Schmeichel, C. Hamilton, R.C. Spencer, Methylphenidate preferentially increases catecholamine neurotransmission within the prefrontal cortex at low doses that enhance cognitive function., Biol. Psychiatrysychiatry. 60 (2006) 1111–20.

[22] S. Kempton, A. Vance, P. Maruff, E. Luk, J. Costin, C. Pantelis, Executive function and attention deficit hyperactivity disorder: stimulant medication and better executive function performance in children., Psychol. Med. 29 (1999) 527–38.

[23] M.A. Mehta, I.M. Goodyer, B.J. Sahakian, Methylphenidate improves working memory and set-shifting in AD/HD: relationships to baseline memory capacity., J. Child Psychol. Psychiatry. 45 (2004) 293–305.

[24] G. Lynch, L.C. Palmer, C.M. Gall, The likelihood of cognitive enhancement., Pharmacol. Biochem. Behav. 99 (2011) 116–29.

[25] D.M. Bannerman, J.N.P. Rawlins, S.B. McHugh, R.M.J. Deacon, B.K. Yee, T. Bast, W.N. Zhang, H.H.J. Pothuizen, J. Feldon, Regional dissociations within the hippocampus--memory and anxiety., Neurosci. Biobehav. Rev. 28 (2004) 273–83.

[26] McNeil Pediatrics, Concerta: Full Prescribing Information. Retrieved from:, (2008) 1–30.

[27] S. T, S.L. Simon, C.P. Domier, K. Richardson, R. Rawson, W. Ling, Cognitive deficits among methamphetamine users with attention deficit hyperactivity disorder symptomology., J. Addict. Disord. 21 (2002) 75–89.

[28] R.D. Rogers, B.J. Everitt, A. Baldacchino, A.J. Blackshaw, R. Swainson, K. Wynne, N.B. Baker, J. Hunter, T. Carthy, E. Booker, M. London, J.F.W. Deakin, B.J. Sahakian, T.W. Robbins, Dissociable deficits in the decision-making cognition of chronic amphetamine abusers, opiate abusers, patients with focal damage to prefrontal cortex, and tryptophan-depleted normal volunteers: evidence for monoaminergic mechanisms., Neuropsychopharmacology. 20 (1999) 322–39.

[29] S.M. Rhodes, J. Park, S. Seth, D.R. Coghill, A comprehensive investigation of memory impairment in attention deficit hyperactivity disorder and oppositional defiant disorder., J. Child Psychol. Psychiatry. 53 (2012) 128–37.

[30] M. Nathanson, New adrenalin-like compounds: their action and therapeutic application, Cal. West. Med. 57 (1942) 301–307.

[31] W. Sargant, J.M. Blackburn, The effect of benzedrine on intelligence scores, Lancet. 228 (1936) 1385–1387.

[32] S.D. Robertson, H.J.G. Matthies, A. Galli, A closer look at amphetamine-induced reverse transport and trafficking of the dopamine and norepinephrine transporters., Mol. Neurobiol. 39 (2009) 73–80.

[33] E. Richelson, M. Pfenning, Blockade by antidepressants and related compounds of biogenic amine uptake into rat brain synaptosomes: most antidepressants selectively block norepinephrine uptake, Eur. J. Pharmacol. 104 (1984) 277–286.

[34] B.K. Madras, G.M. Miller, A.J. Fischman, The dopamine transporter and attention-deficit/hyperactivity disorder., Biol. Psychiatry. 57 (2005) 1397–409.

[35] J.A. Morón, A. Brockington, R.A. Wise, B.A. Rocha, B.T. Hope, Dopamine uptake through the norepinephrine transporter in brain regions with low levels of the dopamine transporter: evidence from knock-out mouse lines., J. Neurosci. 22 (2002) 389–95.

[36] S.G. Anagnostaras, S. Maren, M.S. Fanselow, Temporally graded retrograde amnesia of contextual fear after hippocampal damage in rats: within-subjects examination., J. Neurosci. 19 (1999) 1106–14.

[37] G.D. Gale, S.G. Anagnostaras, B.P. Godsil, S. Mitchell, T. Nozawa, J.R. Sage, B. Wiltgen, M.S. Fanselow, Role of the basolateral amygdala in the storage of fear memories across the adult lifetime of rats., J. Neurosci. 24 (2004) 3810–5.

[38] R. Kuczenski, D.S. Segal, Effects of methylphenidate on extracellular dopamine, serotonin, and norepinephrine: comparison with amphetamine., J. Neurochem. 68 (1997) 2032–7.

[39] R. Kuczenski, D.S. Segal, Exposure of adolescent rats to oral methylphenidate: preferential effects on extracellular norepinephrine and absence of sensitization and cross-sensitization to methamphetamine., J. Neurosci. 22 (2002) 7264–71.

[40] A.M.J. Young, R.G. Ahier, R.L. Upton, M.H. Joseph, J.A. Gray, Increased extracellular dopamine in the nucleus accumbens of the rat during associative learning of neutral stimuli, Neuroscience. 83 (1998) 1175–1183.

[41] C.W. Berridge, J.S. Shumsky, M.E. Andrzejewski, J.A. McGaughy, R.C. Spencer, D.M. Devilbiss, B.D. Waterhouse., Differential Sensitivity to Psychostimulants Across Prefrontal Cognitive Tasks: Differential Involvement of Noradrenergic alpha1- and alpha2-Receptors, Biol. Psychiatry. 71 (2012) 467–473.

[42] A.F. Arnsten, A.G. Dudley, Methylphenidate improves prefrontal cortical cognitive function through alpha2 adrenoceptor and dopamine D1 receptor actions: Relevance to therapeutic effects in Attention Deficit Hyperactivity Disorder., Behav. Brain Funct. 1 (2005) 2.

[43] K.M. Tye, L.D. Tye, J.J. Cone, E.F. Hekkelman, P.H. Janak, A. Bonci, Methylphenidate facilitates learning-induced amygdala plasticity, Nat. Neurosci. 13 (2010) 475–481.

[44] E.J. Dommett, E.L. Henderson, M.S. Westwell, S.A. Greenfield, Methylphenidate amplifies long-term plasticity in the hippocampus via noradrenergic mechanisms., Learn. Mem. 15 (2008) 580–6.

[45] M.G. Packard, J.L. Mcgaugh, Quinpirole and d-amphetamine administration posttraining enhances memory on spatial and cued discriminations in a water maze, Psychobiology. 22 (1994) 54–60.

[46] T.M. Engber, S.A. Dennis, B.E. Jones, M.S. Miller, P.C. Contreras, Brain regional substrates for the actions of the novel wake-promoting agent modafinil in the rat: comparison with amphetamine, Neuroscience. 87 (1998) 905–11.

[47] I. Akirav, G. Richter-Levin, Mechanisms of amygdala modulation of hippocampal plasticity., J. Neurosci. 22 (2002) 9912–21.

[48] J.A. Rosenkranz, A.A. Grace, Dopamine-mediated modulation of odour-evoked amygdala potentials during pavlovian conditioning., Nature. 417 (2002) 282–7.

[49] T. Kienast, A.R. Hariri, F. Schlagenhauf, J. Wrase, P. Sterzer, H.G. Buchholz, M.N.Smolka, G. Grunder, P. Cumming, Y. Kumakura, P. Bartenstein, R.J. Dolan, A. Heinz, Dopamine in amygdala gates limbic processing of aversive stimuli in humans., Nat. Neurosci. 11 (2008) 1381–2.

[50] I. Ehrlich, Y. Humeau, F. Grenier, S. Ciocchi, C. Herry, A. Lüthi, Amygdala inhibitory circuits and the control of fear memory., Neuron. 62 (2009) 757–71.

[51] W. Schultz, Multiple dopamine functions at different time courses., Annu. Rev. Neurosci. 30 (2007) 259–88.

[52] T. Inoue, X. Bai Li, T. Abekawa, Y. Kitaichi, T. Izumi, S. Nakagawa, T. Koyama, Selective serotonin reuptake inhibitor reduces conditioned fear through its effect in the amygdala, Eur. J. Pharmacol. 497 (2004) 311–316.

[53] S.A. Carmack, S.C. Wood, S.G. Anagnostaras, Amphetamine and extinction of cued fear., Neurosci. Lett. 468 (2010) 18–22.

[54] A.F.T. Arnsten, Toward a new understanding of attention-deficit hyperactivity disorder pathophysiology: an important role for prefrontal cortex dysfunction., CNS Drugs. 23 Suppl 1 (2009) 33–41.

[55] T. Spencer, J. Biederman, T. Wilens, M. Harding, D. O'Donnell, S. Griffin, Pharmacotherapy of attention-deficit hyperactivity disorder across the life cycle., J. Am. Acad. Child Adolesc. Psychiatry. 35 (1996) 409–32.

[56] N. Volkow, Role of Dopamine, the Frontal Cortex and Memory Circuits in Drug Addiction: Insight from Imaging Studies, Neurobiol. Learn. Mem. 78 (2002) 610–624.

[57] S. V Faraone, J. Buitelaar, Comparing the efficacy of stimulants for ADHD in children and adolescents using meta-analysis., Eur. Child Adolesc. Psychiatry. 19 (2010) 353–64.

[58] K.C.F. Fone, D.J. Nutt, Stimulants: use and abuse in the treatment of attention deficit hyperactivity disorder., Curr. Opin. Pharmacol. 5 (2005) 87–93.

[59] J.H. Newcorn, C.J. Kratochvil, A.J. Allen, D. Ph, C.D. Casat, D.D. Ruff, R.J. Moore, D. Michelson, Atomoxetine and Osmotically Released Methylphenidate for the Treatment of Attention Deficit Hyperactivity Disorder : Acute Comparison and Differential Response, Am. J. Psychiatry. 165 (2008) 721–730.

[60] S.B. Wigal, J.J. McGough, J.T. MdcCracken, J. Biederman, T.J. Spencer, K.L. Posner, T.L. Wigal, S.H. Kollins, T.M. Clark, D.A. Mays, Y. Zhang, S.J. Tulloch, A laboratory school comparison of mixed amphetamine salts extended release (Adderall XR) and atomoxetine (Strattera) in school-aged children with attention deficit/hyperactivity disorder., J. Atten. Disord. 9 (2005) 275–89.

[61] J.S. Markowitz, B.J. Brinda, A pharmacokinetic evaluation of oral edivoxetine hydrochloride for the treatment of attention deficit-hyperactivity disorder., Expert Opin. Drug Metab. Toxicol. (2014) 1–11.

[62] J. Swanson, R.D. Baler, N.D. Volkow, Understanding the effects of stimulant medications on cognition in individuals with attention-deficit hyperactivity disorder: a decade of progress., Neuropsychopharmacology. 36 (2011) 207–26.

[63] N.D. Volkow, G. Wang, J. Newcorn, J.S. Fowler, F. Telang, M. V Solanto, J. Logan, C. Wong, Y. Ma, J.M. Swanson, K. Schulz, K. Pradhan, Brain dopamine transporter levels in treatment and drug naïve adults with ADHD, Neuroimage. 34 (2007) 1182–1190.

[64] G.E. Torres, R.R. Gainetdinov, M.G. Caron, Plasma membrane monoamine transporters: structure, regulation, and function, Nat. Rev. Neurosci. 4 (2003) 13–25.

[65] I.D. Waldman, D.C. Rowe, a Abramowitz, S.T. Kozel, J.H. Mohr, S.L. Sherman, H.H. Cleveland, M.L. Sanders, J.M.C. Gard, C. Stever, Association and linkage of the dopamine transporter gene and attention-deficit hyperactivity disorder in children: heterogeneity owing to diagnostic subtype and severity., Am. J. Hum. Genet. 63 (1998) 1767–76.

[66] R. Chen, M.R. Tilley, H. Wei, F. Zhou, F.-M. Zhou, S. Ching, N. Quan, R.L. Stephens, E.R. Hill, T. Nottoli, D.D. Han, H.H. Gu, Abolished cocaine reward in mice with a cocaine-insensitive dopamine transporter., Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 9333–8.

[67] M.R. Tilley, H.H. Gu, The Effects of Methylphenidate on Knockin Mice with a Methylphenidate-Resistant Dopamine Transporter, 327 (2008) 554–560.

[68] R. Chen, M.R. Tilley, H. Wei, F. Zhou, F.-M. Zhou, S. Ching, N. Quan, R.L. Stephens, E.R. Hill, T. Nottoli, D.D. Han, H.H. Gu, Abolished cocaine reward in mice with a cocaine-insensitive dopamine transporter., Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 9333–8.

[69] B. Giros, M. Jaber, S.R. Jones, R.M. Wightman, M.G. Caron, Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter, Nature. 379 (1996) 606–612.

[70] S.R. Jones, R.R. Gainetdinov, M. Jaber, B. Giros, R.M. Wightman, M.G. Caron, Profound neuronal plasticity in response to inactivation of the dopamine transporter., Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 4029–34.

[71] B.A. Rocha, F. Fumagalli, R.R. Gainetdinov, S.R. Jones, R. Ator, B. Giros, G.W. Miller, M.G. Caron, Cocaine self-administration in dopamine-transporter knockout mice., Nat. Neurosci. 1 (1998) 132–7.

[72] F. Napolitano, A. Bonito-Oliva, M. Federici, M. Carta, F. Errico, S. Magara, G. Martella, R. Nistico, D. Centonze, A. Pisani, H.H. Gu, N.B. Mercuri, A. Usiello, Role of aberrant striatal

dopamine D1 receptor/cAMP/protein kinase A/DARPP32 signaling in the paradoxical calming effect of amphetamine., J. Neurosci. 30 (2010) 11043–56.

[73] F. Xu, R.R. Gainetdinov, W.C. Wetsel, S.R. Jones, L.M. Bohn, G.W. Miller, Y.M. Wang, M.G. Caron, Mice lacking the norepinephrine transporter are supersensitive to psychostimulants., Nat. Neurosci. 3 (2000) 465–71.

[74] T. Beuming, J. Kniazeff, M.L. Bergmann, L. Shi, L. Gracia, K. Raniszewska, A.H. Newman, J.A. Javitch, H. Weinstein, U. Gether, C.J. Loland, The binding sites for cocaine and dopamine in the dopamine transporter overlap., Nat. Neurosci. 11 (2008) 780–9.

[75] S.G. Anagnostaras, G.G. Murphy, S.E. Hamilton, S.L. Mitchell, N.P. Rahnama, N.M. Nathanson, A.J. Silva, Selective cognitive dysfunction in acetylcholine M1 muscarinic receptor mutant mice., Nat. Neurosci. 6 (2003) 51–8.

[76] U. Frey, H. Matthies, K.G. Reymann, The effect of dopaminergic D1 receptor blockade during tetanization on the expression of long-term potentiation in the rat CA1 region in vitro., Neurosci. Lett. 129 (1991) 111–4.

[77] J. Lisman, A.A. Grace, E. Duzel, A neoHebbian framework for episodic memory; role of dopamine-dependent late LTP., Trends Neurosci. 34 (2011) 536–47.

[78] R.L. Redondo, R.G.M. Morris, Making memories last: the synaptic tagging and capture hypothesis., Nat. Rev. Neurosci. 12 (2011) 17–30.

[79] J.I. Rossato, L.R.M. Bevilaqua, I. Izquierdo, J.H. Medina, M. Cammarota, Dopamine controls persistence of long-term memory storage., Science. 325 (2009) 1017–20.

[80] F. Napolitano, A. Bonito-Oliva, M. Federici, M. Carta, F. Errico, S. Magara, Martella, R. Nistico, D. Centonze, A. Pisani, H.H. Gu, N.B. Mercuri, A. Usiello, Role of aberrant striatal dopamine D1 receptor/cAMP/protein kinase A/DARPP32 signaling in the paradoxical calming effect of amphetamine., J. Neurosci. 30 (2010) 11043–56.

[81] F.J.S. Lee, S. Zue, L. Pei, B. Vukusic, N. Chery, Y. Wang, Y. Wang, H.B. Niznik, X. Yu, F. Liu, Dual regulation of NMDA receptor functions by direct protein-protein interactions with the dopamine D1 receptor, Cell. 111 (2002) 219–230.

[82] A. Citri, R.C. Malenka, Synaptic plasticity: multiple forms, functions, and mechanisms., Neuropsychopharmacology. 33 (2008) 18–41.

[83] M.S. Sonders, S.G. Amara, Channels in transporters., Curr. Opin. Neurobiol. 6 (1996) 294–302.

[84] S.L. Ingram, B.M. Prasad, S.G. Amara, Dopamine transporter-mediated conductances increase excitability of midbrain dopamine neurons., Nat. Neurosci. 5 (2002) 971–8.

[85] E.R. Kandel, Y. Dudai, M.R. Mayford, The molecular and systems biology of memory., Cell. 157 (2014) 163–86.

[86] P.S. Goldman-Rakic, Regional and cellular fractionation of working memory., Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 13473–80.

[87] A.J. Silva, R. Paylor, J.M. Wehner, S. Tonegawa, Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice., Science (80-.). 257 (1992) 206–11.

[88] A.J. Silva, C.F. Stevens, S. Tonegawa, Y. Wang, Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice., Science (80-.). 257 (1992) 201–6.

[89] C.L. Hull, The problem of stimulus equivalence in behavior thoeyr, Psychol. Rev. 46 (1939) 9.

[90] N.J. Mackintosh, A theory of attention: Variations in the associability of stimuli with reinforcement., Psychol. Rev. 82 (1975) 276–298.

[91] J.M. Pearce, G. Hall, A model for Pavlovian learning: variations in the effectiveness of conditioned but not of unconditioned stimuli., Psychol. Rev. 87 (1980) 532–52.

[92] M.R. Roesch, D.J. Calu, G.R. Esber, G. Schoenbaum, Neural correlates of variations in event processing during learning in basolateral amygdala., J. Neurosci. 30 (2010) 2464–71.

[93] M.A. Gluck, R.F. Thompson, Modeling the neural substrates of associative learning and memory: a computational approach., Psychol. Rev. 94 (1987) 176–91.

[94] R. Rescorla, A. Wagner, A Theory of Pavlovian Conditioning : Variations in the Effectiveness of Reinforcement and Nonreinforcement IIT, in: A.H. Black, W.F. Prokasy (Eds.), Class. Cond. II Curr. Res. Theory, Appleton-Century-Crofts, New York, 1972: pp. 64–99.

[95] C.R. Gallistel, P.D. Balsam, Time to rethink the neural mechanisms of learning and memory., Neurobiol. Learn. Mem. 108 (2014) 136–44.

[96] P. Redgrave, T.J. Prescott, K. Gurney, Is the short-latency dopamine response too short to signal reward error?, Trends Neurosci. 22 (1999) 146–51.

[97] R. Spanagel, F. Weiss, The dopamine hypothesis of reward: past and current status., Trends Neurosci. 22 (1999) 521–7.

[98] G. Aston-Jones, C. Chiang, T. Alexinsky, Discharge of noradrenergic locus coeruleus neurons in behaving rats and monkeys suggests a role in vigilance., Prog. Brain Res. 88 (1991) 501–20.

[99] J.E. Lisman, A.A. Grace, The hippocampal-VTA loop: controlling the entry of information into long-term memory., Neuron. 46 (2005) 703–13.

[100] L.R. Halladay, S.D. Iñiguez, F. Furqan, M.C. Previte, A.M. Chisum, C.A. Crawford, Methylphenidate potentiates morphine-induced antinociception, hyperthermia, and locomotor activity in young adult rats, Pharmacol. Biochem. Behav. 92 (2009) 190–196.

[101] P.W. Frankland, S.A. Josselyn, S.G. Anagnostaras, J.H. Kogan, E. Takahashi, A.J. Silva, Consolidation of CS and US representations in associative fear conditioning., Hippocampus. 14 (2004) 557–69.

[102] J.D. Berke, S.E. Hyman, Addiction, dopamine, and the molecular mechanisms of memory., Neuron. 25 (2000) 515–32.

[103] P. Waelti, A. Dickinson, W. Schultz, Dopamine responses comply with basic assumptions of formal learning theory., Nature. 412 (2001) 43–8.

[104] C.W. Berridge, D.M. Devilbiss, Psychostimulants as Cognitive Enhancers: The Prefrontal Cortex, Catecholamines and Attention Deficit Hyperactivity Disorder, Biol. Psychiatry. 69 (2011) e101–e111.