THE ROLE OF LATERODORSAL TEGMENTAL ACETYLCHOLINE IN NEUROCHEMICAL AND BEHAVIORAL MEASURES OF METHAMPHETAMINE REWARD

By

Lauren K. Dobbs

A DISSERTATION

Presented to the Department of Behavioral Neuroscience
and the Oregon Health & Science University School of Medicine
in partial fulfillment of
the requirements for the degree of Doctor of Philosophy

June 2012

Department of Behavioral Neuroscience

School of Medicine

Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. dissertation of

Lauren K. Dobbs

has been approved

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Mentor

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Co-Mentor

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Member

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Member

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LIST OF ABBREVIATIONS

3V	Third ventricle
5-HT	5-Hydroxytryptamine (serotonin)
ACh	Acetylcholine
AChE	Acetylcholinesterase
aCSF	Artificial cereberospinofluid
AMPA	Alpha-amino-hydroxy-methyl-isoxazolepropionate
Ca^{++}	Calcium
CA1	CA1 region of the hippocampus
CA3	CA3 region of the hippocampus
ChAT	Choline acetyltransferase
CPA	Conditioned place aversion
CPG	Central pattern generator
CPP	Conditioned place preference
CS	Conditioned stimulus
CS-	Conditioned stimulus negative
CS+	Conditoned stimulus positive
CTA	Condtioned taste aversion
d4 ACh	Deuterated acetylcholine
DA	Dopamine
DAT	Dopamine transporter
DG	Dentate gyrus
DHβE	Dihydro-beta-erythroidine
DOPAC	3,4-dihydroxyphenylacetic acid
DR	Dorsal raphe
FR	Fixed ratio
fr	Fasiculus retroflexus
GABA	γ-aminobutyric acid
HPLC	High perfomance liquid chromatography
IC	Inferior colliculus
ICSS	Intra-cranial self-stimulation
ICV	Intracerebroventricular
IHC	Immunohistochemistry
IP	Intraperitoneal
LC	Locus coeruleus
LC-MS/MS	Liquid-chromatography tandem mass spectrometry
$_{L}$-Dopa	Levodopa
LDT	Laterodorsal tegmentum
LTB	Low-threshold bursting

LIST OF ABBREVIATIONS

LTP	Long-term potentiation
LV	Lateral ventricle
mAChR	Muscarinic acetylcholine receptor
MAHDR	Methamphetamine High Drinking
MALDR	Methamphetamine Low Drinking
MAO	Monoamine oxidase
METH	Methamphetamine
$mGlu_{2/3/5}$	Metabotropic glutamate (group 2, 3, or 5) receptor
ml	Medial lemniscus
MLR	Mesencephalic locomotor region
mPFC	Medial prefrontal cortex
mRF	Medial reticular formation
NAc	Nucleus accumbens
nAChR	Nicotinic acetylcholine receptor
NE	Norepinephrine
NMDA	N-methyl-D-aspartate
OXO	Oxotremorine sesquifumarate
PBS	Phosphate buffered saline
PE	Polyethylene
PPT	Pedunculopontine tegmentum
PR	Progressive ratio
PRF	Pontine reticular formation
REM	Rapid eye movement
SN	Substantia nigra
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
TH	Tyrosine hydroxylase
THP	Trihexyphenidyl
TTX	Tetrodotoxin
UR-II	Urotensin II receptor
US	Unconditioned stimulus
VMAT-2	Vesicular monoamine transporter-2
VP	Ventral pallidum
VTA	Ventral tegmental area

ACKNOWLEDGMENTS

As I sit down to write the “acknowledgments” section, I find the task of thanking the key individuals who contributed to my development as a scientist to be rather daunting. Over the last six years, I have had the wonderful opportunity to work with several outstanding scientists. Moreover, these individuals have made the completion of my dissertation an enjoyable process. First, I would like to thank Greg Mark, for mentoring me in my first years as a graduate student. Thank you for sharing your knowledge about operant behavior, microdialysis and the cholinergic system. I enjoyed my time in your lab and truly wish you the best.

I would also like to thank Chris Cunningham, my mentor over the last two years, who agreed to undertake a senior student in a bit of a bind. I feel I have learned more in the last two years under your tutelage than I have in a lifetime. I sincerely appreciate how you challenged me and provided honest evaluations of my work. Thank you for sharing your knowledge, sense of humor and M&Ms over the last two years.

The work in my dissertation would definitely not have been possible without the valuable feedback from my committee. Thank you for attending the multitude of meetings and reading this gargantuan document. Thank you, Charlie Meshul, for being my co-mentor and sharing your seemingly inexhaustible knowledge of neurocircuitry and your excitement for science. Thank you Kathy Grant for asking thoughtful questions about my behavioral experiments and making me think about my research from different points of view. The intravenous self-administration experiments would not have been possible without the excellent guidance from Amanda Roberts and her laboratory staff. Finally, thank you, Tamara Phillips, for providing guidance during my first year as a

graduate student and then coming back onto my committee as the “ad hoc” member. I cannot think of anyone I would rather have to “bookend” my graduate career.

Additionally, I would like to thank Tammie Painter and Mireille LaFont for their assistance in the microdialysis and self-administration experiments and for just being awesome. In addition, I would like to thank Jenny Luo and Dr. Dennis Koop for the use of their tandem mass spectrometer and their assistance with the associated sample analysis for the microdialysis experiments.

Finally, I do not think I could have completed the last six years with my sanity intact without the loving support of my husband, Jarod Horn. Thank you for encouraging me and laughing with me throughout this journey.

The experiments in this dissertation were funded by the NIDA Methamphetamine Research Center grant (P50 DA018165), DA014639, a grant from the American Psychological Association, and a Tartar Foundation Fellowship. L.K.D. was supported by T32 DA07262, a Pre-doctoral NRSA (DA27295) and a scholarship from the Achievement Rewards for College Scientists (ARCS). I would especially like to thank Caron and Larry Ogg for donating the scholarship and encouraging my scientific endeavors.

ABSTRACT

Methamphetamine (METH) reward is, in part, mediated by the ability of METH to release dopamine (DA) within the nucleus accumbens (NAc). The DA cell bodies located in the ventral tegmental area (VTA) receive acetylcholine (ACh) projections from the laterodorsal (LDT) and pedunculopontine tegmental nuclei (PPT). These ACh projections mediate DA cell firing, DA release, reward and psychostimulant locomotor activity and stereotypy. However, it is unknown if METH acts through the LDT or PPT to induce neurochemical changes or reward. Therefore, experiments sought to characterize the role of the LDT and PPT in METH-induced levels of ACh and DA within the mesolimbic pathway and determine if mesopontine ACh projections are important for METH reward.

Chapter 2. In these experiments, *in vivo* microdialysis in the mouse was used to test METH's effects on ACh and somatodendritic DA levels within the VTA. Following a systemic METH injection, or an intra-VTA perfusion of METH, DA levels were significantly increased in the VTA. Systemic METH injection induced a significant increase in ACh which persisted for 2 to 3 h post injection. However, intra-VTA METH perfusion did not affect VTA ACh levels. These experiments suggest that METH is acting outside of the VTA to induce increases in extracellular ACh levels within the VTA.

Chapter 3. The aim of these experiments was to characterize the role of PPT- and LDT-derived ACh in METH–induced (1) ACh levels within the VTA, (2) DA levels within the NAc, and (3) locomotor activity. Reversible inhibition of the LDT ACh neurons via microinjection of the M2-type preferring receptor agonist oxotremorine sesquifumarate (OXO) significantly inhibited locomotor activity following an intraperitoneal (IP) injection

of METH or saline. Intra-LDT OXO microinjections dose-dependently attenuated METH-induced increases in ACh within the VTA, but had no effect on DA levels within the NAc. Conversely, intra-PPT OXO microinjections had no effect on METH-induced ACh levels in the VTA or DA levels in the NAc.

Chapter 4. These experiments examined the contribution of the LDT cholinergic neurons in METH reward, as measured by conditioned place preference (CPP). Bilateral electrolytic lesions of the LDT significantly decreased the number of LDT ACh neurons compared to sham operated controls. LDT lesion was negatively correlated with locomotor activity, but had no effect on the expression or acquisition of METH preference. In addition, there was no effect of LDT lesion on the extinction or reconditioning of METH CPP.

These experiments showed that METH indirectly activates the LDT, but not the PPT, to stimulate increases in ACh levels within the VTA; however, this effect is independent of METH-induced DA levels in the NAc and METH reward. Although it is well known that ACh can alter DA release and stimulate reward, METH can induce DA release independently of neuronal stimulation. This likely masks any DA-potentiating effects of LDT ACh on METH-induced DA levels and reward.

Chapter 1

General Introduction

1. Introduction to methamphetamine

The following is a review of the psychostimulant methamphetamine (METH), including a brief history and prevalence of use statistics, its pharmacodynamics and pharmacokinetics, and an introduction to the classification of METH addiction.

Background and prevalence of methamphetamine use. METH is a derivative of amphetamine and a highly addictive psychostimulant. It was originally synthesized from ephedrine by Akira Ogata in 1919 and initially used to treat bronchoconstriction associated with asthma and the swelling of nasal mucosa associated with congestion (Anglin et al. 2000). During its initial use in the general population, beneficial side effects of METH were noted, such as wakefulness and decreased appetite, and later exploited during World War II, during which METH was administered to soldiers to increase vigilance in the field (Anglin et al. 2000). The illicit production of METH began after the withdrawal of the marketed form of METH, Desoxyn, in California in the early 1960s, which resulted in tighter federal restrictions on the manufacture, distribution, and prescription of amphetamine compounds (Vearrier et al. 2012).

The federal government has passed an abundance of legislation since 1988 to combat the illicit manufacture of METH, with the most recent being the Combat Methamphetamine Epidemic Act in 2005, which limited the retail over-the-counter sale of drugs containing pseudoephedrine. However, despite these federal efforts, METH abuse has steadily increased and swept across the country just recently reaching the Atlantic coast (System 2008). While the number of self-reported METH users declined between 2006 and 2008 (SAMHSA 2010a), the rate of METH related hospital emergency intakes increased by more than 50% between 1995 and 2002 (NIDA 2006). Furthermore, METH represented 8% of all drug treatment admissions in 2004, which

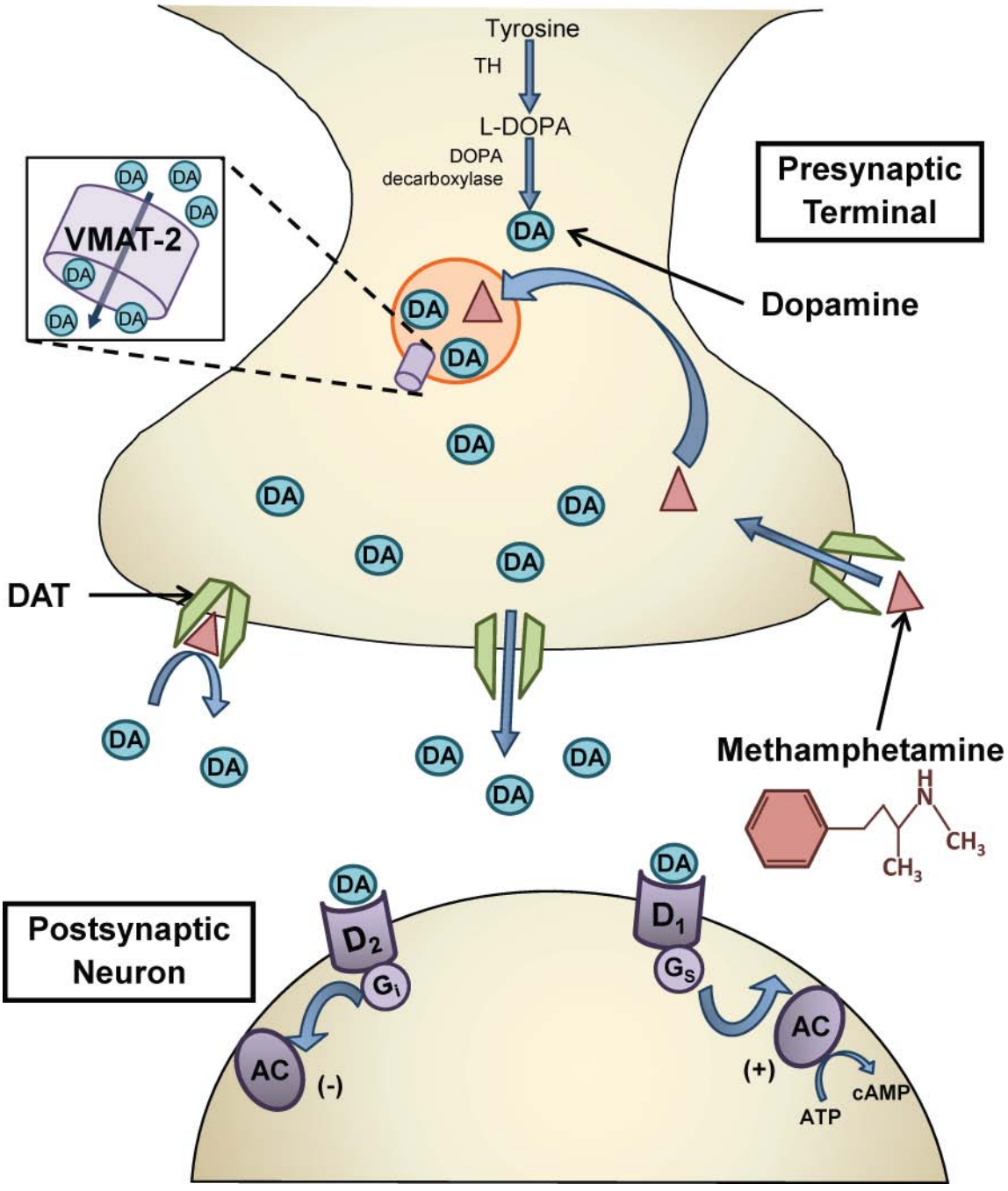
was a 7% increase compared to 1992 (NIDA 2006). Although it may appear that the negative impact of drug addiction is limited to the addicted individual and their immediate family and friends, drug abuse exacts a grievous toll on the economy and society at large. In 2002, the United States reported spending an estimated 181 billion dollars combating addiction, with the largest proportion (129 billion dollars) attributed to a loss of productivity and potential resources (ONDCP 2004). While this estimate clearly illustrates the costly nature of addiction, it remains a conservative estimate since it fails to include the impact of addictions on the quality of life of the drug abuser, his/her family and friends and victims of the drug abuser. METH use can result in violent behavior and has been associated with increased violent crime and property crime (Darke et al. 2010; Degenhardt et al. 2008; Gizzi and Gerkin 2010). Furthermore, the illicit production of METH in what are termed "METH labs" is a hazardous process that often results in explosions, chemical burns and toxic exposures, often to children housed in the area (Vearrier et al. 2012).

Methamphetamine pharmacodynamics, pharmacokinetics and behavioral effects.

METH is a lipid soluble, sympathomimetic compound within the phenylethylamine class of psychostimulants and more readily crosses the blood-brain barrier compared to amphetamine. METH induces the release of biogenic amines, such as dopamine (DA), norepinephrine (NE) and serotonin (5-HT) (Figure 1) (Nordahl et al. 2003). METH can stimulate DA, NE or 5-HT release through its actions on each transmitter's monoamine transporter and the vesicular monoamine transporter-2 (VMAT-2). It is a more potent

Figure 1. Diagram of METH's actions at a DA terminal. DA is synthesized from a conversion of tyrosine into L-DOPA via the enzymes tyrosine hydroxylase (TH) and DOPA decarboxylase. METH is taken up into the presynaptic DA terminal via the DAT. In the DA vesicles METH disrupts the proton gradient and causes a redistribution of DA into the cytoplasm via the VMAT-2. Cytoplasmic DA diffuses down its concentration gradient into the synaptic cleft via a METH reversed DAT. METH also blocks the re-uptake of DA back into the terminal. In the synapse, DA acts on DA type D1 and D2 receptors to excite or inhibit the postsynaptic neuron. D1 receptors activate adenylyl cylclase (AC) and facilitate the formation of cyclic adenosine monophosphate (cAMP). D2 receptors inhibit AC and lead to hyperpolarization of the neuron. D1 and D2 receptors are not known to be on the same postsynaptic neuron, but are shown so for simplicity. The chemical structure for METH is shown below its title. Adapted in part from Cooper et al., 2003.

Figure 1


Tyrosine
TH
L-DOPA
DOPA decarboxylase
Presynaptic Terminal
VMAT-2
DA
Dopamine
DAT
Methamphetamine
H
N
CH3
CH3
Postsynaptic Neuron
D2
Gi
AC
(-)
D1
Gs
AC
(+)
ATP
cAMP

substrate for the NE transporter compared to the DA transporter (DAT) and has the lowest potency for the 5-HT transporter (Han and Gu 2006; Rothman et al. 2001). For a discussion on the role of DA, NE and 5-HT (as well as other common neurotransmitters in the reward pathway) in METH reward, the reader is referred to **section 3**.

Under normal conditions, the DAT functions to transport DA from the synaptic cleft back into the DA terminal. From there, DA is transported back into the vesicles via the VMAT. METH is a substrate for the DAT and VMAT-2 and also inhibits DA reuptake (Eshleman et al. 2001; Schenk 2002; Volz et al. 2007; Zaczek et al. 1991). Once inside the cytosol, METH transports into the DA vesicles and binds free protons, thus disrupting the proton gradient and causing a redistribution of DA from the vesicles to the cytosol. Via a reversal of the DAT, METH induces reverse transport of DA into the synapse in a nomifensine (a DAT inhibitor) inhibitable manner (Sulzer et al. 1995; Sulzer et al. 2005).

Within the human, METH has a half-life of approximately 12 h, however the half-life in the rat is much shorter (approximately 70 min) (Cho et al. 2001). Additionally, in the rat, METH is rapidly converted to amphetamine in the liver, which leads to amphetamine levels exceeding METH levels (Cho et al. 2001). METH's wide distribution throughout the body, long half-life and slow elimination from the brain are thought to contribute to its toxic effects on the pulmonary and cardiovascular systems and brain (Cruickshank and Dyer 2009; Volkow et al. 2010a).

The short-term effects of METH include euphoria, increased activity, hyperthermia, anorexia and increased blood pressure and heart rate (Hart et al. 2001; Makisumi et al. 1998). Long-term effects of METH use can lead to addiction, psychosis (including hallucinations and paranoia), cardiovascular damage, violent behavior, and

neurological damage (Cruickshank and Dyer 2009; London et al. 2004; Thompson et al. 2004; Volkow et al. 2001).

Methamphetamine addiction is a dysregulation of motivational learning. METH addiction is a chronic, relapsing neurological disease. The American Psychiatric Association defines amphetamine dependence as a pattern of maladaptive use that causes the user significant impairment (2000). To make a diagnosis of amphetamine dependence, the user must manifest 3 out of the following 7 criteria in the same 12 month period: (1) tolerance, (2) withdrawal, (3) administration of the drug in greater amounts or longer than originally intended, (4) inability, despite desire, to decrease or control drug use, (5) significant amounts of time dedicated to drug procurement and use, (6) reduction in time spent in other important social, occupational or recreational activities, and (7) persistence of drug use despite negative consequences (Association 2000). Tolerance is characterized by a decrease in the effectiveness with continued use of the same amount of a drug or the need for increased amounts of the drug to achieve the same effects. Withdrawal is defined as the presence of aversive symptoms specific to the drug of abuse upon the cessation of drug use. METH withdrawal is most often characterized by METH craving, depressed or anxious mood, impaired cognition and disturbed sleep (Kalechstein et al. 2003; Watson et al. 1972). The role of various neurotransmitters (such as DA and NE) in aspects of METH use and dependence is discussed below (**section 3**).

The empirical, preclinical study of drugs of abuse has been popular for decades. Research on the neurobiological effects of drugs of abuse has led to debate over the definition of “reward”. Indeed, drugs of abuse and natural substances such as food are often colloquially called “rewards” or are referred to as “rewarding”; however, since this

term can be very subjective, it is important, especially in drug abuse research, to define this term operationally. A reward is a stimulus that elicits a positive affective state (i.e., "liking"), such as pleasure or euphoria, within the subject and that also possesses a motivational or incentive component (i.e., "wanting") (Berridge 1996; 2009). Thus, a stimulus that is rewarding elicits feelings of pleasure and can motivate the subject to obtain the reward or approach the stimulus that is associated with the reward. In addition to being rewarding, drugs of abuse also serve as reinforcers. That is, drugs of abuse increase the probability that the subject will perform a response to obtain the drug. This property of drugs of abuse is often measured in animal studies using the self-administration procedure (this is described in detail in **section 5**). While a substance can be rewarding and reinforcing, it is also possible for a drug to be reinforcing but not rewarding, and vice versa. For instance, experienced morphine users self-administered a low dose of morphine, but reported subjective effects similar to that of saline (Lamb et al. 1991). Healthy human subjects also self-administered a low dose of methamphetamine in the absence of "liking" (Hart et al. 2001). However subjects reported a "good drug effect" with a higher METH dose, which also supported self-administration (Hart et al. 2001).

While human subjects can provide self-report of whether a substance "feels good," animals cannot. We can, however, infer whether an animal finds a substance rewarding by observing behavior, 1) in the presence of the reward itself or, 2) in the presence of cues that previously signaled the presence or delivery of the reward. For instance, rats and human infants display affective reactions to sweet solutions characterized by distinctive patterns of mouth, tongue and forelimb (in the rat only) movements (for a review see Berridge 1996). Natural rewards, such as food, have adaptive inhibitory neural feedback processes, which, in the example of feeding behavior, indicate satiety, interrupt food seeking and consumption, and can alter the

"liking" of the food, as indexed by the affective reaction to the tastant (for a review see Berridge 1996). Drugs of abuse lack natural adaptive modulation and create abnormally robust associations between cues and contexts associated with drug seeking and taking (Everitt and Robbins 2005; Sulzer 2011). For instance, exposure to cues associated with drug taking, such as drug paraphernalia, can incite uncontrolled craving, drug-seeking and drug-taking in the subject (Price et al. 2010; Tolliver et al. 2010). Additionally, these behaviors often persist in the face of negative consequences. Thus, drug addiction is often considered a dysregulation of motivated behavior. Recent advances in our understanding of the neural mechanisms underlying reward have revealed that DA responses within what is colloquially termed the reward pathway are important for reward and the motivated behavior that rewards elicit (see **section 2**). Drugs of abuse are capable of inducing persistent and mal-adaptive drug-seeking behavior by co-opting the reward pathway and inducing neuroadaptations within it.

2. The mesocorticolimbic reward pathway

Connectivity and physiology of dopamine neurons. The rewarding aspect of drugs of abuse is commonly attributed to their actions on DA levels within the mesocorticolimbic reward pathway. The first experiment to determine that the pathway stemming from the midbrain DA neurons to the NAc, known as the medial forebrain bundle, is rewarding was performed by Olds and Milner (Olds and Milner 1954). They found that electrical stimulation of the lateral hypothalamus, which in turn stimulates the medial forebrain bundle, is reinforcing (i.e., subjects self-administered stimulation) and rewarding (i.e., subjects showed a preference for the environment in which they received the stimulation). The DA neuronal regions A9 and A10 that make up the heart of this pathway are located in the ventral midbrain and provide major efferents to cortical and

limbic structures. The A9 cell group, better known as the substantia nigra (SN), is involved in initiating motor behaviors, and has robust projections to the dorsolateral striatum and the frontotemporal cortex (German and Manaye 1993). The A10 region is referred to as the ventral tegmental area (VTA) and has dense projections to the nucleus accumbens (NAc), medial prefrontal cortex (mPFC) and various limbic structures such as the amygdala and bed nucleus of the stria terminalis (Hasue and Shammah-Lagnado 2002). Figure 2 shows selected projections of the mesocorticolimibic pathway. These specific projections are depicted because they emphasize a reciprocal circuit in which METH can act to indirectly stimulate neurons within the laterodorsal tegmental nucleus (LDT) that go on to project to the VTA. The METH-induced increase in DA in the mPFC could transynaptically activate (via DA receptors located on the glutamate neuron soma) the mPFC glutamate neurons that project to the LDT. Alternatively, METH-induced increases in DA within the NAc could inhibit γ-aminobutyric acid (GABA) projection neurons (via DA D2 receptors located on the GABA neuron soma) and thus disinhibit mPFC glutamatergic projections to the LDT. *For more information on the afferent and efferent connections of the LDT, the reader is referred to* ***section 4*** *of this chapter. The reader is also referred to the experiments in* ***Chapter 2****, the results of which led to my hypothesis that METH was acting in this circuit to stimulate increases in VTA acetylcholine (ACh) levels.*

Midbrain DA neurons fire at different frequencies. They produce single spikes of action potentials, which is referred to as tonic activity, or trains of 2 – 6 action potentials, which is referred to as phasic burst firing. Tonic pacemaker firing maintains synaptic DA within a narrow concentration range while burst firing is important for high amplitude DA release (Dugast et al. 1994; Suaud-Chagny et al. 1992). High amplitude DA release is

Figure 2

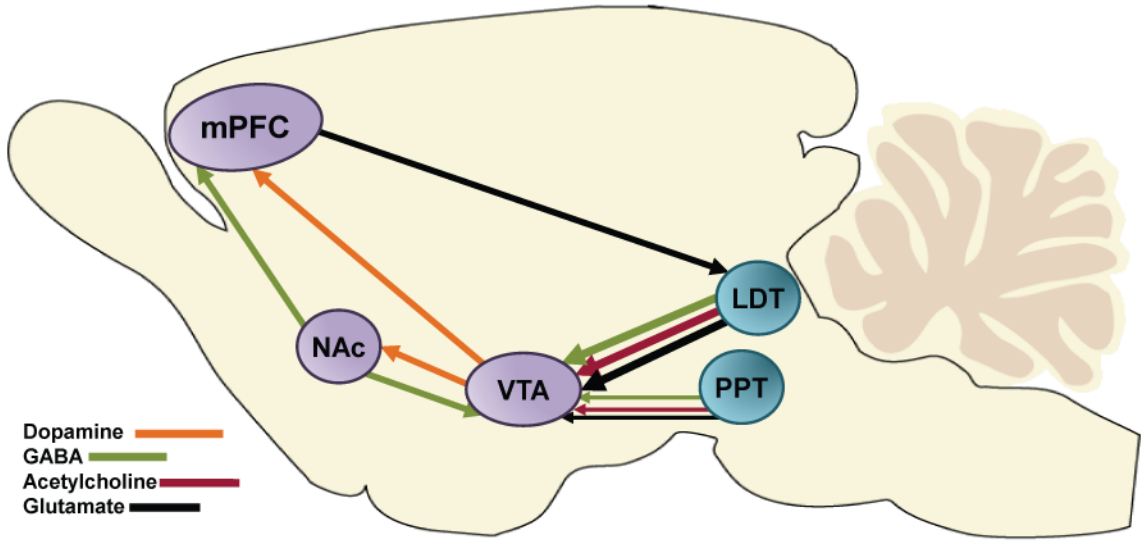


Figure 2. Selected projections of the mesocorticolimbic reward pathway are depicted to emphasize a hypothetical circuit in which METH can act to indirectly stimulate LDT neurons projecting to the VTA. Midbrain DA neurons (orange arrows) in the VTA project to limbic regions like the NAc and cortical regions like the mPFC. In the NAc, γ-aminobutyric acid (GABA; green arrows) projects to the mPFC and VTA to modulate glutamate and DA firing, respectively. The mPFC sends glutamate (black arrows) projections to the LDT in the mesopontine tegmentum. The LDT and pedunculopontine tegmental nucleus (PPT) send neurochemically diverse projections to the midbrain DA neurons; however the LDT has stronger projections to the VTA than the PPT, as indicated by the weight of the arrows. Conversely, the PPT sends stronger projections to the DA neurons in the substantia nigra (SN), while the LDT sends relatively weak projections to the SN (not shown). METH is thought to activate DA release in terminal regions, such as the mPFC, which (via DA receptors) transynaptically activates the glutamate projections to the LDT.

due to the additive nature of DA release as each successive spike builds on the previous (Chergui et al. 1994). However, unlike terminal regions where burst stimulation is equal to the summation of pulses, at the soma, burst firing results in sub-additive somatodendritic DA release, but has a similar time course compared to terminal release (Beckstead et al. 2007). Somatodendritic DA release appears to share several qualities with terminal release in animal models. DA release at the terminal has a similar temporal release pattern compared to release at the soma. In addition, DA release at

the terminal and soma is calcium (Ca^{++})-dependent and subject to neurotransmitter depletion (Kalivas et al. 1989a; Kalivas and Duffy 1991; Kita et al. 2009). Further, DA release at the terminals and soma occurs via tetrodotoxin (TTX)-sensitive exocytosis from vesicles or vesicle-like organelles (John and Jones 2006). Importantly, burst firing triggers a substantial increase in DA release when compared to single-spaced spikes in both areas. The major difference between DA release in the soma and terminal appears to be the quantity of release; with the terminal being more sensitive to burst firing and releasing more DA in general compared to the soma.

Once DA is released from the terminal it acts upon DA receptors on the postsynaptic neuron or on autoreceptors located on the DA soma, dendrites or terminals. There are 2 classes of DA receptors, D_1 and D_2, which are coupled to GTP-binding proteins and classified based on their biochemical characteristics (Cooper et al. 2003; Vallone et al. 2000). Both kinds of receptors are found in the striatum and NAc (Jackson and Westlind-Danielsson 1994). D_2 receptors located on presynaptic dendrites and soma in the SN and VTA function as inhibitory autoreceptors (Jackson and Westlind-Danielsson 1994). D_1 receptors stimulate adenylyl cyclase through activation of the G_S protein, which facilitates formation of cyclic adenosine monophosphate (Cooper et al. 2003; Vallone et al. 2000). This facilitates the activation of protein kinase A which leads to neurotransmitter release. Conversely, D_2 receptors inhibit adenylyl cyclase activity via a G_i protein, which leads to hyperpolarization of the neuron.

<u>Dopamine and reward.</u> Decades of research after the pioneering work of Olds and Milner, we know that DA signaling and release within this pathway is important for drug and food reward, reward prediction, energizing goal-directed behaviors (such as drug-

seeking), and in creating Pavlovian associations between conditioned stimuli and natural or drug rewards (Baler and Volkow 2006; Day and Carelli 2007; Schultz 2010; Wise 1982). Within the VTA, DA neurons fire in response to novel stimuli and unexpected rewards. This DA responding underlies the learning that occurs when novel stimuli are paired with and become predictive of rewards (Fiorillo et al., 2003, Schultz, 2007). After several pairings of a motivationally positive stimulus (unconditioned stimulus; US) with an initially neutral stimulus, the neutral stimulus becomes a predictor of the US (i.e., a conditioned stimulus; CS) and able to evoke behaviors similar to that of the US itself. As this learning occurs, DA neurons shift from firing in the presence of the US to firing in the presence of the CS and stimulate approach behaviors toward the CS.

Ultimately, drugs of abuse modify stimulus processing and reward-related behaviors by usurping the reward pathway to affect the burst firing of midbrain DA neurons and subsequent DA release in terminal regions (Kalivas 1993). Drugs of abuse have been shown to induce somatodendritic as well as terminal DA release (Bradberry and Roth 1989; Rahman et al. 2003; Zhang et al. 2001) and DA activation of the D_2 DA receptor in the VTA has been implicated in the reinstatement of cocaine seeking (Xue et al. 2011). Thus, somatodendritic as well as terminal DA levels may be important in mediating the rewarding aspects of drugs of abuse. The evidence presented suggests that mesolimbic DA responses are necessary for the creation of associations of drug-related cues with the rewarding aspects of drugs. Moreover, presentation of drug-related cues induces sensitization of DA transmission, which has been suggested to facilitate behavioral arousal, approach and instrumental responding (Di Chiara 2002).

Although the classical view of reward has tended to be “dopaminocentric”, there are several additional neurotransmitters and neuropeptides within the reward pathway

that also contribute to goal-directed behaviors and the creation of CS-US associations. In the following sections, I will describe some of the more common neurotransmitters and neuroanatomical sites that influence DA responding and reward.

Effect of glutamate on dopamine activity and release and reward. Midbrain DA neurons (and the DA terminals within the striatum) receive glutamate projections from several areas of the brain including the mesopontine tegmentum, amygdala, hippocampus and mPFC (Bardo 1998). Glutamate is an excitatory amino acid that regulates DA release within the NAc through its actions on the inhibitory metabotropic glutamate group 2 and 3 ($mGlu_{2/3}$) receptors (Karasawa et al. 2006). Agonists of the two ionotropic glutamate receptors, N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), perfused through the VTA caused an increase in DA levels in the PFC and NAc and induced DA burst firing (Kalivas et al. 1989b; Suaud-Chagny et al. 1992; Westerink et al. 1998; Westerink et al. 1996). In addition, a structure in the mesopontine tegmentum, the LDT, is required for glutamate-induced burst firing of DA neurons (Lodge and Grace 2006).

The actions of glutamate within the reward pathway are also involved in the associative learning that occurs between cues and drug rewards and the habit formation associated with prolonged drug exposure. The formation of long-term potentiation (LTP), a type of synaptic plasticity that underlies learning, is driven by the action of glutamate at NMDA and AMPA receptors (for a review see Philibin et al. 2011). In addition, LTP is associated with changes in the dendrite spine morphology and number that accompanies prolonged drug use (Alvarez et al. 2007; Dobi et al. 2011).

Glutamate projections from specific neuroanatomical sites have been identified that contribute to different aspects of reward learning. For instance, hippocampal glutamatergic projections to the VTA are important for relaying information about the context in which the reward was delivered, while glutamatergic projections from the orbital frontal cortex and amygdala are necessary for conditioned approach behavior, especially when there is a delay between the presentation of the CS and delivery of the reward (Everitt and Robbins 2005). Closer investigation of the DA response in relation to conditioned approach behavior reveals that the DA response is not time-locked with the initiation of an appetitive behavior towards the CS (Day et al. 2007). Co-release of glutamate with DA originating in the SN has been proposed as a mechanism to relay fast signals about rewards and conditioned cues for the initiation of approach behavior (Lavin et al. 2005). While glutamate co-release has been more readily observed with DA from the SN, glutamate has also been suggested to co-release with DA originating in the VTA (Lapish et al. 2007; Lapish et al. 2006).

Effect of γ-aminobutyric acid on dopamine activity and release and reward. GABA is an inhibitory amino acid neurotransmitter and a very important regulator of DA signaling within the reward pathway. Within the VTA are GABA interneurons as well as GABA neurons that project to the NAc (Carr and Sesack 2000; Van Bockstaele and Pickel 1995) (Figure 3). In addition, the NAc, ventral pallidum (VP) and mesopontine tegmentum send GABA projections to DA cell bodies in the VTA (Cornwall et al. 1990; Geisler and Zahm 2005; Semba and Fibiger 1992). Symmetrical, presumed inhibitory GABAergic projections from the LDT synapse onto mesoprefrontal and mesoaccumbens projecting VTA GABA neurons (Omelchenko and Sesack 2005). Thus, these presumed GABAergic projections may function to disinhibit the mPFC and NAc. Additionally, LDT inhibitory projections synapse onto mesoprefrontal, but not mesoaccumbens, DA

neurons in the VTA, supporting the idea that the LDT excites VTA mesoaccumbens DA neurons (Omelchenko and Sesack 2005).

Much of the research involving the GABAergic system in reward has focused on ethanol and sedative-hypnotic drug reward. Intra-VTA administration of baclofen, a $GABA_B$ receptor agonist, attenuated the expression of ethanol conditioned place preference (CPP) in mice (Bechtholt and Cunningham 2005) and systemic baclofen administration prevented acquisition of ethanol drinking in a selected line of alcohol preferring rats (Colombo et al. 2002). The other GABA receptor, $GABA_A$, has also been implicated in ethanol reward. $GABA_A$ receptor antagonists bicuculline and picrotoxin enhanced the acquisition of ethanol CPP in mice (Chester and Cunningham 1999).

Effect of acetylcholine on dopamine activity and release and reward. Acetylcholine (ACh) is an important mediator of DA release and the reinforcing characteristics of drugs of abuse in the mesocorticolimbic pathway (Lacey et al. 1990; Mereu et al. 1987). Cholinergic neurons located in the mesopontine tegmentum project to the VTA and SN and provide the cholinergic tone within the ventral midbrain (Oakman et al. 1995; Omelchenko and Sesack 2005). For a detailed description of these connections, see **section 4**. In addition, cholinergic interneurons are present within the dorsal and ventral striatum, and they help regulate DA signaling via actions at GABA neurons (Mansvelder et al. 2003; Zhang et al. 2002).

Within the VTA, ACh acts upon nicotinic (nAChR) and muscarinic (mAChR) ACh receptors localized on DA neurons to increase DA release within key structures of the reward pathway, such as the NAc and mPFC (Miller and Blaha 2005; Westerink et al. 1998; Westerink et al. 1996). In addition, application of ACh to the DA cell body induces

somatodendritic DA release in rat SN dendrosome preparations (Marchi et al. 1991). The M5-subtype mAChR has been localized on VTA DA neurons (Reever et al. 1997; Vilaro et al. 1990; Weiner et al. 1990) and this receptor has been linked to prolonged DA release in the NAc. Electrical stimulation of the cholinergic neurons that project to the VTA results in a tri-phasic DA response in the NAc (Forster et al. 2002b). The first phase is a fast excitatory DA response controlled by nAChRs and glutamate receptors in the VTA. The second phase is inhibitory and dependent on the M2 mAChR located on the ACh soma. The third component is a prolonged excitatory DA release dependent on the M5 mAChR in the VTA. Mice with a genetic deletion of the M5-subtype mAChR showed a suppression of this prolonged DA release (Forster et al. 2002b). Thus, the M5-subtype mAChR mediates prolonged excitatory effects on midbrain DA neurons.

The interaction between DA and ACh via these cholinergic receptors also mediates motivated behaviors. Perhaps the most well-known rewarding cholinergic agonist is nicotine; the chemical found in cigarettes that potentiates the reinforcing qualities of smoking. Nicotine's reinforcing effect is due to its actions on nAChRs in the VTA that regulate DA neuron firing (Mansvelder et al. 2003). In addition, rodent self-administration models in combination with pharmacological manipulation of specific neuroanatomical sties have revealed brain areas that mediate cholinergic reward. For example, rats self-administered the indirect ACh agonist neostigmine or the mixed mAChR/nAChR agonist carbachol into the VTA, which was blocked by co-infusion of mAChR or nAChR antagonists (Ikemoto and Wise 2002). Additionally, intra-VTA microinjection of the mAChR antagonist scopolamine inhibited the acquisition of an operant to obtain a food reward (Ikemoto and Wise 2002; Redgrave and Horrell 1976; Yeomans and Baptista 1997) and knock-out of M5 mAChR specifically in the VTA

inhibited rewarding brain stimulation while intra-VTA infusion of ACh enhanced it (Ikemoto and Wise 2002; Sharf et al. 2006; Yeomans et al. 2001).

3. The effect of methamphetamine on the reward pathway

METH, like other drugs of abuse, acts on the mesocorticolimibic reward pathway to exert its rewarding effects. The following is a brief review of the actions of METH on the specific neurotransmitters NE, 5-HT, DA, ACh, glutamate and GABA.

Norepinephrine. METH is a potent inhibitor of the NE transporter and was reported to increase NE release *in vitro* in striatal synaptosome preparations (Rothman et al. 2001) and *in vivo* in the rat hippocampus (Kuczenski et al. 1995). Additionally, the NE system appears related to METH-induced DA levels and METH toxicity. Rats with a neurotoxic lesion of locus coeruleus (LC)-derived NE axons (via systemic injection of *N*-(2-choroethyl)-*N-ethyl*-2-bromobenzylamine) showed an exacerbation of METH-induced striatal DA depletion and showed inhibited clearance of METH from the striatum (Fornai et al. 1998; Fornai et al. 1999). The lesion of NE axons or inhibition of NE synthesis was also related to enhanced METH locomotor behavior, stereotypies and increased METH neurotoxicity (as indexed by the formation of reactive oxygen species, the presence of striatal gliosis, and reductions in striatal DA levels) (Weinshenker et al. 2008). These studies suggest that NE may be neuroprotective against the toxic effects of METH on striatal DA neurons.

Activation of the NE system via administration of the α_2 receptor antagonist yohimbine has been implicated in stress-induced relapse to METH-seeking behavior (Shepard et al. 2004). Additionally, cue-induced reinstatement of METH-seeking was

inhibited by pretreatment with mirtazepine, a mixed antagonist of NE, histamine and 5-HT receptors (Graves and Napier 2011). Further, inhibition of the NE system by administration of the α_1 receptor antagonist prazosin attenuated the development of amphetamine locomotor sensitization (Vanderschuren et al. 2003). The effect of NE receptor or transporter inhibition on METH self-administration has not been reported; however, loss of the NE neurons or administration of NE receptor antagonists did not affect cocaine self-administration (Roberts et al. 1977; Wee et al. 2006; Woolverton 1987). Additionally, the selective NE transporter inhibitors desipramine and atomoxetine were not self-administered by rhesus monkeys, suggesting that blockade of the NE transporter alone is not reinforcing (Wee et al. 2006; Wee and Woolverton 2004).

Serotonin. Although METH is a weak inhibitor of the 5-HT transporter (Rothman et al. 2001), it has been reported to increase 5-HT release *in vivo* in the rat caudate and NAc (Kuczenski et al. 1995; Segal and Kuczenski 1997). A single high dose treatment or multiple high dose treatments of METH decreased 5-HT transporter function, as measured by [^{3}H]5-HT uptake in striatal synaptosome preparations (Fleckenstein et al. 1999). Despite the effect of METH on 5-HT transporter function and 5-HT release, pretreatment with the selective 5-HT reuptake inhibitor fluoxetine, paroxetine or fluvoxamine had no effect on METH CPP (Takamatsu et al. 2011; Takamatsu et al. 2006a). Additionally, randomized, placebo-controlled clinical trials reported that treatment with reuptake inhibitors selective for 5-HT or 5-HT receptor antagonists failed to decrease METH use in METH dependent individuals (Johnson et al. 2008; Karila et al. 2010; Piasecki et al. 2002). However, one study found that sertraline, a selective 5-HT reuptake inhibitor, actually increased METH craving and use in dependent individuals (Zorick et al. 2011). These data indicate that manipulation of the 5-HT system does not

attenuate METH's rewarding effects, and in some cases might even exacerbate METH craving.

Dopamine. Amphetamines compete with DA to bind to the DAT and bind at a site separate from the cocaine binding site (Fleckenstein et al. 2007). Several studies, using a variety of preparations including anesthetized rats (Izawa et al. 2006), awake freely moving rats (Camp et al. 1994; Shoblock et al. 2003; Tsai and Chen 1994; Zhang et al. 2001), and awake freely moving mice (Shimosato et al. 2003), have found that METH causes DA release within the ventral striatum. Additionally, evidence suggests that amphetamines act on the VMAT-2 located on tubulovesicular organelles found in DA dendrites to increase somatodendritic release of DA in the VTA and NAc (Fleckenstein et al. 2007; Nirenberg et al. 1996).

Reports on the effects of METH at the DA cell body within the VTA have been mixed. Some studies have found that intravenous METH administration inhibits DA neuron cell firing in the SN pars compacta (SNc) and VTA in anesthetized rats; however, no control (i.e. saline administration) was tested in either experiment (Kamata and Kameyama 1985; Kawashima et al. 1999). In contrast, one study found that intravenous administration of cocaine, amphetamine or METH induces an increase in DA firing rate and bursting as well as a slow rhythmic oscillation of DA firing (Shi et al. 2004). The authors speculated that this slow oscillation in firing underlies the reinforcing qualities of psychostimulants since the non-rewarding indirect DA agonist levodopa ($_L$-DOPA) failed to induce it. In addition, a 7 day systemic METH regimen sensitized rat VTA DA neurons to acute METH application as indexed by *in vitro* oscillations in cytosolic Ca^{++} current (Uramura et al. 2000). Interestingly, rats treated with saline for 7 days showed METH-induced increases in DA neuron Ca^{++} current, but no oscillations were present.

Exposure to METH also has effects on the levels of tyrosine hydroxylase (TH), the enzyme critical for the synthesis of tyrosine to $_{L}$-DOPA (which is then converted to DA). Forty-eight h after the last METH dose of a 2-week escalating METH dose regimen or binge exposure in rats increased DA and TH tissue content in the VTA, but decreased it in the NAc (Keller et al. 2011). Similarly, rats with a history of extended access to METH self-administration (9 h/day for 10 days) showed an increase in TH mRNA levels within the VTA and SNc 1 day after forced METH abstinence; however, TH levels had returned to normal 30 days after forced METH abstinence (Shepard et al. 2006).

The ability of METH to affect the mesolimbic DA system has also been implicated in its reinforcing and rewarding effects. Lobeline, a nAChR agonist and VMAT-2 ligand, has been shown to inhibit DA uptake into vesicles and induce [^{3}H]DA release in striatal slices (Teng et al. 1997). The latter effect was not blocked by mecamylamine, a nAChR antagonist, suggesting that lobeline's effects on DA release were due to its actions on the VMAT-2 (Teng et al. 1997). Acute pretreatment or chronic treatment with lobeline inhibited intravenous METH self-administration in rats (Harrod et al. 2001b). Furthermore, a different VMAT-2 inhibitor (GZ-793A) also decreased METH self-administration and blocked the acquisition of METH CPP (Beckmann et al. 2012). Similarly, pretreatment with a DA D3 receptor antagonist reduced the breakpoint for responding to receive intravenous METH under a progressive ratio (PR) schedule of reinforcement and attenuated cue-induced reinstatement of METH seeking behavior (Higley et al. 2011a; Higley et al. 2011b).

The abundance of evidence clearly indicates that METH acts upon the midbrain dopaminergic system to robustly increase DA levels within the terminal. In addition,

METH has effects on DA neuron firing and activity; however, these data are mixed and may reflect the different preparations used (*in vivo* recordings in anesthetized rats vs. *in vitro* measurements) and different dependent measures used (single unit recording vs. digital imaging of cytosolic Ca^{++} current) between the studies. Furthermore, the effects of METH on the DA system appear to underlie its reinforcing and rewarding qualities. While these studies have clarified the role of acute METH exposure on DA firing or prior systemic METH exposure on DA or TH tissue content, to my knowledge, there have been no studies to date to investigate the effect of acute METH exposure on levels of DA in the VTA in awake freely moving mice. In addition, these previous studies have used intravenous administration of METH (either experimenter or self-administered), but none have assessed the effect of METH directly applied to the DA cell bodies. *One of the goals of this dissertation is to measure the effect of (1) systemic injection and (2) intra-VTA perfusion of METH on somatodendritic DA levels within the VTA in awake freely moving mice (**Chapter 2**).*

Glutamate. There are only a few reports on the actions of rewarding (as opposed to neurotoxic) doses of METH on extracellular glutamate levels. These reports indicate that systemic administration of METH (1 or 2 mg/kg) increased glutamate in the PFC, caudate and putamen and decreased glutamate levels in the VTA and SNc (Qi et al. 2009; Shoblock et al. 2003; Zhang et al. 2001). The authors suggest that METH increased DA release in the PFC, striatum, VTA and SNc and thereby transynaptically stimulated or inhibited PFC glutamate neurons and terminals via DA D1 or D2 receptors, respectively. However, METH did not affect glutamate levels in the NAc. Interestingly, the effect of METH on extracellular glutamate levels is distinct from the effects of other psychostimulants such as cocaine and amphetamine. For instance, METH increased glutamate in the PFC significantly more than amphetamine (Shoblock et al. 2003). The

authors speculate that this difference in neurochemical responding (in addition to the ability of amphetamine to induce more PFC DA release than METH) might contribute to differential activation of PFC "reward inhibition" pathways and underlie METH's profoundly reinforcing and rewarding effects.

High dose METH is toxic to striatal DA neurons and glutamate has also been shown to induce excitotoxicity (Golembiowska et al. 2002; Ohmori et al. 1996). Therefore, it is not surprising that the majority of glutamate research in regards to METH has focused on METH neurotoxicity. In these studies, very high doses of METH are typically repeatedly administered (7.5 mg/kg to 15 mg/kg, 3 or 4 times, 2 h apart) in order to induce neurotoxicity. *In vivo* microdialysis in the rat indicated that METH induces large increases in extracellular glutamate levels within the ventral hippocampus, striatum and caudate putamen (Abekawa et al. 1994; Bustamante et al. 2002; Nash and Yamamoto 1992; Rocher and Gardier 2001; Stephans and Yamamoto 1994). Furthermore, competitive and non-competitive NMDA antagonists prevented METH-induced DA neurotoxicity. In addition, METH did not increase glutamate levels in the NAc, an area that shows no toxic DA damage following high dose METH administration.

GABA. GABA has been a relatively neglected target of METH research and the limited data regarding the effect of METH on the GABAergic system within the reward pathway is mixed. Several studies testing a range of METH doses (1.0, 2.5, or 15 mg/kg) have found no effect of acute METH administration on the levels of GABA in the SN, mPFC, NAc, VP, striatum, hippocampus, or hypothalamus (Bustamante et al. 2002; Herrold et al. 2011; Kaiya et al. 1983). However, low to moderate doses of METH (0.25 – 2.0 mg/kg) increased GABA neuron firing rate *in vivo* in the VTA (Steffensen et al. 2008). The authors speculate that METH disinhibited GABA neuron firing by increasing DA

levels which then acted on DA D2 receptors located on GABA neurons. Chronic high dose METH administration decreased the expression of the $GABA_A$ α2 receptor subunit in the NAc, but increased its expression in the caudate and putamen (Zhang et al. 2006). Additionally, rats trained to self-administer METH on a PR schedule had lower breakpoints when pretreated with the $GABA_B$ agonist baclofen (Ranaldi and Poeggel 2002). This investigation suggests that the $GABA_B$ receptor may play a role in METH reward and be a possible therapeutic target. Additionally, a handful of studies have investigated the effect of GABA transaminase inhibitors, which inhibit the metabolism of GABA, on METH neurochemical responses and reward. Administration of GABA transaminase inhibitors attenuated METH-induced increases in DA levels within the NAc and METH-induced decreases in TH within the striatum (Gerasimov et al. 1999; Hotchkiss and Gibb 1980). However, administration of γ-vinyl-γ-aminobutyric acid, a marketed anti-epileptic and GABA transaminase inhibitor, to METH users had no effect on self-reported METH craving, or feelings of "high" or "any drug effect" (De La Garza et al. 2009). Thus, METH appears to affect the GABA system within the reward pathway and might be a useful target for the treatment of METH dependent individuals.

Acetylcholine. The effect of METH on ACh levels in the striatum has been fairly well characterized. Systemic administration of METH indirectly stimulated the cholinergic interneurons within the striatum to induce significant increases in striatal ACh levels as indexed by striatal homogenates and *in vivo* microdialysis (McGeer et al. 1974; Taguchi et al. 1998; Tsai and Chen 1994). McGreer and colleagues (1974) speculated that METH induced an increase in striatal DA, which then acted on inhibitory DA D2 receptors located on ACh neurons to decrease ACh *release.* Released ACh is rapidly catabolized by acetylcholinesterase (AChE) and would likely not be detected 2 h later when the rats were euthanized and striatal homogenates prepared for analysis.

Therefore, the authors hypothesized that decreased ACh *release* would result in increased ACh *tissue levels* when measured in homogenized striatal tissue. Interestingly, the METH-induced increase in ACh tissue levels was completely blocked by co-administration of a DAT inhibitor and α-methyl-p-tyrosine (a TH inhibitor), suggesting that the DA (or other monoaminergic) system(s) is involved in this effect. In a separate study using *in vivo* microdialysis, systemic METH injection increased striatal ACh levels, and this was partially blocked by pretreatment with haloperidol (a DA D2 agonist) (Taguchi et al. 1998). This suggests that METH indirectly stimulated ACh release by increasing DA levels, which in turn probably acted on the excitatory DA D1 receptor located on the ACh neuron soma. Furthermore, pretreatment with reserpine (a DAT inhibitor) and α-methyl-p-tyrosine inhibited the METH-induced increase in striatal ACh levels. Conversely, there was no increase in ACh levels following intra-striatal perfusion of METH (Taguchi et al. 1998). However, the authors used "reverse microdialysis" as opposed to a bolus microinjection to administer METH into the striatum. Since this process relies on diffusion across the permeable dialysis membrane, it is possible that METH levels in the striatum were too low to induce a significant increase in DA and ultimately affect ACh levels. In contrast to the study by Taguchi and colleagues, a binge pattern of METH exposure in rats led to a decrease in ACh levels within the caudate and putamen, but not the ventral striatum (Kuczenski and Segal 2001). This finding further supports the hypothesis that METH's effects on striatal DA levels are mediating striatal ACh levels, since binge dosing with METH also decreases striatal DA levels (Keller et al. 2011).

To my knowledge, there have been no investigations on the effect of METH on intra-VTA cholinergic responses. However, a recent study found that intravenous *cocaine* self-administration in rats induced two distinct cholinergic responses within the

VTA: one associated with cocaine seeking and one associated with cocaine delivery (You et al. 2008). In this study, presentation of cocaine-predictive cues induced a significant increase in ACh levels within the VTA of cocaine-trained, but not yoked control rats. Administration of cocaine induced a significant and prolonged increase in VTA ACh levels in rats previously trained to self-administer cocaine and in yoked control rats. One interpretation of these findings is that the cholinergic response in the VTA that occurs upon cue exposure energizes drug seeking behavior. This suggests that intra-VTA ACh levels may be an important factor in the creation of drug-predictive cues and maintenance of drug seeking behaviors.

Recent studies also suggest that the cholinergic system may be a useful target to decrease the likelihood of relapse to METH seeking. Nicotine administration during withdrawal from METH self-administration or administered during a reinstatement test inhibited METH-primed reinstatement of METH seeking in a rat model and this effect was blocked by the nicotinic antagonist mecamylamine (Hiranita et al. 2004; Hiranita et al. 2006). Furthermore, in a human population of METH users, administration of the indirect ACh agonist rivastigmine decreased self-reported levels of “anxious” and “desire” following an acute investigator-administered METH exposure (De La Garza et al. 2008).

These studies show that METH increases ACh within the striatum, and suggest this occurs by METH increasing DA within the striatum, which then acts on DA receptors located on ACh neurons. In addition, cholinergic tone within the VTA appears to be important in mediating reward of another psychostimulant, cocaine. The cholinergic system has also been investigated as a potential therapeutic target for METH addiction. These experiments in combination with the evidence that ACh increases DA release and

mediates reward through its actions at the DA neuron soma suggest that cholinergic tone within the VTA is a key mechanism of action through which psychostimulants can induce reward. *However, there have been no investigations on (1) the effect of METH on ACh levels within the VTA, or (2) the effect of VTA ACh levels on METH reward. One of the aims of this dissertation includes determining the effect of METH on ACh levels within the VTA (**Chapter 2**). In addition, this dissertation will investigate the role of VTA ACh tone in METH reward (**Chapter 4**). **Section 4** will elaborate on the source of the cholinergic tone within the VTA, the mesopontine tegmentum, and its potential role in reward.*

4. The ventral tegmental area receives cholinergic projections from mesopontine structures

In this section I will discuss the connections and constituents of the mesopontine tegmentum and how it relates to the reward pathway. Since the focus of this dissertation is on the cholinergic projections of the mesopontine tegmentum, I will first introduce the six cholinergic cell groups that have been identified in the brain.

Neural cholinergic cell groups. The brain's cholinergic system is divided into six cell groups, defined as Ch1 – Ch6. While these groups do not solely contain ACh neurons, they have been defined as cholinergic distinct regions that provide major cholinergic projections to several brain areas in the monkey and rat (Mesulam et al. 1983b). The Ch1 and Ch2 groups are located in the medial septum and vertical limb of the diagonal band, respectively, and provide the major cholinergic projections to the hippocampus. ACh neurons in these two regions make up 50% and 75% of the total neuronal type (Mesulam et al. 1983a; Mesulam et al. 1983b). The Ch3 group is located in the

horizontal limb of the diagonal band and consists of up to 75% and 25% cholinergic neurons within its medial and lateral sections, respectively (Mesulam et al. 1983b). The Ch3 group provides cholinergic projections to the olfactory blub. The majority of cholinergic afferents to the amygdala and neocortex stems from Ch4, the nucleus basalis of Meynert, which consists of approximately 90% ACh neurons (Mesulam et al. 1983a; Woolf and Butcher 1982). The mesopontine tegmentum contains the Ch5 and Ch6 groups termed the pedunculopontine tegmentum (PPT) and LDT, respectively. These groups provide the principle cholinergic afferents to the thalamus (Mesulam et al. 1983b; Semba and Fibiger 1992). Their additional projections and constituents will be discussed in more detail below.

Connections of the mesopontine tegmentum. As discussed earlier, midbrain DA neurons send projections to several targets in the limbic and cortical regions, and also receive afferents from several structures (see **section 2**). Thus, the VTA is ideally situated for integrating neurochemical information and initiating behavioral output to natural and conditioned rewards. Anterograde and retrograde tracing studies have confirmed topographical projections stemming from the neurochemically diverse mesopontine structures, the LDT and PPT. These structures make monosynaptic connections with the mesoaccumbens- and mesoprefrontal-projecting neurons in the VTA (Bolam et al. 1991; Geisler and Zahm 2005; Omelchenko and Sesack 2005). Major neuronal sub-populations in the LDT and PPT are cholinergic, GABAergic and glutamatergic (*these are discussed in more detail in the next sub-section*).

The VTA receives the majority of its cholinergic input from the dense cholinergic fields of the LDT and the posterior PPT, with a smaller percentage of afferents originating in anterior regions of the PPT. Separate populations of LDT cholinergic neurons

preferentially target VTA DA mesoaccumbens neurons or GABA neurons with either mesoaccumbens or mesocortical projections (Figure 3) (Oakman et al. 1995; Omelchenko and Sesack 2006). LDT cholinergic neurons also receive sensory input from the superior colliculus and respond to sensory stimuli (Koyama et al. 1994; Satoh and Fibiger 1986). In contrast to the LDT, the PPT ACh neurons have more robust projections to the SN (Oakman et al. 1995). Recent investigations suggest the posterior PPT receives polymodal sensory input from areas such as the superior and inferior colliculus, lemniscal nuclei, parabrachial and trigeminal nucleus and that the majority of cells within the posterior PPT respond to auditory and/or visual stimuli (Pan and Hyland 2005; Winn 2008). The anterior PPT receives input from forebrain structures, such as the globus pallidus, sub-thalamic nucleus and SN pars reticulata (SNr), and synapses onto dorsal striatal structures. This pathway is more involved in habitual stimulus-response action. Conversely, neurons projecting from the LDT and posterior PPT synapse onto more ventral mesolimbic structures, such as VTA mesoaccumbens DA neurons, and are more involved in goal-directed approach behaviors (Winn 2006; 2008).

Just as the LDT and PPT send afferents to dopaminergic cell bodies of the VTA, DA is able to indirectly influence the mesopontine region through its projections to the mPFC. Using retrograde and anterograde tracing techniques, major efferents have been identified originating in the mPFC and lateral habenula projecting to the LDT (Satoh and Fibiger 1986). The cortical regions that project to mesopontine cholinergic neurons are innervated by midbrain DA neurons, thus providing the necessary neurocircuitry for METH to affect cholinergic output to the VTA.

The PPT and LDT receive cholinergic input from their respective contralateral site as well as the ipsilateral LDT and PPT, respectively. The vesicular ACh transporter is

Figure 3

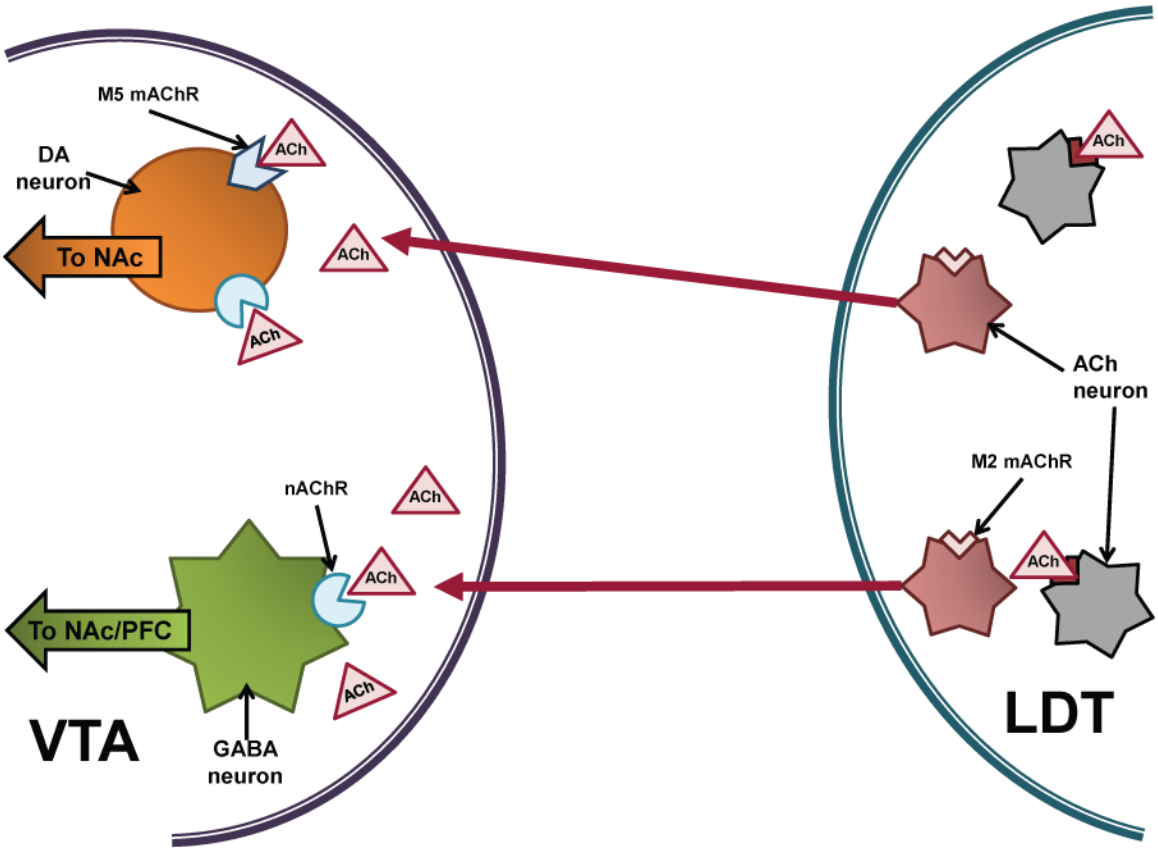


Figure 3. Schematic diagram of cholinergic input to the VTA. Different populations of ACh neurons in the LDT (red stars) project to VTA DA (orange circle) or GABA (green star) neurons. ACh acts on muscarinic ACh receptors (mAChR) or nicotinic ACh receptors (nAChR) in the VTA to stimulate post-synaptic activity. One population of ACh neurons synapse onto VTA GABA neurons that project to the NAc or PFC. Another population of ACh neurons synapse onto VTA DA neurons that project to the NAc. ACh neuron activity in the LDT is in part controlled by M2 type mACh autoreceptors (M2 mAChR) located on ACh soma. Binding of ACh to these autoreceptors inhibits neuronal activity, indicated by the grey ACh neurons.

located on cholinergic terminals and dendrites and is responsible for ACh uptake for local storage into vesicles within the dendrites and terminals (Garzon and Pickel 2000). The cholinergic neurons in these areas possess autoinhibitory M2-subtype mAChRs, which have been shown to activate a TTX-insensitive hyperpolarization of cholinergic neurons (Luebke et al. 1993). Although the majority of muscarinic receptors in

brainstem structures appear to be the M2 autoreceptor type (Leonard and Llinas 1994; Li et al. 1991), evidence shows that M2 mRNA is not restricted to choline acetyltransferase (ChAT)-containing (i.e., cholinergic) neurons (Luebke et al. 1993; Vilaro et al. 1992). Thus, the M2 autoreceptor may also be located on other neuronal sub-types, such as GABA and glutamate. Inhibition of cholinergic firing via administration of a M2 receptor agonist decreased DA firing and release in the VTA (Lodge and Grace 2006) while mAChR antagonists administered into the LDT increased the number of active neurons in the VTA (Mena-Segovia et al. 2008). The latter effect likely occurs as a consequence of blocking the inhibitory ACh autoreceptors, which then disinhibit ACh projections to the VTA. Cholinergic neurons within the LDT also increased firing following bath application of nicotine *in vitro* (Ishibashi et al. 2009). This activation was blocked by nAChR antagonists suggesting the presence of nAChRs as well as mAChRs within the LDT.

The PPT and LDT also receive input from the NE-containing neurons of the LC and the 5HT-containing neurons of the dorsal raphe nucleus (DR). Projections from the DR were reported to inhibit cholinergic cells in the PPT and LDT associated with rapid eye movement (REM) sleep (termed REM-on cells) through actions on inhibitory postsynaptic $5HT_{1A}$ and $5HT_{1B}$ receptors (Monti 2011). The LC can also inhibit the REM-on cholinergic cells of the PPT and LDT through actions at postsynaptic inhibitory α_2 receptors (Samuels and Szabadi 2008). Iontophoresis of NE or 5-HT into the LDT of anesthetized rats inhibited two-thirds of neurons measured, but NE also excited a few neurons while 5-HT never did (Koyama and Kayama 1993). In unanesthesized cats, NE increased tonic discharge of LDT cholinergic neurons associated with REM sleep or wakefulness and switched the ACh neurons associated with REM sleep from bursting to tonic activity (Koyama and Sakai 2000). In this study, though, application of 5-HT to the

LDT had no effect on cholinergic neuron activity. NE from the LC is thought to excite the population of cholinergic neurons in the PPT and LDT associated with wakefulness through actions at excitatory postsynaptic α_1 receptors (Samuels and Szabadi 2008). It is unknown whether NE or 5-HT release in the LDT stimulates LDT cholinergic projections to the VTA. Additionally, to my knowledge there have been no investigations on whether METH stimulates PPT or LDT ACh neurons (and ultimately affects ACh release within the VTA or SN) via action at the NE or 5-HT transporters on terminals within the PPT or LDT. However, given that METH is a substrate for the NE and 5-HT transporters, it is reasonable to hypothesize that METH could also be inducing NE and 5-HT release within the LDT, which in turn could have inhibitory and excitatory affects on the LDT cholinergic neurons.

Lesion studies and pharmacological or electrical manipulation of the mesopontine region further support the existence of topographic projections from the LDT/PPT to the VTA/SN. Lesioning the LDT inhibited neostigmine-induced increases in terminal DA levels within the NAc; however, lesions of the PPT had no effect on neostigmine-induced increases in NAc DA levels (Blaha and Winn 1993). Furthermore, electrical stimulation of the LDT induced increases in intra-VTA ACh, which activated DA neurons and stimulated DA output in the mesocorticolimbic pathway via nAChRs and mAChRs (Forster and Blaha 2000; Forster et al. 2001; Lodge and Grace 2006). As one would expect given its more dorsal-striatal connections, stimulation of the PPT, via injection of the muscarinic antagonist scopolamine, increased DA burst firing and efflux in the dorsal striatum and behavioral activation (Chapman et al. 1997; Lokwan et al. 1999; Yeomans et al. 1993).

Constituents of the mesopontine tegmentum. Located within the PPT and LDT are neurons that contain ACh, GABA, glutamate, and neuropeptides like substance P (for a review see Winn 2006). Distribution of the neuronal subtypes is not homogeneous in either region and it is estimated that ACh neurons account for approximately 23% of all neurons in the PPT and 32% in the LDT (Wang and Morales 2009).

The PPT is considered analogous in structure to the SN, with a pars compacta and pars reticulata; however, the borders between the two areas (and neuronal subtypes) remain indistinct (Winn 2006). Cholinergic, glutamatergic, and GABAergic neurons were intermingled within each subdivision of the PPT (Wang and Morales 2009). Within the pars compacta, the glutamatergic neurons were 1.5 times more concentrated than the cholinergic neurons and GABAergic neurons were the minority. However, in the pars reticulata the GABAergic neurons dominated and were about 2 times more concentrated than the cholinergic minority.

Similar to the PPT, cholinergic, GABAergic and glutamatergic neurons were intermixed and non-homogenously distributed throughout the LDT (Wang and Morales 2009). Within the rostral LDT glutamate neurons dominated and cholinergic neurons were the minority. The area that showed the most concentrated cholinergic presence was the medial portion (32% of total neurons); however, GABA and glutamate neurons were similar in concentration to the cholinergic population. GABAergic neurons were the majority in the caudal portion of the LDT, with cholinergic neurons representing the minority.

There was little co-expression of glutamate or GABA within ChAT-immunoreactive (i.e. cholinergic) neurons in the PPT or LDT; however there might be co-

expression of ACh with glutamate or GABA at PPT or LDT terminals. Although the cholinergic neurons were not the majority in either structure, they are important for the maintenance of several physiological functions and behaviors (*this is discussed in more detail in the next sub-section*).

Function of the pedunculopontine tegmental nucleus. An abundance of evidence suggests the PPT is important for learning. Through its connections with midbrain DA neurons, the PPT supports the formation of action-outcome associations, such as operant behaviors. The PPT has also been implicated in attention, sensory processing, stereotypy and Parkinson's disease; however, these are beyond the scope of this introduction. The reader is referred to the general discussion (**Chapter 5**) and to a review by Phillip Winn for a more detailed description of the role of the PPT in these processes (Winn 2006).

Several studies have investigated the role of the PPT in various types of learning by performing PPT lesions either pre- or post-exposure to the behavioral procedure. Of most relevance to the current dissertation are the studies that have evaluated the effect of PPT lesions on reward-related learning, including CPP and operant self-administration measures. Some studies have also evaluated the role of the PPT on maze learning (Dellu et al. 1991; Keating and Winn 2002; Taylor et al. 2004).

Rats with a bilateral excitotoxic lesion of the PPT failed to discriminate between a conditioned stimulus that had been associated with food delivery (conditioned stimulus positive or CS+) and a conditioned stimulus that was never associated with a motivational event (conditioned stimulus negative or CS-), as indexed by conditioned approach behavior (Inglis et al. 2000). In the same study, rats with a bilateral PPT lesion

were trained in a classical conditioning procedure in the first phase, where a CS+ predicted the delivery of a sucrose reward (US). The second phase of training required the rats to discriminate between two levers: depression of one triggered presentation of the CS+ and depression of the other had no consequence. Rats with a PPT lesion did not display conditioned approach behavior in the first phase and could not discriminate between the reinforced and non-reinforced levers in the second phase (Inglis et al. 2000). Similarly, in a separate study, rats with a bilateral lesion of the PPT failed to discriminate between the non-reinforced and reinforced levers in a conditioned approach procedure (Inglis et al. 1994b). Importantly, the PPT lesioned rats' performance was not due to differences in locomotor activity, compared to sham operated controls (Inglis et al. 1994b). These findings suggest that the PPT is necessary for forming CS-US associations or for discriminating between a CS+ and CS-. A more general interpretation of these findings is that the PPT is necessary for selection of the appropriate behavioral output.

Bilateral lesion of the PPT also produced deficits in acquiring other operant behaviors, such as operant lever pressing for rewarding intra-cranial brain stimulation (Lepore and Franklin 1996) and intravenous amphetamine administration (Alderson et al. 2004). In addition, PPT lesion attenuated the acquisition of a place preference for morphine (Bechara and van der Kooy 1989) and amphetamine (Olmstead and Franklin 1994). In a more recent experiment, Alderson and colleagues found that the posterior portion of the PPT in particular is important for intravenous nicotine self-administration in rats (Alderson et al. 2006).

While an abundance of data suggests the PPT is necessary to learn a CS-US association, the PPT does not appear to be required for the expression of a previously

learned association. For instance, pre-training of CS-US association (Bechara and van der Kooy 1989) or an operant response associated with food delivery (Alderson et al. 2004) prior to making a bilateral PPT lesion did not affect post-surgical expression of preference or self-administration, respectively. In these studies, rats were trained on morphine CPP or to press a lever to receive food. After training was complete, rats received a bilateral PPT lesion. Post-surgery, PPT-lesioned rats successfully expressed morphine place preference or transitioned from operant food self-administration to intravenous amphetamine self-administration. These findings indicate that the PPT is not necessary for the expression of an already learned association. Furthermore, since PPT-lesioned rats expressed normal morphine CPP and self-administration of amphetamine, this suggests that the subjects still found the stimuli rewarding or reinforcing. Thus, it is likely that the PPT lesion did not diminish the rewarding or reinforcing value of the stimuli.

Interestingly, rats that were trained to self-administer food pellets prior to a PPT lesion acquired responding for intravenous amphetamine on a fixed ratio (FR), but the same PPT-lesioned subjects showed significantly fewer amphetamine infusions and a lower breakpoint when tested on a PR schedule, when compared to sham operated controls (Alderson et al. 2004). Compared to the FR schedule, the PR schedule is more cognitively and motorically demanding because the contingency increases throughout the procedure. So, while no deficit was detected in pre food-trained rats on a FR schedule, the more demanding PR schedule revealed the presence of a deficit in PPT lesioned rats. This deficit could be explained by a failure of the rats to learn the more cognitively demanding contingency or reflect an impairment of motor behavior; however, PPT lesions did not appear to have an effect on motor behavior (see below). These experiments therefore suggest that the PPT is important for learning.

Additionally, the PPT is not only necessary for learning contingencies that involve positive reinforcers, but also for learning associations between cues and negatively valenced reinforcers, such as a foot shock. Rats with a bilateral PPT lesion had a significantly shorter latency to enter the dark compartment of a shuttle box, which had previously been paired with a foot shock, compared to sham operated controls (Fujimoto et al. 1992). In addition, PPT lesioned rats had a significantly lower avoidance rate in an active avoidance procedure compared to sham operated controls (Fujimoto et al. 1992; Fujimoto et al. 1989). However, similar to self-administration and CPP procedures with drugs of abuse, rats that were trained in the active avoidance task *prior* to receiving bilateral PPT lesions displayed avoidance rates comparable to sham operated controls (Fujimoto et al. 1992). Interestingly, PPT lesioned rats that failed to acquire the active avoidance procedure also had significantly lower ACh levels in the medial thalamus and SN, which are projection targets of PPT cholinergic neurons (Fujimoto et al. 1989).

The ability of the PPT to affect learning of stimulus-outcome associations does not appear to be due to reward devaluation or due to a deficit in motivation or locomotor activity. PPT lesions actually increased consumption of a sucrose solution and had no effect on home cage feeding behavior (Alderson et al. 2001; Olmstead et al. 1999). Additionally, in a separate study, PPT-lesioned rats allowed to consume 20% sucrose reduced their home cage food intake and maintained caloric intake equal to that of sham operated controls (Keating et al. 2002). Interestingly, PPT lesions failed to affect acquisition of sucrose CPP, across multiple concentrations of sucrose (Alderson et al. 2001). The results of this study are in direct opposition to other studies suggesting the PPT is necessary for acquisition of a CPP. The authors suggest that methodological differences in the lesion procedure (and the ultimate extent of the lesion) could account

for the different results between studies. Additionally, rats in this study were food restricted in order to motivate them to consume sucrose during conditioning trials. Therefore, the motivational state of the animal might influence whether a PPT lesion disrupts acquisition or expression of a place preference. These studies indicate that 1) the motivational state of the animal might influence whether a PPT lesion disrupts acquisition or expression of a place preference, and 2) lesions of the PPT do not affect the ability to perceive a sucrose reward.

Several studies have also shown that PPT lesions do not affect acute amphetamine hyperactivity or spontaneous locomotor activity (Inglis et al. 1994b; Olmstead and Franklin 1994). However, rats with PPT lesions developed abnormal orofacial stereotypies in response to amphetamine (Inglis et al. 1994a) and showed a general inability to suppress inappropriate movements during a conditioned reinforcement procedure that required rats to press and hold a lever until a trigger stimulus signaled to stop (Florio et al. 1999). This perseverative behavior may explain the increase in sucrose consumption in PPT lesioned rats that previous studies have reported (see above).

These data indicate that the PPT is important for the formation of positively- and negatively-valenced action-outcome or stimulus-outcome associations and in selecting the appropriate behavioral output. The rewarding and reinforcing qualities of drugs of abuse facilitate strong associations with stimuli that signal their delivery and with actions that produce their delivery. Further, presentation of stimuli previously associated with drug delivery can induce drug-seeking behavior and compulsive drug-seeking is a hallmark of drug dependence (see **section 1**). Therefore, understanding the neurochemical underpinnings of this this kind of associative learning might reveal novel

therapeutic targets to treat aspects of drug-dependence, such as chronic drug-seeking behavior. Given the neurochemical connections between the PPT and SN DA neurons and the aforementioned behavioral studies, it is hypothesized that the PPT serves as a relay between incoming sensory information and DA neurons so the organism selects appropriate motor outcomes. *This background suggests that PPT ACh projections should not be a primary mediator of dopaminergic responses within the NAc, which is the primary dopaminergic site mediating METH reward. However, no studies have investigated the role of the PPT in METH-induced neurochemical responses in the mesocorticolimbic reward pathway. One goal of this dissertation is to determine the contribution of PPT ACh neurons in METH-induced DA responses in the NAc and ACh responses in the VTA (see* ***Chapter 3****).*

Function of the laterodorsal tegmental nucleus. The LDT has been implicated in a variety of behaviors and physiological functions including wakefulness and REM sleep, sensorimotor gating and psychosis, locomotor activity and stereotypy and midbrain DA responses and reinforcement. Since the majority of these functions are beyond the scope of this introduction, the reader is referred to the general discussion (**Chapter 5**) for a more in depth review. This sub-section will focus on reviewing the role of the LDT in DA signaling within the mesocorticolimbic pathway, spontaneous and stimulant induced locomotor activity, sensitization and reinforcement. In contrast to the literature on the function of the PPT, however, there have been no studies regarding the role of the LDT in a CPP procedure.

Bilateral LDT lesions in rats attenuated sensitization of locomotor activity to nicotine (Alderson et al. 2004). However, the literature regarding the role of the LDT in amphetamine-induced locomotor activity and stereotypy is mixed and seems to be due

to differences in methods between studies. One investigation reported that rats with a bilateral LDT lesion exhibited attenuated sensitization of amphetamine-induced stereotypy, but not locomotor activity (Laviolette et al. 2000). In contrast, an investigation using a different sensitization procedure found no effect of bilateral LDT lesion on the sensitization of amphetamine stereotypy, but did note an inhibition of post-stereotypy amphetamine hyperactivity (Nelson et al. 2007). Another study indicated that bilateral LDT lesion led to increased amphetamine stereotypy and exaggerated amphetamine-induced DA response in the NAc (Forster et al. 2002a).

Lesions of the LDT also result in changes in neurochemical responding within the mesocorticolimbic reward pathway. Excitotoxic lesion of the rat LDT, but not the PPT, attenuated neostigmine-induced increases in NAc DA levels (Blaha et al. 1996). In addition, electrical stimulation of the LDT increased DA efflux in the NAc (Forster and Blaha 2000). Furthermore, systemic administration of cocaine significantly enhanced LDT-stimulated levels of DA in the NAc and this was blocked by microinjection of scopolamine into the VTA (Lester et al. 2010). This suggests that the LDT-to-VTA cholinergic projection is important in modulating cocaine's effects on NAc DA release.

Despite agreement in the literature that a functional connection exists between the LDT and the dopaminergic reward pathway, there is a paucity of investigations on the role of the LDT in reward-related behaviors. However, one study tested the effect of reversible inhibition of the LDT cholinergic neurons via microinjection of the mAChR agonist, OXO, on a rat model of intravenous cocaine self-administration. Intra-LDT OXO microinjection decreased intravenous cocaine self-administration under a FR schedule and the breakpoint for cocaine responding under a PR schedule of reinforcement (Shabani et al. 2010). In addition, intra-LDT OXO microinjection attenuated food self-

administration (Shabani et al. 2010). This study suggests that the LDT cholinergic projection may be involved in cocaine and food reinforcement. *However, no studies have investigated the contribution of the LDT cholinergic neurons on METH-induced neurochemical responding or reward. One aim of this dissertation is to determine the role of LDT ACh neurons in METH-induced DA responses in the NAc and ACh responses in the VTA (**Chapter 3**). Another aim is to characterize the role of the LDT in METH reward (**Chapter 4**).*

5. Animal models of methamphetamine reward

In this section I will review methods commonly used to assess drug reward using rodent models: self-administration, intra-cranial self-stimulation (ICSS), conditioned reinforcement and Pavlovian approach. The CPP procedure will be discussed in detail in **section 6**. These procedures have been used to characterize the neurotransmitters, receptors and neuroanatomical sites underlying METH reward.

Self-administration. Operant self-administration is a method often used in rodents to assess the reinforcing effects of drugs. This procedure has high face validity for human drug abuse and allows the investigator to individually assess the appetitive and consummatory phases of drug taking. The procedure typically requires the subject to operate a manipulandum, such as a lever or a nose-poke hole, in order to receive an automatic infusion of the drug or access to consume the drug. Operation of the manipulandum is often accompanied by the presentation of a discrete conditioned cue, such as a light, to signal the availability or delivery of the drug. Subjects may be required to operate the manipulandum a certain amount of times before drug is delivered. This requirement is termed a schedule of reinforcement and is pre-

determined by the investigator. Drug self-administration can occur via an indwelling intravenous jugular catheter, “by mouth” (i.e., drinking), intragastrically, or intracranially into a specific neuroanatomic site or the cranial ventricle (termed intracerebroventricular, or ICV, administration). These routes of administration require surgery and time for post-operative recovery before the behavioral training can begin. Ethanol self-administration is typically indexed using the oral route of administration; however, there are exceptions. For instance, recent ethanol studies have used an intragastric route of administration for ethanol consumption in mice and rats (Fidler et al. 2006; Fidler et al. 2011). In addition, mice have been selectively bred for high or low drinking of a METH solution (Shabani et al. 2011; Wheeler et al. 2009).

METH self-administration has been reported in rodent models using a variety of routes of administration including drinking, ICV administration and intravenous administration. However, most investigations of METH self-administration (especially using an intravenous route of administration) use a rat rather than a mouse model. This is likely due to the increased difficulty of the surgical procedure, of catheter patency maintenance and with animal survival over a prolonged time using a mouse model. In addition, it is possible that the optimum set of parameters (e.g. unit METH dose, manipulandum selection, stimuli selection, session length, etc.) have not been reliably established for intravenous METH self-administration in a mouse model.

Studies in rats have investigated the effect of behavioral stressors as well as neural receptors implicated in stress on the acquisition and reinstatement of intravenous METH self-administration (Moffett and Goeders 2005; 2007; Shepard et al. 2004). Blockade of the anxiogenic corticotropin-releasing hormone receptor attenuated the reinstatement of METH seeking (Moffett and Goeders 2007). Conversely, administration

of the anxiogenic compound yohimbine induced METH reinstatement (Shepard et al. 2004); however, behavioral stress or administration of corticosterone did not facilitate METH self-administration (Moffett and Goeders 2005). Additionally, administration of the $GABA_B$ receptor agonist baclofen dose-dependently inhibited METH self-administration in rats (Ranaldi and Poeggel 2002). The effect of cholinergics has also been evaluated in rat models of METH self-administration. Treatment with nicotine during METH withdrawal attenuated METH-primed reinstatement of METH seeking (Hiranita et al. 2004). In addition, pretreatment with lobeline, a mixed VMAT-2 ligand and nAChR agonist, attenuated METH self-administration across a range of METH doses (Harrod et al. 2001b).

There are only 5 published studies of intravenous METH self-administration using a mouse model, and 4 of the 5 come from the same laboratory. The first published intravenous METH self-administration study tested a variety of drugs of abuse in C57BL/6J mice (Carney et al. 1991). From their graphs it appears that mice administered an average of 17 mg/kg METH per 23 h session. Compared to the other mouse studies (and even the rat studies), this is quite a large amount of METH. However, mice in this study had practically continual METH access (23 h/day) and these data were only from a total of 3 subjects. The more recent studies used either the C57BL/6J inbred strain or mutant mouse lines that either have (1) a partial reduction in the expression of glial cell line-derived neurotrophic factor or (2) a complete knock out of tumor necrosis factor alpha (Yan et al. 2012; Yan et al. 2006; Yan et al. 2007a; Yan et al. 2007b). In daily 3 h self-administration sessions mice administered an average of 1 to 3 mg/kg METH. However, in these experiments METH-prime did not consistently reinstate extinguished METH seeking.

Current literature on METH intravenous self-administration suggests that METH is reinforcing in both the mouse and rat. This method allows investigators to explore the neurochemical substrates of METH reinforcement and to test potential therapeutic treatments on drug taking and seeking behaviors. While this method has high face validity for human METH abuse it also has limitations. METH has a biphasic reward curve, with higher doses eliciting aversion. This can make selection of a successful unit dose difficult, as I have discovered in unpublished experiments. In these experiments, it was necessary to initially train the mice to respond on an operant to receive a food pellet before introducing METH self-administration. In other words, in my hands, the mice were not "capable of learning" to lever press to receive intravenous METH without prior food training. For the full description of methods and results of the intravenous METH self-administration experiment, the reader is referred to **Appendix II.** In addition, a recent review evaluated the methods used in published mouse self-administration experiments (Thomsen and Caine 2011). It revealed that commonly used methods, including initial food training, for mouse drug self-administration can lead to the false-positive result that the subjects find the drug reinforcing. In other words, the initial food training required to establish METH self-administration makes it difficult to interpret whether lever pressing behavior in response to the cue reflected a food-cue or METH-cue association.

METH self-administration using a drinking route of administration has been recently characterized in a series of studies (Shabani et al. 2012a; Shabani et al. 2012b; Shabani et al. 2011; Wheeler et al. 2009). In these studies, mice were selectively bred for either high METH drinking (MAHDR) or low METH drinking (MALDR) using a 2-bottle choice procedure (Shabani et al. 2011; Wheeler et al. 2009). MAHDR mice showed greater consumption of different concentrations of METH and a higher METH

consumption preference compared to MALDR mice. Furthermore, the MAHDR mice showed a significant METH *place* preference while the MALDR mice did not. Additionally, in operant self-administration procedures MAHDR mice showed more operant responding for METH (for oral access and ICV) and greater consumption of oral METH compared to MALDR mice (Shabani et al. 2012a). These experiments showed that mice will self-administer METH across several routes of administration and suggest a genetic underpinning to METH reinforcement. The accumulation of literature reported in this section indicates that investigators have used a variety of self-administration preparations in rats and mice to evaluate the reinforcing qualities of METH. However, other methods have also been used and are discussed below.

Intra-cranial self-stimulation. Olds and Milner established that electrical stimulation of the medial forebrain bundle and septal brain area supports self-administration and is therefore reinforcing (Olds and Milner 1954). Additionally, they found that subjects returned to the environment in which they received electrical stimulation of these brain areas, suggesting that the stimulation was also rewarding. In the ICSS procedure, subjects perform an operant (e.g., lever press) to receive an electrical pulse or train of pulses into an area of the brain (usually the medial forebrain bundle or lateral hypothalamus) (for a brief review of the methods see Wise 1996). Several of the neuroanatomical sites that mediate the reinforcing and rewarding effects of ICSS lie within the mesocorticolimbic reward pathway and overlap with those that mediate the rewarding aspects of drugs of abuse (Cheer et al. 2007; Fiorino et al. 1993; Zacharko et al. 1990). Additionally, mice conditioned a place preference for the environment in which they had access to ICSS (Ikeda et al. 2001). It is therefore not surprising that ICSS is often considered a measure of reward in addition to reinforcement and used to index the rewarding effects of drugs of abuse.

To assess the ability of a drug to alter ICSS, subjects are first trained on the ICSS procedure. Once stable responding for intracranial stimulation is attained, subjects are administered the test drug and allowed to respond to receive a range of stimulation frequencies. Typically, the stimulation frequency is varied while the intensity, pulse number and pulse width are held constant. This enables the experimenter to change the number of action potentials elicited while maintaining a constant number of stimulated neurons (Wise 1989). If the drug synergizes with the rewarding and reinforcing effects of ICSS, it will produce a leftward shift in the rate-frequency curve. In other words, there is a decrease in the frequency threshold that previously supported behavior. Conversely, when a higher frequency threshold is required to support behavior, there is a rightward shift the rate-frequency curve. This suggests that the drug antagonized the rewarding and reinforcing effects of ICSS. Drugs of abuse, such as amphetamines, typically cause a leftward shift in this curve and can negate the reward antagonistic effects of drugs (usually DA antagonists) that induce rightward shifts in the curve.

Multiple studies have reported a synergistic effect of the administration of drugs of abuse, such as amphetamines (Schaefer and Michael 1988; Wise and Munn 1993), morphine (Bauco et al. 1993) and cocaine (Katsidoni et al. 2011), with the rewarding effects of ICSS. Pre-treatment with METH also induces a leftward shift in the rate-frequency curve, suggesting that METH enhances ICSS (Higley et al. 2011b; Miyata et al. 2011; Spiller et al. 2008). Interestingly, presentation of cues that were previously predictive of cocaine or morphine delivery also reduced the threshold for ICSS (Hayes and Gardner 2004). This experiment suggests that the Pavlovian association between the cue and the rewarding effects of the drugs was responsible for the decrease in ICSS threshold.

Techniques based on conditioned stimulus – unconditioned stimulus drug associations. Environmental stimuli can exert significant control over behavior, especially when those stimuli have been previously paired with drugs of abuse. Clinical studies of human populations dependent on methamphetamine (Price et al. 2010; Tolliver et al. 2010), cocaine (Fox et al. 2006; Volkow et al. 2010b) and alcohol (Hammarberg et al. 2009; Ooteman et al. 2007) reported increased subjective craving scores following exposure to drug related cues (e.g., pictures or videos of people using drugs, drug paraphernalia). The rewarding effects of drugs of abuse are therefore often inferred from their ability to enter into Pavlovian (stimulus-outcome) associations with environmental cues. In the laboratory, this procedure involves pairing a previously neutral cue (CS), such as a cue light, with a stimulus that has inherent motivational value (US), like a drug of abuse. Following formation of this association several techniques can index whether the US is rewarding. Conditioned place preference is one such method. Since this is the method I selected for the experiments presented in **chapter 4**, I have provided a detailed description of it in a separate section (**section 6**).

Another way to assess the rewarding value of a US is to test the ability of the CS to be a conditioned reinforcer, that is, its ability to facilitate the acquisition of an instrumental response in the absence of the US. During the CS-US pairings, it is thought that the CS acquires some of the incentive motivational properties of the US, thereby gaining motivational control over instrumental behavior (for a review see Everitt et al. 2001). Drugs of abuse can enhance the ability of a CS to function as a conditioned reinforcer. In one experiment, thirsty rats were initially trained to associate presentation of a light with water delivery. After establishing this association, administration of amphetamine into the NAc enhanced responding on a novel lever that resulted in

presentation of the conditioned reinforcer (i.e., the light) (Taylor and Robbins 1984; 1986). Additionally, drugs of abuse have also been used as the US in conditioned reinforcer experiments. A CS previously paired with intravenous administration of cocaine (Di Ciano et al. 2007; Di Ciano and Everitt 2004; Di Ciano et al. 2008) or heroin (Di Ciano and Everitt 2004) facilitated a new lever-press response for that CS in absence of the drug.

Pavlovian approach is another technique used to assess the rewarding value of a US after establishing a CS-US association. This is measured in autoshaping or "sign-tracking" experiments and involves observing the subject's discriminative approach behavior to a CS that has been previously paired with a reward, as opposed to a CS that was never associated with a reward. The CS elicits approach behavior because it attains motivational value through pairing with a reward. Approach to the CS previously paired with the reward is thought to be mediated by the ability of the rewarding US to imbue the CS with motivational incentive value (Everitt et al. 2001). For example, rats approached a CS (white vertical rectangles on a screen) that had been paired with the delivery of food, even though this response was noncontingent on reward delivery (Cardinal et al. 2002). Additionally, cues paired with the administration of cocaine (Uslaner et al. 2006) and ethanol (Cunningham and Patel 2007) have also been shown to elicit approach behavior in rats and mice, respectively. Thus, Pavlovian approach provides a means to assess the rewarding value of a drug based on a CS-US drug association.

There are additional techniques that involve establishing a CS-US drug association, such as Pavlovian-to-instrumental transfer and second order schedules, but these are not necessarily used to assess the rewarding value of a US or a simple CS-US

association. Instead, these procedures address larger questions, such as the ability of a CS to maintain motivation and responding during complex reinforcement contingencies (for reviews of these procedures see Holmes et al. 2010; Schindler et al. 2002).

6. Conditioned place preference

Place preference is a form of stimulus-outcome associative learning that assesses the motivational value of a stimulus through the observation of choice behavior. The basic procedure involves pairing a distinct CS (the CS+) with a rewarding or aversive US. Additionally, a separate distinct CS (the CS-) is paired with a neutral stimulus (e.g. saline). As the subject learns an association between the CS+ and the US, the CS+ begins to elicit conditioned approach behavior (or conditioned avoidance if the US was aversive). The amount of place preference or aversion is measured in a drug-free choice test, in which the animal is allowed access to both the CS+ and CS-, and the time spent in each is measured.

Place preference is often used in rodent models to infer the rewarding value of drugs of abuse by measuring how much time the subject spends in contact with the CS+ (Tzschentke 1998). In rodent place preference studies evaluating drug reward, the CS is typically a compartment with distinct tactile (e.g., floor textures), visual (e.g., colors of the walls or brightness), or olfactory (e.g., almond extract) cues. The animal is given an injection of the drug and placed in one of the distinct compartments for a given amount of time. Typically 24 h later, the animal is given an injection of saline or no injection and placed in the opposite distinct compartment. These pairings (termed conditioning trials) continue to alternate until the animal has experienced usually 2 – 4 of each; however, the number of pairings depends on several variables, including the drug being tested. Following the conditioning trials, a drug-free preference test is administered in which the

animal is given an injection of saline and placed in the apparatus with access to both compartments. A place preference is represented by the animal approaching and staying in contact with the drug-paired compartment (the CS+) more than the saline-paired compartment (the CS-). Conversely, a conditioned place aversion (CPA) is when the animal avoids the CS+ and spends most of its time in contact with the CS-.

The place preference procedure allows the investigator to assess different stages of associative learning. For instance, a manipulation may be made during or before the conditioning trials to assess the effect of the manipulation on the acquisition of place preference. Alternatively, the investigator may wait to apply a manipulation until the preference test, which would test the expression of the place preference. Similar to operant self-administration procedures, you can also evaluate extinction and reinstatement of the preference behavior. Extinction is achieved by administering repeated pairings of the previously drug-paired CS in the absence of the drug (US). Following extinction training, the subject no longer shows a preference for the originally drug-paired CS. A variety of stimuli have been shown to induce reinstatement of an extinguished place preference, including priming doses of the drug itself and stress (e.g. foot shock or physical restraint) (Aguilar et al. 2009; Cruz et al. 2010; Qi et al. 2009).

An important consideration in the place preference procedure is apparatus bias, and this topic was recently discussed in a review (Cunningham et al. 2003). Initial preference for one compartment of the apparatus prior to conditioning can produce a ceiling effect, which results in the unconditioned preference obscuring the conditioned preference. In addition, pairing a rewarding drug with an initially non-preferred side leads to problems in the interpretation of a CPP. If the non-preferred side initially induced anxiety (which may be observed in white or brightly lit compartments), a CPP

may reflect anxiolytic rather than rewarding properties of the drug. A related concern is subject assignment bias, which is when the investigator systematically pairs one compartment with the test drug and the other compartment with vehicle. If one compartment is better at conditioning a drug effect than the other, this bias can lead to a false positive or false negative preference. Subject assignment bias is especially troublesome when compounded by an existing apparatus bias.

In contrast to intravenous self-administration procedures, CPP experiments do not require lengthy pre-training or technically demanding surgical procedures and thus take less time to complete. In addition, between subjects preference/reward comparison is less confounded due to each subject receiving the same amount of drug. Furthermore, the drug-free preference test allows for assessing the dependent variable in the absence of drug-induced effects on cognition, motor behavior, or attention. However, the CPP procedure lacks the face validity of the self-administration procedure since the experimenter administers the drug. Subjects may also display novelty-seeking in the CPP procedure, which can confound interpretation of a side preference during the test phase. Despite these limitations, CPP provides a useful measure of the ability of environmental cues previously paired with drugs of abuse to elicit drug-seeking behavior and allows the investigator to make inferences about the rewarding value of a drug.

Substrates of methamphetamine conditioned place preference. Everitt and Robbins theorize that psychostimulants are capable of enhancing the salience of environmental stimuli, thereby creating abnormal CS-US associations (Everitt and Robbins 2005). Place conditioning is useful for assessing the strength of CS-US associations and several studies have investigated the ability of METH to establish a CPP in rodent models. Previous studies have evaluated the genetic correlates of METH reward by

evaluating METH CPP in inbred mouse strains, the F2 generation from the cross of inbred strains, and in mice selectively bred for METH consumption phenotypes (Cunningham and Noble 1992; Shabani et al. 2011; Wheeler et al. 2009). In addition, investigators have used a mouse model of METH CPP to evaluate specific neuroanatomical sites and neurochemical substrates of METH reward. For instance, Fos activation of the mPFC and NAc core was observed following conditioning of a METH place preference in C57BL/6J mice (Chiang et al. 2009). The following sub-sections will review the current literature to address the roles of important neurotransmitters within the reward pathway (DA, glutamate, GABA and ACh) in METH CPP.

Dopamine in methamphetamine conditioned place preference. Several studies have investigated the role of the DA receptors in conditioning a METH place preference in mice and rats. Administration of sertindole, a mixed $5HT_2$ and D_2 receptor antagonist, during conditioning inhibited METH CPP in rats (Suzuki and Misawa 1995). In addition, ICV or systemic administration of a D_2 (sulpiride) or D_1 (SCH23390) receptor antagonist prior to METH conditioning trials inhibited acquisition of a METH preference in mice (Kurokawa et al. 2010a; Kurokawa et al. 2010b). Furthermore, systemic administration of SCH23390 or raclopride (D_2 receptor antagonist) immediately before the preference test inhibited the expression of METH CPP in rats (Mizoguchi et al. 2004). However, in this study it was not clear whether the antagonists administered prior to the preference had effects on locomotor activity. Expression of a place preference requires maintaining contact with one of the chambers. Therefore, it is not surprising that enhanced locomotor activity during the preference test can disrupt the expression of a place preference (Gremel & Cunningham, 2007). Despite this confound, the studies generally

indicate that the D_1 and D_2 receptors are necessary for the acquisition and expression of METH place preference.

Glutamate in methamphetamine conditioned place preference. A recent study found a decrease in surface expression and increase in intracellular expression of $mGlu_5$ receptors in the mPFC of rats that express a METH place preference (Herrold et al. 2011). In general, studies have found that the blockade of glutamate receptors inhibits the acquisition of METH place preference in rodent models. Systemic or ICV administration of a range of NMDA receptor antagonists prior to METH conditioning trials attenuated the acquisition of METH place preference in mice and rats (Kim and Jang 1997; Miyatake et al. 2005; Mizoguchi et al. 2004). Likewise, systemic administration of the non-competitive NMDA antagonist, dextromethorphan, inhibited the acquisition of METH preference in rats (Yang et al. 2006). In addition, ICV administration of an AMPA or $mGlu_5$ receptor antagonist prior to METH conditioning trials attenuated acquisition of METH preference in mice (Miyatake et al. 2005).

GABA in methamphetamine conditioned place preference. There are limited data on the role of GABA in METH preference, and the majority of studies have focused on the $GABA_B$ receptor. The $GABA_B$ receptor agonist baclofen attenuated the acquisition and expression of METH place preference and facilitated the extinction of METH place preference in rats (Li et al. 2001; Voigt et al. 2011a). Positive allosteric modulators of the $GABA_B$ receptor (e.g., GS39783 and CGP7930) also interfered with the maintenance of METH place preference when administered after the first preference test (Voigt et al. 2011b). Inhibition of the catabolism of GABA through administration of racemic gamma-vinyl-GABA inhibited METH-primed reinstatement of METH CPP (DeMarco et al. 2009). Importantly, this treatment did not have effects on locomotor activity. Although these

studies suggest that the $GABA_B$ receptor may be involved in METH CPP, a recent study revealed no change in the distribution of $GABA_B$ receptors between the surface and intracellular space in the mPFC, NAc, and VP of rats expressing a METH place preference (Herrold et al. 2011). These studies suggest that GABA receptors may mediate METH reward, and that receptor internalization is not responsible for this effect.

Acetylcholine in methamphetamine conditioned place preference. To my knowledge there are 3 published studies investigating the role of ACh in METH CPP (however, one of the publications is in the Japanese language (Kawamura et al. 2005)). Systemic administration of the mAChR antagonist trihexyphenidyl (THP) attenuated expression of METH CPP, but a different mAChR antagonist, scopolamine, did not (Shimosato et al. 2001). Since THP is also a NMDA receptor antagonist (McDonough and Shih 1995), it is possible that THP's effect on METH CPP was not mediated by the mAChR, but by the NMDA receptor. Another study administered AChE inhibitor donzepil (which increases extracellular ACh levels) prior to METH conditioning trials (Takamatsu et al. 2006b). Although they reported no effect of donzepil on the acquisition of METH CPP, the statistical analyses are suspect. It appears instead of an ANOVA to assess between group (vehicle or donzepil pretreated) differences the authors used individual paired t-tests to compare the pre-conditioning preference to the post-conditioning preference within each treatment group. Therefore, it is not possible to compare the amount of preference between groups, and it is difficult to conclusively state whether donzepil had an effect on the acquisition of METH CPP. *The paucity of investigations of ACh in METH CPP makes it difficult to draw any conclusions about the role of the cholinergic system in METH reward. Furthermore, there have been no studies to investigate the role of a specific cholinergic cell group or site of action in METH CPP. The experiments*

*in this dissertation sought to elucidate the role of LDT cholinergic neurons on METH CPP (**Chapter 4**).*

Selection of conditioned place preference to measure the formation of a CS-US drug association. One of the goals of this dissertation is to characterize the role of the LDT in the formation of an association between METH and discriminative cues paired with METH (**Chapter 4**). In the behavioral experiments of this dissertation I chose to measure the formation of a METH-cue (CS-US) association using the CPP procedure because this procedure was optimal to test my hypothesis that the LDT is involved in the formation of an association between a cue and the rewarding effects of METH (see **section 7**). Other procedures, such as Pavlovian approach (i.e., autoshaping or sign-tracking) or conditioned reinforcement, that involve establishing a CS-US association have also been used to index the rewarding value of drugs of abuse. Pavlovian approach, however, relies on the CS to elicit approach behavior. This can result in differences between subjects in the amount of time spent in contact with the CS and can add unwanted error variance, thus making comparisons between groups difficult . In contrast, our procedure for CPP forces the subject to remain in contact with the CS during the conditioning trial, so that all subjects receive an equal amount of time experiencing the CS during the conditioning trial. Conditioned reinforcement establishes a CS-US association like CPP, but uses a different index to measure that association, i.e., the ability of the CS to enhance acquisition of an operant behavior versus observation of choice behavior in presence of the CS+ and CS-. Given my difficulties in establishing operant behavior in a mouse model (see the self-administration sub-section above and **Appendix II**) I felt the CPP procedure was better suited to test my hypothesis.

Procedures that involve performance of an instrumental response, such as drug self-administration and ICSS are also often used as measures of drug reward. However, both of these procedures require the subject to acquire an operant response to receive the US, and in previous experiments I was not able to establish operant intravenous METH self-administration in mice without initial food training (**Appendix II**). Initial food training, especially when the same discriminative stimulus is used to signal food and METH, confounds interpretation of the subject's drug seeking behavior. Moreover, prior food training can lead to a false positive result of the subject finding the drug reinforcing (Thomsen and Caine 2011).

In contrast to the mouse model, there is an abundance of literature on intravenous METH self-administration in the rat. While it may have been easier to establish METH self-administration and ultimately measure deficits in the acquisition of a METH-cue association using a rat model, the previous neurochemical experiments (**Chapters 2** and **3**) used a mouse model. The findings of these experiments (that METH stimulates LDT ACh neurons to induce a prolonged increase in VTA ACh levels) in part led to my hypothesis for the behavioral experiments. However, it is not clear whether METH stimulates the LDT cholinergic neurons (by indirectly activating the LDT via activation of PFC glutamatergic projections; Figure 2) to induce increased ACh levels within the VTA of the rat.

Based on this rationale, the use of a mouse model and the CPP procedure was the optimal method to assess the role of the LDT in the acquisition of a Pavlovian association between a cue and the rewarding effects of METH.

7. Summary, rationale and dissertation goals

METH abuse is a costly public health concern and understanding its neurobiology is imperative to establish pharmacologic treatments. Similar to other drugs of abuse, METH acts within the reward pathway of the brain to induce DA release in the NAc, which is rewarding and facilitates the creation of associations between METH-related cues (e.g. drug paraphernalia) and METH reward (Baler and Volkow 2006; Day and Carelli 2007; Wise 1982). Exposure to drug-conditioned cues can elicit craving and drug-seeking behavior. Within the reward pathway the neurotransmitter ACh acts on DA cell bodies within the VTA to modulate downstream DA release and reward (Ikemoto and Wise 2002; Westerink et al. 1998; Westerink et al. 1996). Intra-VTA ACh originates in the LDT and PPT of the mesopontine tegmentum (Oakman et al. 1995; Omelchenko and Sesack 2005), and these areas have also been implicated in DA release and reward-related learning (Alderson et al. 2004; Forster and Blaha 2000). In addition, cocaine delivery or cues associated with cocaine delivery induced ACh release in the VTA of rats previously trained to self-administer cocaine (You et al. 2008). This presumably occurred by cocaine indirectly stimulating the LDT cholinergic neurons through increased DA levels within the mPFC, thereby activating glutamatergic projections to the LDT. Furthermore, increased VTA ACh levels were associated with cocaine seeking, suggesting that intra-VTA ACh release may signal DA neurons and energize drug-seeking behavior. While the literature reports the effect of METH on ACh levels within the striatum (McGeer et al. 1974; Taguchi et al. 1998; Tsai and Chen 1994), it is unknown whether METH induces ACh release in the VTA. Given previous research on cocaine, a METH-induced increase in VTA ACh may be related to increased DA levels within the NAc and METH reward. The main goals of this dissertation are to characterize the effect of METH on ACh and DA levels within the reward pathway and

determine the role of mesopontine-derived ACh in METH reward. My general hypothesis is that METH increases VTA ACh levels and this increase facilitates making associations between METH-related cues and METH reward. I suggest that METH activates the LDT cholinergic projections to the VTA indirectly. That is, METH induces DA release within the mPFC, which stimulates mPFC glutamate projections (via action on DA receptors) to the LDT. Alternatively, METH-induced increases in NAc DA levels could inhibit GABA neurons projecting to the mPFC (by binding to D2 receptors located on the GABA soma) and thereby disinhibit the mPFC glutamatergic projection to the LDT.

The first experiments presented in **Chapter 2** used *in vivo* microdialysis in awake freely moving mice to measure METH-induced changes in neurotransmitters in the VTA. Evidence suggests that within the SN and VTA, DA is stored in tubulovesicular organelles in DA dendrites that express the VMAT-2 (Nirenberg et al. 1996). *In vitro* studies suggest that amphetamines act upon the VMAT-2 to increase DA release (Fleckenstein et al. 2007). Additionally, previous research suggests that a different psychostimulant, cocaine, may increase VTA ACh levels and be involved in drug-seeking behavior (You et al. 2008). Therefore, the effect of METH on ACh levels and somatodendritic DA levels was measured simultaneously in the VTA. To clarify whether METH was acting within or outside of the VTA to induce changes in DA and ACh levels, two routes of METH administration were tested: (1) perfusion of METH directly into the VTA, and (2) IP METH injection. I hypothesized that, (1) METH would increase extracellular levels of DA and ACh within the VTA and (2) METH was acting outside of the VTA to induce the increase in ACh levels.

The LDT and PPT send cholinergic projections to mesolimbic DA neurons in the VTA; however, tracing studies suggest that the primary source of ventral tegmental ACh is the LDT (Omelchenko and Sesack 2005). Furthermore, stimulation of LDT cholinergic neurons increases NAc DA release, while inhibition attenuates DA levels and amphetamine-induced stereotypy (Blaha et al. 1996; Forster and Blaha 2000; Laviolette et al. 2000). The experiments in **Chapter 3** tested whether reversible inhibition of LDT or PPT cholinergic neurons affected METH-induced levels of DA within the NAc and ACh within the VTA. LDT or PPT cholinergic cells were reversibly inhibited by microinjection of the M2-subtype preferring mAChR agonist OXO. Intra-LDT or PPT OXO agonized the inhibitory M2-subtype mACh autoreceptor located on cholinergic neurons, thus inhibiting cholinergic output. I hypothesized that reversible inhibition of the LDT, but not the PPT, would attenuate METH-induced levels of ventral tegmental ACh and accumbens DA.

Based on the results of the experiments presented in Chapter 3, the experiments in **Chapter 4** focused on the role of the LDT, but not the PPT, cholinergic neurons in METH reward. LDT cholinergic neurons respond to sensory stimuli (Koyama et al. 1994). This information together with the results from Chapters 2 and 3 in this dissertation suggest that LDT ACh neurons might convey sensory information to midbrain DA neurons and guide goal-directed behavior. Therefore, the experiments in **Chapter 4** were designed to test the role of the LDT in learning a Pavlovian association between a METH related cue and the rewarding aspects of METH, as indexed by CPP. In these experiments, I performed a bilateral electrolytic lesion of the LDT in mice prior to METH conditioning trials. Immunohistochemistry was performed for ChAT to quantify the number of cholinergic cells in LDT lesioned and sham operated mice. I hypothesized that LDT cholinergic neurons were involved in forming a Pavlovian (CS-

US) association between METH-related cues and METH's rewarding effects and that a LDT lesion would attenuate METH CPP.

Chapter 2

COMPARISON OF SYSTEMIC AND LOCAL METHAMPHETAMINE TREATMENT ON ACETYLCHOLINE AND DOPAMINE LEVELS IN THE VENTRAL TEGMENTAL AREA IN THE MOUSE

This chapter was adapted from:

Dobbs, L.K. & Mark, G.P. Comparison of Systemic and Local Methamphetamine Treatment on Acetylcholine and Dopamine Levels in the Ventral Tegmental Area in the Mouse. *Neuroscience* 2008; 156(3): 700-711, with permission from Elsevier.

ABSTRACT

ACh is an important mediator of DA release and the reinforcing characteristics of drugs of abuse in the mesocorticolimbic pathway. Within the VTA, the interaction of DA with ACh appears to be integral in mediating motivated behaviors. However, the effects of METH on VTA ACh and DA release remain poorly characterized. The current investigation performed *in vivo* microdialysis to evaluate the effects of METH on intra-VTA levels of ACh and DA. Male C57BL/6J mice received counterbalanced administration of an IP injection (saline, 2 mg/kg, or 5 mg/kg) and an intra-VTA infusion (vehicle, 100 µM or 1 mM) of METH. Locally perfused METH (100 µM or 1 mM) or vehicle slightly increased extracellular ACh. This effect was transient and likely due to disturbing the animal while attaching the pulse to the microdialysis line. Locally perfused METH (100 µM and 1 mM) induced an increase in somatodendritic DA levels in the 20 min post perfusion, compared to vehicle. Systemic METH (5 mg/kg) administration increased somatodendritic DA levels in the 60 min after METH injection compared to this dose group's baseline and compared to saline-treated mice in the post-METH time bin. IP METH (2 mg/kg and 5 mg/kg) or saline increased extracellular ACh levels compared to each dose group's baseline. Additionally, 5 mg/kg METH significantly increased ACh levels compared to the saline and 2 mg/kg METH groups in the 140 min post-METH time bin. IP saline and 2 mg/kg METH produced a transient increase in ACh, while treatment with 5 mg/kg METH induced a prolonged increase in ACh levels (up to 160 min post injection). These data suggest that METH acts in the VTA to induce a robust and short-lived increase in extracellular DA release but acts in an area upstream from the VTA to produce a prolonged increase in ACh release in the VTA. We conclude that METH may activate a recurrent loop in the mesocorticolimbic DA system to stimulate pontine cholinergic nuclei and produce prolonged ACh release in the VTA.

INTRODUCTION

The midbrain DA cell groups A9 and A10 provide major ascending dopaminergic tone to the telencephelon and have been implicated in a wide variety of tasks (Wise, 1982, Oades and Halliday, 1987, German and Manaye, 1993, Robbins and Everitt, 1996, Schultz, 2006). The A9 cell group, thought to be involved in initiating motor behaviors, projects from the SN to the dorsolateral striatum in rodents (German and Manaye 1993) and collateral projections in primates reach dorsolateral prefrontal cortex (Porrino and Goldman-Rakic 1982). Similarly, the A10 cell group within the ventromedial mesencephalon, often referred to as the VTA, has dense mesocortical and mesolimbic connections that terminate in the NAc, medial prefrontal and temporal cortices and basal forebrain structures such as the amygdala, bed nucleus of the stria terminalis and lateral septum (Hasue and Shammah-Lagnado 2002). These connections collectively form the mesocorticolimbic pathway and play a critical role in diverse motivational processes including procuring and consuming food and addiction-like behaviors (Wise, 1982, Koob, 1992, Salamone et al., 2007). Since drug addiction is often characterized as a dysfunction of motivational processes it is not surprising that the majority of addiction research has focused on the role of DA within the structures of the mesocorticolimbic system (Baler and Volkow 2006; Di Chiara et al. 1999; Koob and Le Moal 1997; Wise 2004).

Within the VTA, DA neurons fire in response to novel stimuli, conditioned stimuli and non-predicted rewards (Fiorillo et al. 2003; Schultz 2010). Different firing frequencies determine the rate of postsynaptic DA release and enable various behaviors. Tonic pacemaker firing maintains synaptic DA within a narrow concentration range and is associated with movement, cognition and motivation while phasic burst firing results in rapid increases in synaptic DA and enables reward prediction error

(Lapish et al. 2007; Schultz 1998; 2002; Waelti et al. 2001). Drugs that affect the burst firing of DA neurons and synaptic concentrations of DA can ultimately modify stimulus processing and reward-related behaviors (Kalivas 1993; Schultz 2006).

Several neurotransmitters, such as glutamate, GABA, and ACh modulate DA cell firing and DA release from dendrites and in terminal fields (Bardo 1998; Kalivas 1993; Kalivas et al. 1989b; Lacey et al. 1990; Mereu et al. 1987; Rahman and McBride 2002; Zhang et al. 2002). The source of the cholinergic tone within the VTA originates in the PPT and LDT within the mesopontine tegmentum (Oakman et al. 1995; Omelchenko and Sesack 2005; 2006; Satoh and Fibiger 1986). Administration of cholinergic agonists in the VTA increased DA release in the NAc and PFC (Gronier et al. 2000; Westerink et al. 1998; Westerink et al. 1996). Moreover, the intra-VTA ACh appears to be integral in mediating motivated behaviors. Administration of cholinergic agonists in the VTA is rewarding as indexed by intra-VTA self-administration (Ikemoto and Wise 2002), CPP (Ikemoto and Wise 2002; Yeomans et al. 1985), and enhancement of brain stimulation reward (Redgrave and Horrell 1976; Yeomans and Baptista 1997; Yeomans et al. 2001).

METH is a powerful behavioral reinforcer that supports self-administration in humans (Hart et al. 2001), mice (Shabani et al. 2012a; Yan et al. 2006; Yan et al. 2007a) and rats (Moffett and Goeders 2005; Roth and Carroll 2004). METH induces DA release at terminal regions within the mesocorticolimbic pathway (Izawa et al. 2006; Shoblock et al. 2003) in part through its actions on the DAT and VMAT-2 (Fleckenstein et al. 2007; Sulzer et al. 2005). Furthermore, studies using a rat model showed that METH increases somatodendritic DA levels in the VTA (Zhang et al. 2001) and SN (Bustamante et al. 2002). In addition to its effect on midbrain DA levels, METH has effects on striatal cholinergic responses. Administration of a single low dose

administration of METH increased striatal ACh levels (Taguchi et al. 1998). However, treatment with an escalating dose METH regimen interspersed with multiple METH binges decreased striatal ACh levels and METH stereotypy and enhanced post-stereotypy locomotor activity (Kuczenski and Segal 2001). Furthermore, it appears that midbrain DA neurons are necessary for the METH-induced increase in striatal ACh levels since a 6-hydroxydopamine lesion of the SN abolished the METH-induced increase in striatal ACh levels (Taguchi et al. 1998). Therefore, METH may act upon midbrain DA neurons to affect striatal ACh levels and locomotor responses.

The locomotor stimulant properties of METH are a valuable correlate to its effects on the neurochemical environment. The characteristic increase in dopaminergic transmission and firing between the VTA and NAc that facilitates the reinforcing effects of drugs also contributes to appetitive behaviors and sensitization of the locomotor stimulant response (Wise and Bozarth 1987). Several studies highlight the importance of DA underlying the behavioral stimulation of amphetamine and cocaine (Kelly and Iversen, 1976, Clarke et al., 1988). The DA receptor subtypes D1 and D2, specifically, have been implicated in mediating acute amphetamine- (Narayanan et al., 1996) and cocaine-induced (Karasinska et al., 2005) locomotor activity.

Despite the evidence that METH increases DA levels within the rat mesocorticolimbic pathway and alters ACh levels within the striatum, it remains unknown whether METH affects somatodendritic DA levels and ACh levels within the mouse VTA. The current investigation evaluated extracellular levels of ACh and DA in the VTA in response to METH. We first examined the locus of METH's effects by administering METH directly into the VTA using reverse microdialysis. Next we investigated the effects of systemic METH injection on somatodendritic DA and ACh release in the VTA.

The final experiment assessed prolonged locomotor activity following systemic METH administration.

METHODS

Subjects

Male C57BL/6J mice purchased from The Jackson Laboratory (Sacramento, CA) were used in this study. This strain has been reported to be behaviorally sensitive to the rewarding aspects of psychostimulants (Griffin and Middaugh 2003; Meliska et al. 1995; Orsini et al. 2005; Orsini et al. 2004). We selected this strain in order to characterize its intra-VTA neurochemical response to METH, which might be related to this strain's sensitivity to the rewarding aspects of psychostimulants (METH reward and reinforcement were tested in subsequent experiments and are presented in **Chapter 4** and **Appendix II**, respectively). Animals were experimentally naïve and between 8 and 13 weeks old for all experiments. Mice were group housed until being surgically implanted with guide cannula, after which they were individually housed. For the first two experiments, animals were housed in a temperature and humidity controlled vivarium on a 12 h light-dark cycle with lights on at 0700h. For the third experiment, housing and procedures were carried out in the Portland VA Medical Center in a temperature and humidity controlled vivarium on a 12 h light-dark cycle. Food and water were available *ad libitum* throughout the study, except during the experimental procedures. Animal husbandry conformed to the guidelines set forth in the National Research Council of the National Academies publication regarding the principles of laboratory animal care (Academies 2003) and in a manner approved by the University's Institutional Animal Care and Use Committee at Oregon Health & Science University and the Portland VA Medical Center's Institutional Animal Care and Use Committee.

Drugs

METH hydrochloride was obtained from the National Institute on Drug Abuse drug supply program (Research Triangle Institute, Research Triangle Park, NC). METH was dissolved in sterile, physiological saline to final doses of 2 mg/kg or 5 mg/kg for systemic injection. Systemic METH was delivered in a volume of 0.1 ml/25 g. For intra-VTA perfusion, METH was dissolved in buffered Ringers solution to final concentrations of 100 µM or 1 mM. Neostigmine hydrobromide was purchased from Sigma (St. Louis, MO).

Surgery

Mice were anesthetized with isoflurane (2% in O_2) and unilaterally implanted with a stainless steel guide shaft (21-gauge, 5 mm long) positioned 3.25 mm above the VTA. Coordinates for guide shaft placement were AP: -2.92 mm from bregma; ML: 0.6 mm from midsagittal suture; and DV: 1.3 mm from leveled skull surface (Paxinos and Franklin 2001). Guide shafts were implanted using a Cartesian stereotaxic device (Kopf Instruments) and were secured with dental acrylic along with two, 000-120 cap screws (one anterior to bregma and one posterior to lambda) that served to anchor the dental acrylic to the skull. Patency of guide shafts was maintained throughout the study by inserting a 26-gauge stainless steel wire stylet. Mice were allowed at least one week to recover prior to testing.

Microdialysis Apparatus

Mice were housed in a cylindrical clear polycarbonate chamber (38 cm x 27 cm; Instech Laboratories, Plymouth Meeting, PA) in a sound-attenuated enclosure during *in vivo* microdialysis. A fiber optic lamp on the lowest setting was positioned 30 cm above the polycarbonate chamber and provided illumination during the experiment. A counter-

balanced arm held a single channel swivel extended over the middle of the chamber. A Hamilton syringe (Glenco, 2.5 cc gastight; Sigma, St. Louis, MO) was fitted onto a pump (Razel A99; Braintree Inc, Stamford, CT), which delivered buffered dialysis ringer (142 mM NaCl, 3.9 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.35 mM Na_2HPO_4, 0.3 mM NaH_2PO_4, 0.3 µM neostigmine·Br; pH 7.3) via polyethylene (PE) 20 tubing through the swivel to the microdialysis probe at a rate of 1 µl/min.

Locomotor Activity Apparatus

Locomotor activity data were collected using Accuscan (Columbus, OH) photocell beam activity monitors (40x40x30 cm). Each monitor was housed within an acrylic chamber with sound attenuating foam lining the inside. In addition, a fluorescent light mounted at the top of the rear wall provided illumination and a fan, also located within each chamber, provided ventilation and ambient noise to aid in the masking of outside noises. Eight photocells were located 2 cm above the floor and record beam breaks as the animal moves about the chamber. These beam breaks were translated into distance traveled (cm). The bottom of the chamber was covered in a thin layer of corn cob bedding to absorb urine during the lengthy testing sessions and mimic the environment during experiments 1 and 2. Animals were tested in the same order and at the same time of day and in the same monitor on days 1, 2 and 3. All behavioral testing occurred between 0900h and 1900h.

***In Vivo* Microdialysis Procedure**

One day before testing, a 7.5 mm long microdialysis probe was inserted into the guide shaft so that it extended 2.5 mm beyond the guide shaft into the VTA. Microdialysis probes were constructed in a concentric design described previously (Hoebel et al. 1991). This design consisted of a 10.5 mm long, 26-gauge stainless-steel

tube surrounding a fused silica glass tube (75 μm i.d. X 152 μm o.d., 30 cm long; Polymicro Tech Inc., Phoenix, AZ) with a 0.75 mm long tip of dialysis membrane (218 μm o.d., 9000 MW cutoff; Spectrum Med. Co., Houston, TX) sealed at the end with epoxy cement. To ensure each probe was inserted to the correct depth inside the guide shaft, a 3 mm long 21-gauge collar was soldered onto the 26-gauge probe body to act as a depth-stop. A 10 cm length of PE20 tubing was attached to the probe body via a fluid swivel and served as the inlet line for delivery of the dialysis perfusion medium. The silica glass tubing served as the outlet and was passed inside the PE20 tube through a small slit that was sealed with epoxy cement (Elmers Products, Inc., Columbus, OH). The silica outlet tube ran the length of the probe body and inside of the 0.75 mm dialysis fiber tip.

Microdialysis probes were implanted at least 16 h prior to testing and were perfused overnight with a buffered Ringers solution at a flow rate of 0.5 μl/min. This time allowed the tissue trauma to dissipate and mice to habituate to the testing chamber. The following morning the flow rate was increased and maintained at 1.0 μl/min throughout testing. Food and water were removed prior to testing.

Baseline samples were taken every 10 min for at least 60 min. For each animal, samples were alternately analyzed using high-performance liquid chromatography (HPLC) for ACh and DA, resulting in a 20 min sample interval for each neurotransmitter. Samples to be analyzed for DA were collected into 2 μl of 0.1M HCl / 0.1mM EDTA to prevent oxidation. Following baseline sampling, mice were given either a perfusion of vehicle into the VTA or a saline injection. The vehicle perfusion consisted of intercalating a 20 cm length of PE20 tubing, preloaded with the buffered Ringers

solution, into the input line of the microdialysis probe. Saline was injected IP in a volume of 0.1 ml per 25 g. Samples were collected every 10 min over the next 80 min and alternately analyzed for ACh and DA content. Over the next 6 h, each animal was given a systemic injection and an intra-VTA perfusion of METH. Separate groups of mice received the high doses (5 mg/kg IP and 1 mM intra-VTA) or low doses (2 mg/kg IP and 100 µM intra-VTA) of METH. Order of treatment with respect to the route of administration was counterbalanced.

Locomotor Activity Procedure

Locomotor activity was assessed using experimentally naïve mice in a 3 day procedure. On Day 1 mice were moved in their home cages into the testing room 45-60 min before testing began in order to acclimate to the noises and room environment. After acclimation, mice were weighed and placed into individual holding cages. Within 10 min of weighing, mice were IP injected with 0.9% sterile saline and placed into the center of the activity monitor. Activity was recorded for 5 h in 20 min epochs. At the end of the 5 h period, each animal was immediately removed from the chamber and placed back into its home cage. Once all animals were evaluated, they were returned to their home room. On Day 2, mice were moved in their home cages into the testing room at the same time as they were on Day 1 and all procedures were repeated exactly as described for Day 1. Day 1 testing provided habituation to handling and injection and a measure of locomotion in a novel environment, while Day 2 testing provided baseline activity data in habituated mice. On Day 3 mice were moved into the testing room as on Days 1 and 2. Mice were given an IP injection of saline, 2 mg/kg or 5 mg/kg METH and placed directly into the activity apparatus. Activity was monitored for 5 h and when testing was complete, animals were euthanized by carbon dioxide asphyxiation.

Experiment 1: Measurement of ventral tegmental area acetylcholine and dopamine levels in response to intra-ventral tegmental perfusions of methamphetamine.

Three 10 min samples collected for each neurotransmitter (60 min total) preceding the intra-VTA METH pulse served as baseline. A 20 cm length of PE20 tubing, preloaded with either 1 mM or 100 µM METH dissolved in the buffered Ringers solution (20 µl volume), was intercalated into the input line of the microdialysis probe. Because the dialysis fiber allows approximately 10% of amines similar to METH to pass into tissue (based on unpublished *in vitro* tests with perfusion of DA), the perfusion lines were preloaded with relatively large concentrations of METH. Vehicle, 100 µM or 1 mM METH was perfused into the VTA. Samples were taken every 10 min for 80 – 100 min and alternately measured for ACh and DA. There were 11 animals per dose group for the ACh measurements (total n = 33). The number of animals for the DA measurements was n = 11, n = 11, and n = 12 for the vehicle, 100 µM and 1 mM METH dose groups, respectively.

Experiment 2: Measurement of ventral tegmental area acetylcholine and dopamine levels in response to systemic injections of methamphetamine.

Three 10 min samples collected for each neurotransmitter (60 min total) preceding the IP injection of METH served as baseline. Following baseline, animals were IP injected with saline or METH (2 or 5 mg/kg) in a volume of 0.1 ml per 25 g. Samples were taken every 10 min for 100 – 300 min and alternately measured for ACh and DA. The number of animals for the ACh measurements was n = 11, n = 14, and n = 14 for the saline, 2 and 5 mg/kg METH dose groups, respectively. For the DA measurements, the number of animals was n = 10, n = 13 and n = 11, for the saline, 2 and 5 mg/kg METH dose groups, respectively.

Experiment 3: Effect of systemic methamphetamine on locomotor activity.

Mice (n = 24) were evaluated during two separate passes, for a total of twelve mice per pass and eight mice per dose (saline, 2 mg/kg METH and 5 mg/kg METH). Each pass was counterbalanced for METH dose and across chambers. The first pass began at 0900h and the second pass began at 1400h. This was done to mimic the time of day of injections during the first two experiments. There was no difference in the locomotor response between the two passes (data not shown).

Sample Analysis: High-Performance Liquid Chromatography Methods

Acetylcholine. Dialysates were measured for ACh by reverse phase HPLC with electrochemical detection. This system consisted of a single piston pump and pulse dampener (SSI C., Fisher, St. Louis, MO), a 20 µl sample loop, and an amperometric detector (Waters Corp. Model 464; Milford, MA). Mobile phase for this system consisted of 200 mM potassium phosphate dibasic with 125 mg/L tetramethylammonium hydroxide. Monobasic potassium phosphate was used to adjust the pH to 7.8. ACh and choline were separated on an 8 cm, C18 analytical column (Varian Inc., Walnut Creek, CA) and then converted sequentially to betaine and hydrogen peroxide by an immobilized enzyme reactor (Varian). The resultant hydrogen peroxide was oxidized on a platinum electrode (BAS Inc., West Lafayette, IN) set at 0.5 V with respect to an Ag/AgCl reference electrode (Advanced Measurement Technologies, Oak Ridge, TN). Variability in sensitivity over time was measured by injecting aliquots of a known standard concentration at the beginning and end of each microdialysis session.

Dopamine. Dialysates were analyzed for DA by reverse phase HPLC with electrochemical detection (Epsilon amperometric detector; Bioanalytical Systems, West

Lafayette, IN) with a dual piston pump (BAS, Model PM80). DA was separated on a UniJet microbore column (BAS Inc, 1X100 mm, 3 μm, ODS packing). Mobile phase for this system consisted of 100 mM monochloroacetic acid, 500 μM EDTA, 600 μM octanesulfonic acid and 8.6% v/v acetonitrile (pH 3.2). The mobile phase flow rate was 0.9 ml/min. Aliquots of known standard concentrations were injected into the HPLC system throughout the microdialysis session to provide a measure of variability in sensitivity over time.

Histology and Probe Verification

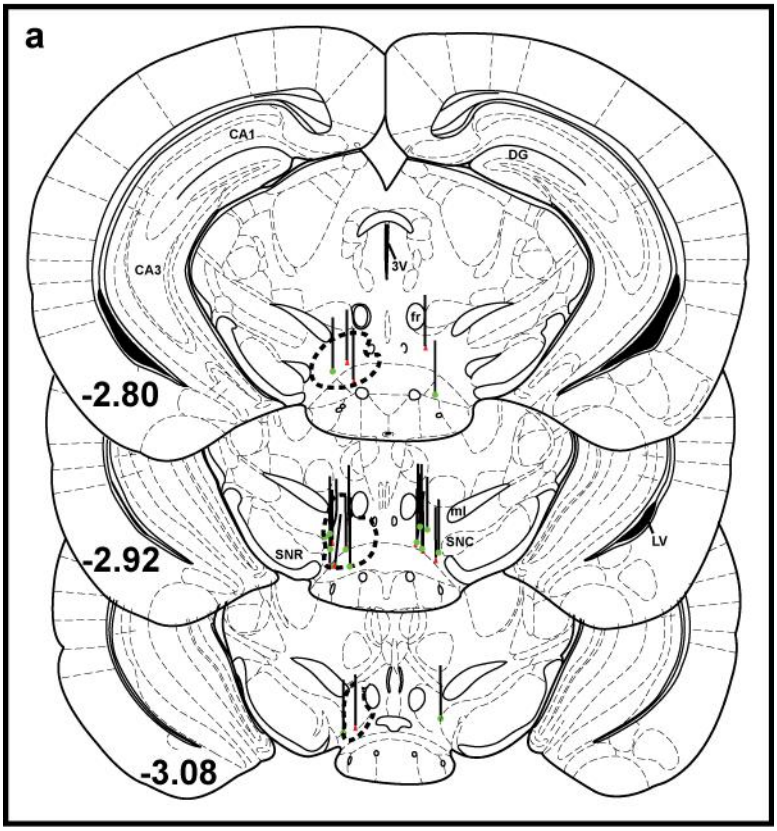
Once microdialysis sampling was complete, animals were overdosed with isoflurane. Brains were fixed with the probe in place in 10% formalin in phosphate buffered saline (PBS) for two weeks and then placed in 20% sucrose, 10% formalin in PBS. Three days prior to sectioning, brains were transferred to 30% sucrose in PBS. Brains were sectioned on a Leica cryostat and 40 μm sections were floated in a mounting solution (300 ml dH_2O, 1.5 g gelatin, 0.6 ml acetic acid, 200 ml 95% ethanol) prior to mounting onto slides. Slides were allowed to air dry before being thionin stained. Figure 4a shows a schematic of the probe locations using histological drawings from the atlas of Paxinos and Franklin (Paxinos and Franklin 2001). For simplicity, probes placed in the left or right hemisphere are shown on the same plate, but each animal only received a unilateral implant. Figure 4b shows a representative photomicrograph of probe placement.

Statistical Analyses

Microdialysis. Peak heights were measured for DA and ACh and then converted to mass (in moles) per 10 μl and 4 μl of sample, respectively, by comparison to standard curves. Absolute levels of ACh and DA were not corrected for probe recovery because

Figure 4. Schematic representation of microdialysis probe placements in the VTA shown on histological plates from the atlas of Paxinos and Franklin (2001) (**a**). The approximate boundaries of the VTA are shown by the dashed line. Black bars indicate the location of each probe and the size of the 0.75 mm dialysis fiber tip. Bars with green tips represent animals that received the low dose of METH (2 mg/kg and/or 100 μM) and bars with red tips represent animals that received the high dose of METH (5 mg/kg and/or 1mM). The numbers on the left-hand side of each section indicate millimeters posterior from bregma. A representative photomicrograph of a thionin stained coronal brain slice shows the microdialysis probe track (**b**). Red arrowheads represent the dorsal and ventral limits of the fiber. CA3 (CA3 region of the hippocampus), CA1 (CA1 region of the hippocampus), DG (dentate gyrus), ml (medial lemniscus), SNR (substantia nigra reticulata), SNC(substantia nigra pars compacta), fr (fasiculus retro-flexus), 3V (3rd ventricle), LV (lateral ventricle)

Figure 4



in vitro tests showed a less than 5% variation in recovery between probes made in our laboratory (Mark, unpublished observations).

DA and ACh content in each sample was converted to a percentage of the mean of three baseline samples. Animals were counterbalanced for order of treatment (systemic injection versus intra-VTA perfusion) and whether DA or ACh was measured first. We thus addressed two important concerns with the repeated measure design: (1) whether the dialysate levels of ACh or DA were consistent when taken at 10 min or at 20 min post perfusion or injection and, (2) whether the first METH treatment received (IP versus intra-VTA METH) affected the dialysate levels of ACh or DA following the second METH treatment. We grouped data by treatment and performed a two-way ANOVA to compare the DA and ACh levels from dialysates taken at 10 min or 20 min. No significant differences were found in DA or ACh levels between the 10 min and 20 min samples, thus we collapsed across time creating 20 min samples. To assess the potential effects of a prior METH treatment on subsequent METH-induced changes in DA and ACh we compared, within each dose, the DA and ACh levels across time with the first METH treatment (intra-VTA or IP METH) as the between-subjects factor. Two-way ANOVA indicated no significant differences in DA or ACh level whether an animal received the intra-VTA pulse first or following a systemic injection, or vice versa. Thus, prior METH treatment had no significant effect on subsequent DA or ACh levels and all analyses were performed collapsed across treatment order.

Dialysis data from systemic and locally applied METH manipulations were analyzed separately using a two-way repeated measure ANOVA with drug dose as the between subjects factor. The between subjects factor levels for experiment 1 were:

vehicle, 100 µM and 1mM. The between subjects factor levels for experiments 2 were: saline, 2 and 5 mg/kg METH. For the within subject factor, time was collapsed into 2 time bins: post-vehicle/saline (i.e., baseline) and post-METH (i.e., post-vehicle, 100 µM or 1mM METH for experiment 1; or post-saline, 2 mg/kg and 5 mg/kg METH for experiment 2). Since some subjects had missing samples, data were collapsed into these two time bins in order to include all subjects in the analyses. Missing samples were due to problems such as the probe coming disconnected during dialysate collection or technical problems with sample injection onto the HPLC column. Post-METH time bins were selected that best captured the peak of the DA or ACh response following the METH injection or perfusion. For both experiments, the baseline time bin consisted of collapsing the 60 min of data following the vehicle perfusion or saline injection, respectively. For experiment 1, the first 20 min sample after METH perfusion was used for the post-METH time bin for the DA and ACh analyses. For experiment 2, different post-METH time bins were created for the DA and ACh data analyses. For the DA analysis, the post-METH time bin consisted of the first 60 min following METH injection. For the ACh analysis, the post-METH time bin consisted of the first 140 min following METH injection. Analysis of DA levels in experiment 2 revealed the presence of an extreme outlier (greater than 3 standard deviations from the mean) in the 5 mg/kg METH dose group. This subject's data was removed from this analysis (n = 11 for the 5 mg/kg group). Significant interactions were followed up with simple main effects analyses and Bonferroni corrected post-hoc tests. Data are expressed as the mean ± SEM and all results were considered at an alpha of 0.05.

Locomotor Activity. Horizontal distance traveled (cm) was analyzed using a two-way repeated measure ANOVA with Day (baseline and test) as the within subjects factor and systemic drug treatment (Drug; saline, 2 or 5 mg/kg METH) as the between subjects

factor. In addition, a two-way repeated measure ANOVA analyzed locomotor activity across the 5 h session for days 2 and 3 separately. For these analyses, the 20 min time bins were the within subjects factor (15, 20 min time bins) and Drug (saline, 2 or 5 mg/kg METH) was the between-subjects factor. Violations to sphericity were corrected with Greenhouse-Geisser. Significant interactions were followed up with simple main effects analyses and Bonferroni corrected post-hoc tests. Data are expressed as the mean ± SEM and all results were considered at an alpha of 0.05.

RESULTS

Determination of baseline values and absolute levels of acetylcholine and dopamine recovered in the ventral tegmental area

Samples were taken during the animal's light cycle between 0900 and 1900 h. Since the 5 mg/kg dose of METH caused robust activation, up to 5 h of recovery samples were necessary for neurotransmitter levels to return to baseline and before making any further manipulations. For all analyses, the reference basal level of ACh and DA was calculated by averaging the peak heights of the last three samples prior to drug treatment. During baseline measurements, the average absolute recovery of ACh and DA (± SEM and not corrected for probe efficiency) across all subjects was 50.0 ± 3.0 fmol/10 µl sample and 4.9 ± 0.07 fmol/4 µl sample, respectively.

Experiment 1: Effects of intra-ventral tegmental methamphetamine on extrasynaptic acetylcholine and dopamine in the ventral tegmental area

Dopamine. Figure 5a shows somatodendritic DA levels over time for each drug group. Two-way repeated measure ANOVA performed on the collapsed data indicated main effects of Time ($F_{1,31} = 18.38$, $p < 0.0005$) and Dose ($F_{2,31} = 6.18$, $p = 0.006$) and a

significant Time x Dose interaction ($F_{2,31} = 6.18$, $p = 0.006$) (Figure 5b). Somatodendritic DA levels did not differ between drug groups at baseline; however within the post-METH time bin, treatment with 1 mM METH, but not 100 µM, induced a significant increase in DA levels compared to vehicle-treated controls ($p = 0.005$). Treatment with 100 µM ($F_{1,10} = 11.60$, $p = 0.007$) or 1 mM ($F_{1,11} = 11.27$, $p = 0.006$), but not vehicle, significantly increased extrasynaptic DA levels compared to each dose group's baseline.

Acetylcholine. Figure 5c shows intra-VTA ACh levels across time for each METH dose group. Analysis of the collapsed data indicated a main effect of Time ($F_{1,30} = 8.15$, $p = 0.008$; Figure 5d). The main effect of Dose and Time x Dose interaction were not significant. Each dose group showed a slight increase in extracellular ACh levels during the post-METH time bin. Animals were generally asleep during baseline measurements and intercalation of the vehicle/METH pulse into the perfusion line often woke them and induced mild locomotor activation. We therefore suspect the general ACh increase was due to disturbing the animal when intercalating the pulse into the perfusion line.

Experiment 2: Effects of systemic methamphetamine on extrasynaptic acetylcholine and dopamine in the ventral tegmental area

Dopamine. Figure 6a shows somatodendritic DA levels across time for each dose group. Analyses of the collapsed time bin data revealed main effects of Time ($F_{1,31} = 13.05$, $p = 0.001$) and Dose ($F_{2,31} = 4.04$, $p = 0.028$) and a significant Time x Dose interaction ($F_{2,31} = 4.04$, $p = 0.028$) (Figure 6b). Follow-up analyses indicated no difference in extracellular DA levels between dose groups at baseline. However, Bonferroni corrected post-hoc tests indicated that mice treated with 5 mg/kg METH had significantly higher DA levels compared to mice treated with saline ($p = 0.028$), but not

Figure 5

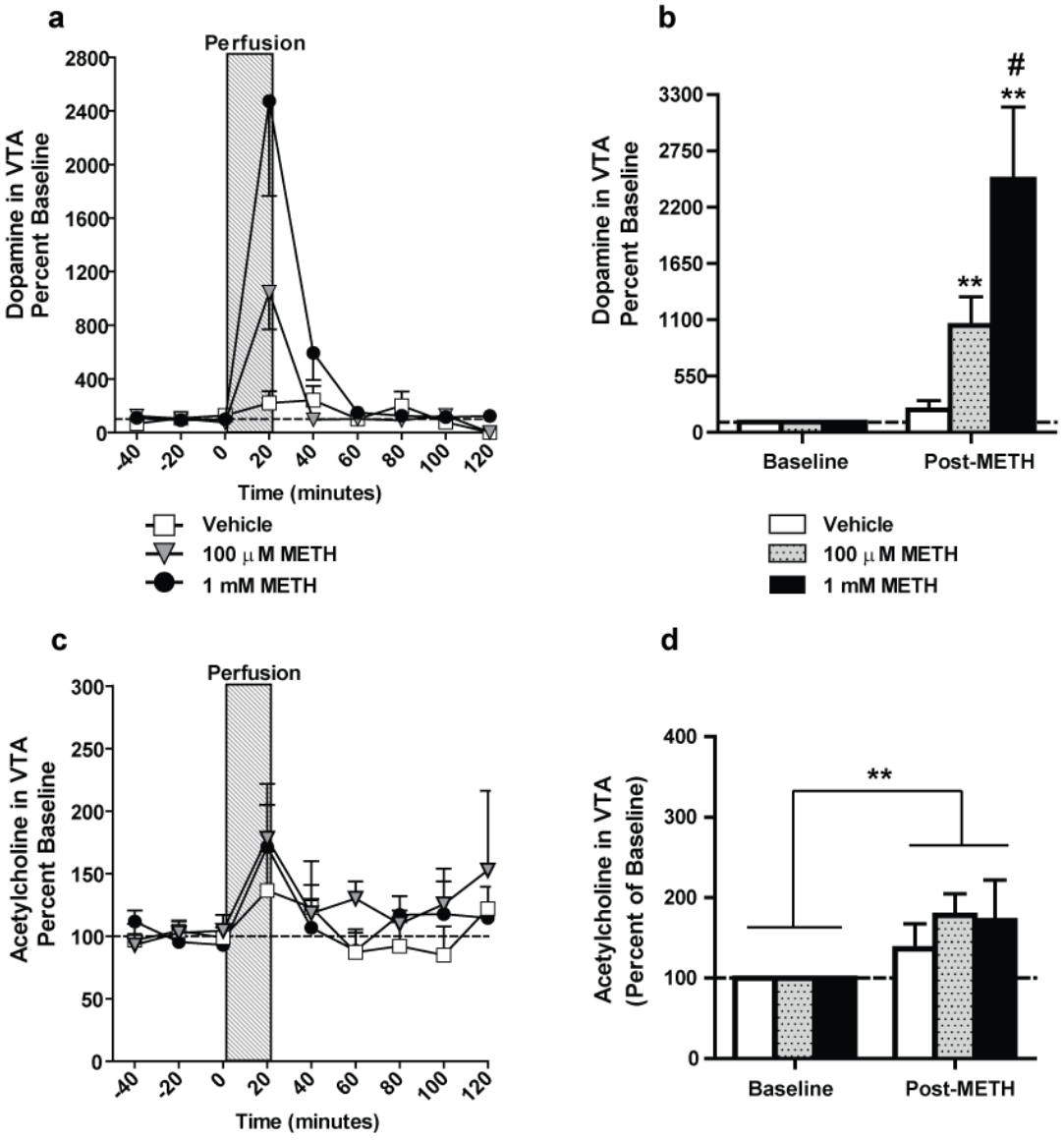


Figure 5. Extracellular DA (**a**) and ACh (**c**) levels in response to local applications of METH (black circles 1 mM ; grey trianlges 100 μM) or vehicle (white squares) in the VTA is shown. The grey hatched bar represents the duration of time that METH or vehicle was perfused into the VTA. DA (**b**) or ACh (**d**) samples were collapsed for each mouse for the 60 min baseline and compared to the first 20 min sample post vehicle or METH perfusion for analysis. DA levels were significantly higher after 100 μM or 1 mM METH compared to baseline. In the first 20 min post-METH, the 1 mM group had higher DA levels compared to the vehicle group. There was an increase in ACh levels between baseline and post-METH for all dose groups. ** $p < 0.01$ vs. same dose at baseline (**b**), or baseline vs. post-METH collapsed across dose group (**d**); # $p < 0.01$ vs. vehicle post-METH.

mice treated with 2 mg/kg METH (p = 0.175). Additionally, mice treated with saline or 2 mg/kg METH did not show a significant increase in DA levels between the baseline and post-METH time bins. However mice treated with 5 mg/kg METH showed a significant increase in DA levels at the post-METH time bin compared to their own baseline (main effect of Time; $F_{1,10}$ = 12.21, p = 0.006).

Acetylcholine. Figure 6c shows intra-VTA ACh levels across time for each dose group. Two-way ANOVA showed significant main effects of Time ($F_{1,36}$ = 112.73, p < 0.0005) and Dose ($F_{2,36}$ = 26.66, p < 0.0005) and a significant Time x Dose interaction ($F_{2,36}$ = 26.62, p < 0.0005; Figure 6d). There were no significant differences in extracellular ACh levels between dose groups at baseline. However, during the 140-min post-METH time bin, mice treated with 5 mg/kg METH had significantly increased ACh levels compared to mice treated with 2 mg/kg METH (p < 0.0005) or saline (p < 0.0005). There was also a trend towards significance for mice treated with 2 mg/kg METH to have higher ACh levels compared to saline-treated controls (p = 0.065). Treatment with saline ($F_{1,10}$ = 5.16, p = 0.046), 2 mg/kg METH ($F_{1,13}$ = 45.03, p < 0.0005) or 5 mg/kg METH ($F_{1,13}$ = 82.33, p < 0.0005) significantly increased intra-VTA ACh levels compared to each dose group's baseline. The more transient increase in ACh levels following saline or 2 mg/kg METH, relative to the increase seen after 5 mg/kg METH, appears similar to the ACh increase seen during the post-METH time bin in experiment 1, suggesting that disturbing the animal can induce a general increase in ACh levels in the VTA. However, compared to saline and 2 mg/kg METH, the highest METH dose (5 mg/kg) appears to induce a prolonged increase in ACh levels.

Experiment 3: Effects of systemic methamphetamine on locomotor activity

Figure 6

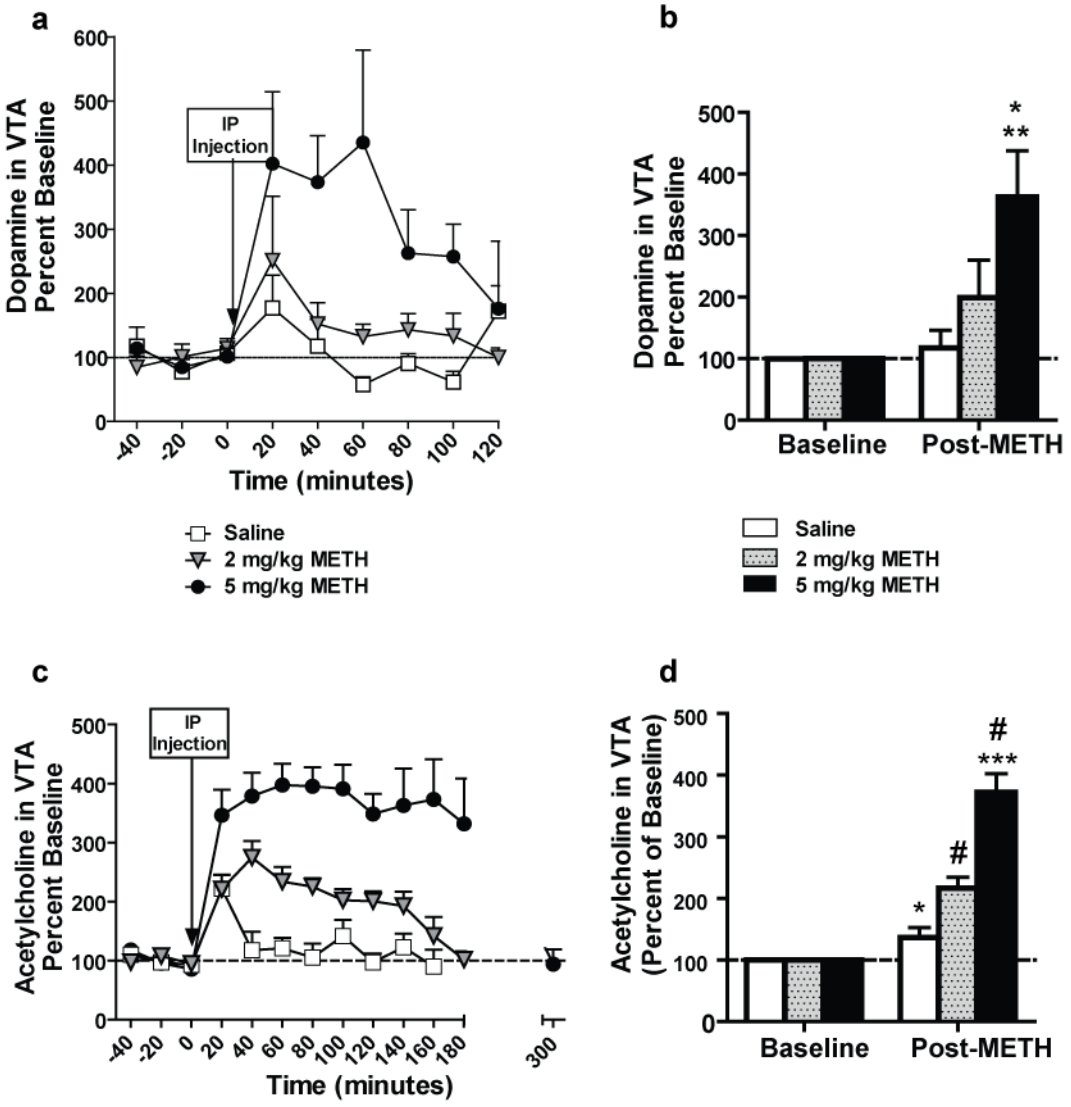


Figure 6. Extracellular DA (**a**) and ACh (**b**) levels were measured in the VTA in response to IP injection of saline (white squares) or METH (2 mg/kg grey triangles; 5 mg/kg, black circles) is shown. The timing of the IP injection is marked with an arrow. DA (**b**) and ACh (**d**) samples were collapsed for each mouse for the 60 min baseline and compared to the first 60 min Post-METH injection for the DA analysis and compared to the first 140 min Post-METH injection for the ACh analysis. 5 mg/kg METH induced a significant increase in extracellular DA levels. There was a dose-dependent increase in extracellular ACh levels in the VTA following METH injection. There were no differences in the amount of DA or ACh between dose groups at baseline. * $p < 0.05$ vs saline at post-METH time bin (**b**), or vs. same dose at baseline (**d**); ** $p < 0.01$ vs same dose at baseline; *** $p < 0.0005$ vs. saline and 2 mg/kg; # $p < 0.0005$ vs. same dose at baseline.

Figure 7

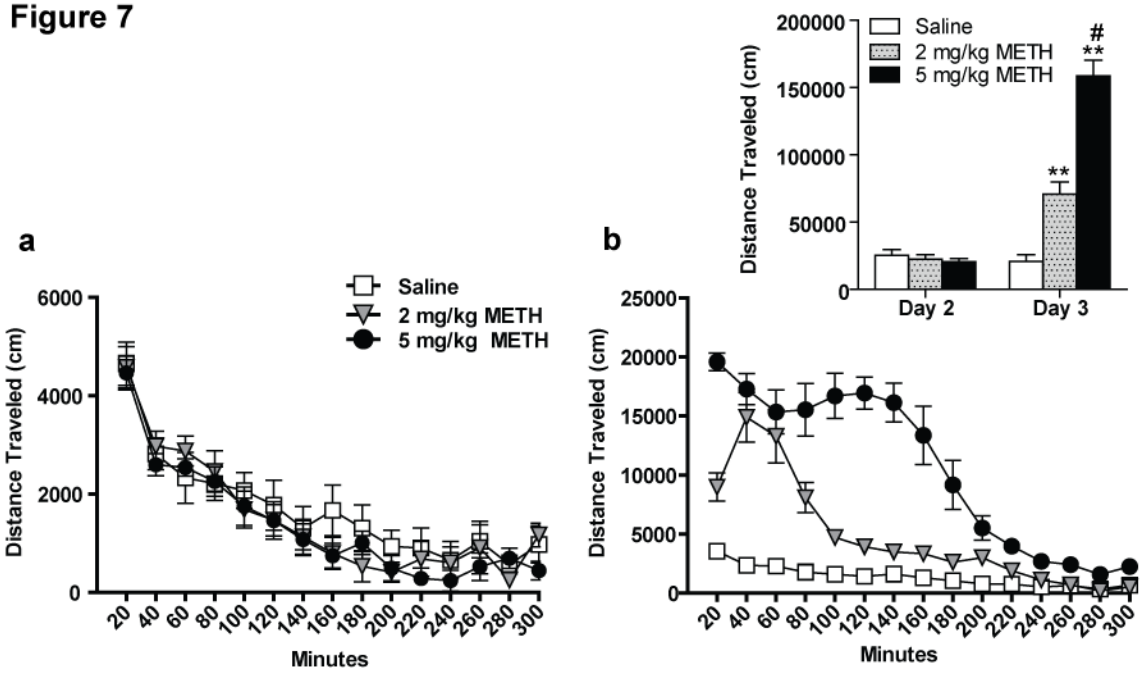


Figure 7. Locomotor activity (cm) for the 5 h test session (shown in 20 min time bins) on baseline (Day 2) (**a**) and test (Day 3) (**b**) is shown. On the baseline day, all subjects received IP saline. There was no difference in activity between groups on baseline day, but subjects showed a decrease in activity over time. On the test day, subjects received IP injection of saline (white squares), 2 mg/kg METH (grey triangles), or 5 mg/kg METH (black circles) before being placed in the locomotor apparatus. Mice treated with 5 mg/kg METH showed significant activation at each time bin compared to saline controls. Mice treated with 2 mg/kg METH showed greater activity for the first 80 min compared to saline controls. The reader is referred to the text for the individual time bin analyses. **Inset:** The total distance traveled during the 5 h session was analyzed between Days 2 and 3. Activity between groups did not differ on Day 2. On Day 3, 2 mg/kg and 5 mg/kg METH induced a significant activation compared to saline controls. In addition, 5 mg/kg METH induced higher activation compared to 2 mg/kg METH on Day 3. ** $p < 0.001$ vs. saline on Day 3; # $p < 0.001$ vs. 2 mg/kg on Day 3.

Two-way repeated measure ANOVA performed on the total distance traveled during the 5 h testing session indicated significant main effects of Day ($F_{1,21} = 211.10$, $p < 0.0001$) and Drug ($F_{2,21} = 32.54$, $p < 0.0001$) and a significant Day x Drug interaction ($F_{2,21} = 99.64$, $p < 0.0001$; Figure 7, inset). Baseline locomotor activity (Day 2) was not significantly different between the drug treatment groups. On Day 3 mice that received 2 mg/kg METH ($p < 0.001$) or 5 mg/kg METH ($p < 0.001$) were significantly more active

compared to saline-treated controls. Additionally, mice that received 5 mg/kg METH were significantly more active than mice that received 2 mg/kg METH ($p < 0.001$). Figure 7a shows locomotor activity divided into 20-min time bins on the baseline day (Day 2). Two-way ANOVA of these data showed a significant main effect of Time ($F_{6.8, 142.85} = 57.85$, $p < 0.0005$), but no significant main effect of Drug or significant Drug x Time interaction. Bonferroni corrected post-hoc tests revealed a general pattern of decreasing activation with each time bin across the entire 5 h session. Evaluation of Day 3 locomotor activity revealed significant main effects of Time ($F_{2.7,56.1} = 57.42$, $p < 0.0005$) and Drug ($F_{2,21} = 58.26$, $p < 0.0005$) and a significant Drug x Time interaction ($F_{5.4,56.1} = 16.30$, $p < 0.0005$; Figure 7b). Mice that received 2 mg/kg METH were significantly more active than saline controls for the first 80 min (p-values ranged from $p < 0.0005$ to $p = 0.025$). An injection of 5 mg/kg METH induced a significant increase in activity at each time bin for the entire 5 h compared to saline controls (p-values ranged from $p < 0.0005$ to $p = 0.033$). The 5 mg/kg METH injection induced significantly higher activity than 2 mg/kg METH in the first 20 min ($p < 0.0005$), from 80 – 180 min post injection (p-values ranged from $p < 0.0005$ to $p < 0.007$), and from 220 – 300 min post injection (p-values ranged from $p = 0.001$ to $p = 0.017$).

DISCUSSION

A major finding of this study was the dissociation between the locus of METH effects on ACh levels in VTA compared to DA. Extracellular DA in the VTA was robustly increased by both systemic injection and local perfusion of METH into the VTA. Additionally, systemic administration of METH induced a prolonged increase in VTA ACh levels. In contrast with systemic injection, a small and transient increase in extracellular ACh levels was seen following an intra-VTA perfusion of METH or vehicle. However, the

increase was similar in magnitude whether subjects received METH or vehicle and returned to baseline immediately after the infusion. Moreover, while intercalating the perfusion pulse into the dialysis line, animals often woke up and began moving about the chamber. Thus, we suspect the non-specific increase in ACh levels was induced by generally disturbing the animal. The VTA receives NE and 5-HT afferents from the LC and DR, respectively (Adell and Artigas 2004). However, since intra-VTA METH did not affect VTA ACh levels, it is unlikely that METH transynaptically stimulated ACh terminals via increased intra-VTA NE or 5-HT release. Thus, it appears that METH functions outside of the VTA to induce increases in VTA ACh levels.

The findings of this study also demonstrate several components of the functional relationship between ACh and DA in VTA. We found that while ACh may participate in METH-induced DA increases in the VTA (i.e., systemic METH increased ACh *and* DA), the METH-induced DA increase in the VTA is not dependent on ACh output. That is, intra-VTA perfusion of METH increased DA output in the absence of a drug-specific ACh response.

The time course of the increase in VTA ACh levels following systemic METH injection was much more prolonged (140 min) compared to the transient increase in somatodendritic DA levels (60 min). Similarly, in experiment 3 mice treated with 5 mg/kg METH exhibited protracted locomotor activation. Previous studies have reported prolonged locomotor activation following administration of IP METH (Riviere et al. 1999) and amphetamine (Conti et al. 1997), but not after intra-VTA administration of amphetamine (Kalivas and Weber, 1988, Vezina and Stewart, 1990). This suggests that intra-VTA administration of METH would not likely produce locomotor activation.

Methamphetamine affects extracellular dopamine levels through several mechanisms

Several studies have performed microdialysis to measure DA output in terminal fields. For instance, extracellular DA increased in the NAc in response to natural rewards (Hernandez and Hoebel 1988) and cholinergic agonists (Westerink et al., 1996, Gronier et al., 2000). The vast majority of drugs with abuse potential in humans cause increase DA transmission in animal models. Rats receiving passive or active systemic administration of cocaine (Bradberry and Roth 1989), amphetamine (Ranaldi et al. 1999) and METH (Izawa et al., 2006) showed an increase in extrasynaptic DA in the NAc. Within the NAc, METH is a potent substrate for the DAT (for a review see Fleckenstein et al. 2007) and causes an increase in extracellular DA levels by reverse transport.

Likewise, *in vivo* electrophysiology and microdialysis studies using rats have evaluated several mediators of somatodendritic DA release in the VTA. Somatodendritic DA release is in part mediated by cholinergic muscarinic (Gronier et al. 2000; Gronier and Rasmussen 1998) and nicotinic (Rahman et al. 2004) receptors. Other studies have focused on how the GABAergic and cholinergic receptors in the NAc mediate VTA somatodendritic DA release (Rahman and McBride 2002), or how intra-VTA serotonergic tone affects somatodendritic DA release in different lines of rats (Liu et al. 2006). Additionally, there is evidence that cocaine (Chen and Reith 1994; Reith et al. 1997) and METH (Zhang et al. 2001) induce an increase in somatodendritic DA output in the rat VTA. However, to our knowledge this is the first published study using microdialysis to assess the effects of METH on somatodendritic DA release in the VTA of mice.

Current evidence suggests METH acts on DA dendrites to affect somatodendritic release similarly to how it triggers DA release in terminal fields. Under normal

conditions, the VMAT-2 transports cytoplasmic DA into reserpine-sensitive tubulovesicular organelles within the dendrites, thereby decreasing the cytoplasmic concentration of DA and preventing monoamine oxidase (MAO)-mediated metabolism (Cooper et al. 2003; Nirenberg et al. 1996). When the METH concentration is high, its lipophilicity facilitates diffusion into the dendrites via the plasmalemmal membrane causing a disruption of the VMAT-2 and the proton gradient, thus preventing the sequestration of DA into vesicles (for reviews see Fleckenstein et al. 2007; Volz et al. 2007). The resulting build-up in cytoplasmic DA causes a reversal of the DAT and induces somatodendritic DA release.

METH may also affect somatodendritic release through its ability to mediate DA cell firing. METH induced oscillations in cytosollic Ca^{++} within VTA DA neurons (Uramura et al. 2000). These oscillations can directly influence DA neuron activation. However, the oscillations were observed following daily METH injections for 7 consecutive days and it is not known whether acute injection of METH, as used in the current study, induces similar Ca^{++} flux. Nevertheless, our data show a rapid and strong increase in somatodendritic DA in response to METH treatment, which is consistent with stimulated release.

Following the increase, DA levels in the VTA were rapidly decreased after METH injection. This quick return to baseline is consistent with other studies which found that over time, psychostimulants, including METH, inhibit VTA DA firing presumably through activation of inhibitory D2 receptors following somatodendritic DA release (Kamata and Kameyama 1985; Shi et al. 2004). The rapid return to baseline following intra-VTA METH may have been due to several factors. First, activation of DA D2 autoreceptors following somatodendritic DA release would cause an inhibition of DA cell firing

(Aghajanian and Bunney 1977; Grace and Bunney 1985) which shuts down further release. However, METH does not require neuronal depolarization to induce DA release and can independently modulate Ca^{++} release. So, it might be that DA storage vesicles were depleted following such a robust response, thereby disabling further somatodendritic DA release.

Intra-ventral tegmental dopamine and acetylcholine interactions

Excitatory cholinergic synapses on DA neurons may affect DA release in terminal fields (Gronier et al. 2000). Muscarinic (Lacey et al. 1990) and nicotinic (Calabresi et al. 1989) receptors in the VTA have been suggested to play a role in controlling VTA DA cell firing and both receptors have been implicated in mediating reinforcement (Yeomans and Baptista 1997). Nicotine increased VTA and SN DA neuron firing, and this was blocked by mecamylamine (Mereu et al. 1987). Likewise, mAChR agonists induced increases in VTA DA release, and this was prevented by pretreatment of the mAChR antagonist n-methylscopolamine (Gronier et al. 2000). Moreover, stimulation of LDT cholinergic neurons caused an increase in DA output in the NAc (Forster and Blaha 2000). Morphological identification of cholinergic asymmetric synapses with VTA DA neurons also suggests that ACh neurons could affect DA output (Garzon et al. 1999; Omelchenko and Sesack 2006).

In the current data, however, the increase in ACh levels was more prolonged compared to the large, transient increase in somatodendritic DA levels. This suggests that while DA neurons habituated to stimulation, the ACh neurons did not. Thus, there is not a simple interaction between ACh and DA neuronal activity. While ACh may contribute to the activation of DA neurons (as described above), secondary mechanisms

such as DA autoreceptor activation or vesicle depletion may act to oppose cholinergic stimulation and rectify DA levels in the VTA.

Methamphetamine acts on cholinergic neurons to affect ventral tegmental area acetylcholine release

Since the METH-induced dose-dependent ACh response only occurred when METH was administered systemically, we conclude that METH did not act directly on ACh terminals in the VTA to cause an increase in ACh levels. Cholinergic innervation of the VTA and SN from the LDT and PPT in the mesopontine region has been previously defined (Oakman et al. 1995; Omelchenko and Sesack 2006; Satoh and Fibiger 1986; Semba and Fibiger 1992). These afferents labeled with vesicular ACh transporter, indicating that these terminals contain vesicles that release ACh (Garzon et al., 1999). Our data suggest that METH directly or indirectly activates these cholinergic projections to the VTA. We propose that METH indirectly stimulates the LDT cholinergic neurons by increasing DA levels within the mPFC, which in turn act via DA receptors to stimulate the glutamatergic projection to the LDT. Alternatively, METH-induced increases in NAc DA levels could inhibit GABA neurons projecting to the mPFC (by binding to D2 receptors located on the GABA soma) and thereby disinhibit the mPFC glutamatergic projection to the LDT. Moreover, the prolonged ACh response to systemic METH suggests that the cholinergic neurons projecting to the VTA do not habituate, but rather respond repeatedly after acute METH administration.

It is presently unknown which neural substrate(s) METH acts upon to cause the prolonged increase in VTA ACh output. Afferent connections to the pontine cholinergic cell bodies have been defined using retrograde and anterograde axonal transport

methods. Projections have been described from the mPFC and habenula to the LDT (Satoh and Fibiger 1986; Semba and Fibiger 1992). The bed nucleus of the stria terminalis and the central nucleus of the amygdala send projections to the PPT (Satoh and Fibiger, 1986, Semba and Fibiger, 1992, Steininger et al., 1992). Thus, the majority of afferents to pontine cholinergic and glutamatergic cell groups come from limbic structures that are innervated by DA. METH may activate a recurrent loop by initiating a cholinergic-mediated stimulation of mesocorticolimbic dopaminergic pathways via nAChRs and mAChRs within the VTA (see also Forster and Blaha, 2000). This suggests a functional pathway upon which METH might act to stimulate the mesolimbic system.

Methamphetamine-induced locomotor activity is potentially related to ventral tegmental area dopamine and acetylcholine

In the current study, the relationship between the METH-induced locomotor activity and extrasynaptic ACh and DA is consistent with the idea that activation of DA fibers within the mesolimbic pathway is required for psychostimulant locomotor activation (Wise and Bozarth 1987). Previous studies have found that METH and cocaine induce acute locomotion and increases in NAc DA levels in rats (Camp et al. 1994; Shoblock et al. 2003). Additionally, ongoing DA receptor activation is required for post-stereotypy locomotion after acute IP administration of amphetamine (Conti et al. 1997) and the DA D1 receptor is necessary for cocaine-induced locomotor activation (Karasinska et al., 2005) and the activating effects of amphetamine (Xu et al. 2000). The DA-dependent nature of psychostimulant-induced locomotor activation suggests that other neurotransmitters, such as ACh, which alter DA neuron firing and release, may also affect psychostimulant-induced locomotor activation. Amphetamine-induced post-stereotypy locomotor activity (measured 140 min after a systemic administration of

amphetamine) was attenuated by treatment with haloperidol, a DA D2 receptor antagonist (Conti et al. 1997). Thus, prolonged psychostimulant locomotion might be dependent on a prolonged DA response. Prolonged DA release in the striatum is regulated by the M5 subtype mAChRs located on DA neurons in the midbrain (Forster and Blaha 2003; Vilaro et al. 1990; Yeomans et al. 2001). Therefore, intra-VTA cholinergic modulation of protracted DA release via the M5 subtype receptor might underlie the prolonged METH-induced locomotor response. However, investigations using mice with a global knock out of the M5 mAChR have shown a wide difference in stimulated locomotor behavior. For instance, one study found that M5 knockout mice exhibited enhanced scopolamine-induced locomotor activity (Chintoh et al. 2003). Other studies report mixed results regarding amphetamine-induced locomotor activity. Investigations have reported that M5 knockouts show increased (Schmidt et al. 2010) (4 mg/kg), decreased (Wang et al. 2004) (4 and 8 mg/kg) or no difference (Yamada et al. 2001) (1, 3, and 10 mg/kg) in amphetamine-induced locomotor activity compared to wild-type mice. While differences in the results between these studies might be due to the different locomotor activity procedures used, these studies all found no difference in spontaneous locomotor activity between M5 knockouts and controls.

These studies, however, only measured locomotor activity up to 60 min after administration of amphetamine. We therefore cannot draw a conclusion about the role of the M5 receptor in *prolonged* amphetamine-induced locomotor activation. Additionally, it is possible that the amphetamine doses used in the previous studies, which induced a difference in locomotor activity between M5 knockouts and wild types, did not induce the magnitude of activation that 5 mg/kg METH did in the current experiment. Finally, in these previous investigations, the M5 receptor deletion was

global, as opposed to VTA-specific. These issues make it difficult to infer whether the intra-VTA M5 receptors regulate prolonged amphetamine or METH locomotor activation.

It is also possible that a non-muscarinic mechanism is influencing prolonged psychostimulant-induced locomotor activity. The nicotinic receptor within the VTA is necessary for nicotine-induced forward locomotion (Panagis et al., 1996, Alderson et al., 2005). Although it is well established that nAChRs in the VTA are required for the locomotor activating effects of nicotine, it is less clear that nicotinic mechanisms are responsible for METH-induced locomotor activity. Previous studies have failed to block the acute stimulating effects of METH and amphetamine with IP administration of subunit non-selective (mecamylamine) and α-7 selective (methyllycaconitine) nicotinic antagonists (Escubedo et al., 2005, Kamens and Phillips, 2008).

In addition to ACh, other neurotransmitters are also thought to be involved in acute psychostimulant activation. For instance, in the VTA inhibition of *tonic* glutamate release resulted in an exaggeration of spontaneous and amphetamine-induced activation (Dalia et al. 1996).

Conclusions

The current study established that systemic administration of METH induces a prolonged increase in extracellular ACh levels within the VTA as well as a robust increase in somatodendritic DA levels. We speculate that the prolonged increase in ACh levels regulates protracted terminal DA responding and locomotor activation. We propose that METH activates a recurrent loop within the mesocorticolimbic pathway to simulate increases in VTA ACh and DA. Future studies will be needed to determine the behavioral sequelae of this form of recurrent activation.

Chapter 3

ACETYLCHOLINE FROM THE MESOPONTINE TEGMENTAL NUCLEI DIFFERENTIALLY AFFECTS METHAMPHETAMINE INDUCED LOCOMOTOR ACTIVITY AND NEUROTRANSMITTER LEVELS IN THE MESOLIMBIC PATHWAY

This chapter was adapted from:

Dobbs L.K. & Mark G.P., Acetylcholine from the mesopontine tegmental nuclei differentially affects methamphetamine induced locomotor activity and neurotransmitter levels in the mesolimbic pathway, *Behavioural Brain Research* 2012; 226(1): 224-234, with permission from Elsevier.

ABSTRACT

METH increases DA levels within the mesolimbic pathway as well as ACh, a neurotransmitter known to increase DA cell firing and release and mediate reinforcement, within the VTA. The LDT and PPT nuclei provide cholinergic input to the VTA; however, the contribution of LDT- and PPT-derived ACh to METH-induced DA and ACh levels and locomotor activation remains unknown. The first experiment examined the role of LDT-derived ACh in METH locomotor activation by reversibly inhibiting these neurons with bilateral intra-LDT microinjections of the M2 receptor agonist OXO. Male C57BL/6J mice were given a bilateral 0.1 µl OXO (0, 1, or 10 nM/side) microinjection immediately prior to IP saline or METH (2 mg/kg). The highest OXO concentration significantly inhibited both saline- and METH-primed locomotor activity. In a second set of experiments we characterized the individual contributions of ACh originating in the LDT or PPT to METH-induced levels of ACh and DA by administering intra-LDT or PPT OXO and performing *in vivo* microdialysis in the VTA and NAc. Intra-LDT OXO dose-dependently attenuated the METH-induced increase in ACh within the VTA but had no effect on DA in NAc. Intra-PPT OXO had no effect on ACh or DA levels within the VTA or NAc, respectively. We conclude that LDT, but not PPT, ACh is important in locomotor behavior and the cholinergic, but not dopaminergic, response to systemic METH.

INTRODUCTION

The mesopontine tegmentum, which contains the LDT and PPT, is a neurochemically and functionally diverse structure that is important for the interpretation of sensory input and selection of an appropriate motor output (Wang and Morales 2009; Winn 2006). The LDT and PPT provide the only known cholinergic projections to midbrain DA neurons in the SN and VTA. Specifically, the cholinergic projection from the LDT preferentially targets VTA DA and GABA neurons (Oakman et al. 1995; Omelchenko and Sesack 2006), while projections from the PPT primarily target DA neurons within the SN. Previous research has used electrical and pharmacological manipulation of the LDT-to-VTA ACh projection to assess its contribution to DA neuron firing and extracellular DA levels in the mesolimbic pathway (Forster and Blaha 2000; Forster et al. 2002b; Lodge and Grace 2006). By measuring the DA oxidation current within the NAc and DA neuron firing in the VTA, this research suggests that LDT ACh can increase DA neuron firing and release through its actions on M5-subtype mAChRs and nAChRs located on DA neurons in the VTA (for a review see Mena-Segovia et al.). Furthermore, specific targeting of the PPT (Yeomans et al. 1993) or LDT (Laviolette et al. 2000) cholinergic projection has implicated mesopontine ACh in exploratory behavior, spontaneous locomotion and the rewarding value of ICSS (Yeomans et al. 1993). In addition, application of cholinergic agonists and antagonists in the VTA suggests that nAChRs and mAChRs on DA neurons in the VTA affect ICSS and extracellular DA levels in terminal regions of the mesolimbic pathway, as measured by microdialysis (Miller and Blaha 2005; Westerink et al. 1998; Westerink et al. 1996). Taken together, these data suggest that mesopontine cholinergic projections to the VTA could be a key contributor to mesolimbic DA responses and DA-mediated reinforcement.

Cholinergic modulation of DA responding is one mechanism drugs of abuse can use to increase DA levels within the mesolimbic pathway (Gerber et al. 2001; Miller and Blaha 2005). METH, a highly addictive psychostimulant, is one such drug that induces DA release in terminal regions such as the NAc and at the cell bodies in the VTA (Dobbs and Mark 2008; Hess et al. 2000; Shoblock et al. 2003; Zhang et al. 2001). In addition, recent evidence suggests that METH also induces a prolonged increase in ACh levels within the VTA (Dobbs and Mark 2008); however, it is unclear whether this METH-induced increase in ACh contributes to increased DA levels within NAc or METH-related behaviors, such as locomotor activation. It is well known that acute administration of METH induces robust locomotor activation (Shoblock et al. 2003) and a recent study in C57BL/6J mice found brain METH concentration to be correlated with locomotor activity (Zombeck et al. 2009). Indeed, it has been hypothesized that a common neural substrate may underlie the rewarding and locomotor activating effects of drugs of abuse (Wise and Bozarth 1987) and recent evidence suggests that mesolimbic DA may mediate the stimulating and rewarding effects of METH (Itzhak et al. 2002; Jones et al. 2007). Thus, METH-induced locomotor activity is a measurable behavior that may be an analog of the rewarding effects of METH. In addition, the cholinergic projection to the mesolimbic pathway from the PPT and LDT has been implicated in reward-related behavior and locomotor behavior. However, little is known about the role of ACh in METH-related behaviors and neurochemical effects. The present study evaluated the contribution of LDT-derived ACh in METH- (or saline) induced locomotor activity. A separate set of experiments used *in vivo* microdialysis to clarify whether the source of ACh primarily originated in the LDT and/or the PPT and whether the resulting intra-VTA cholinergic tone contributes to METH-induced increases in NAc DA levels.

In order to test this, we reversibly inhibited cholinergic neurons in the LDT or PPT via a bilateral microinjection of the mAChR agonist OXO. OXO preferentially binds to M2-subtype ACh receptors, which are inhibitory autoreceptors that activate a TTX-insensitive hyperpolarization thereby inhibiting neuronal activity and terminal ACh release. We hypothesized that reversible inhibition of LDT-, but not PPT-, derived ACh would attenuate METH-induced levels of ACh and DA in the VTA and NAc, respectively. We also hypothesized that inhibition of LDT derived ACh would attenuate METH-induced locomotor behavior.

METHODS

Subjects

Male C57BL/6J mice between 8 – 13 weeks old (Jackson Laboratories, Sacramento, CA) were used in all five experiments. Experiment 1 consisted of 39 experimentally naïve mice, 19 of which were subsequently used in Experiment 2 after a 3-week wash-out period. An additional 15 experimentally naïve C57BL/6J mice were added to finish Experiment 2, for a total of 31 mice used in Experiment 2. Experiments 3, 4 and 5 consisted of 10, 16 and 13 experimentally naïve mice, respectively. All animals were maintained in a climate and humidity controlled vivarium on a 12 h light-dark cycle with lights on at 0600h. Animals were allowed *ad libitum* access to food and water while in their home cage. All experiments were approved by the Oregon Health & Science University Institutional Animal Care and Use Committee and carried out in accordance with the guidelines set forth by the National Research Council of the National Academies (NationalResearchCouncilofthe NationalAcademies 2003).

Drugs

METH hydrochloride was provided by the National Institutes on Drug Abuse drug supply program (Research Triangle Park, NC). Mice received an IP injection of either 2 or 3.5 mg/kg METH in the locomotor or microdialysis experiments, respectively. METH doses were selected by their ability to stimulate an intermediate increase in locomotor activation or ACh levels within the VTA while not producing a floor or ceiling effect (Dobbs and Mark 2008). METH was dissolved in 0.9% saline and administered in a volume of 0.1 ml/25 g. As the control, IP saline was injected in a volume of 0.1 ml/25 g. OXO (Sigma, St. Louis, MO) was dissolved in artificial cereberospinofluid (aCSF) to 1, 5 or 10 nM for bilateral microinfusions of 0.1 µl/side into the LDT or PPT. These concentrations are expressed as the salt and were selected based on competition binding studies in the human and rat pons (OXO for the M2 receptor subtype: *Kd* = 1.45 nM, *Bmax* = 14,093 cpm) (Potter et al. 1991; Vanderheyden et al. 1990) and from previous experiments in the lab administering intra-LDT OXO microinjections in the rat. The aCSF consisted of (in mM): 128 NaCl, 3.9 KCl, 1.2 $CaCl_2$, 1.0 $MgCl_2$, 25 $NaHCO_3$, 0.3 NaH_2PO_4 pH 7.3.

Surgery

Mice were placed under isoflurane anesthesia and stereotaxically implanted with bilateral 6.5 mm long, 23-gauge thin wall guide shafts positioned above the LDT (AP: -4.96 mm, ML: ±0.65 mm, DV: -1. 5 mm) or the PPT (AP: -4.72 mm, ML: ± 1.25 mm, DV: -1.75 mm). In the same surgery, mice were also implanted with a unilateral 5 mm long 21-gauge guide shaft positioned above the VTA (AP: -2.92 mm, ML: ±0.6 mm, DV: -1.3 mm; Experiments 2 and 4) or the NAc (AP: +1.1 mm, ML: ±0.8 mm, DV: -1.5 mm; Experiments 3 and 5). Coordinates were selected from the atlas of Paxinos and Franklin (Paxinos and Franklin 2001). A stylet was kept in the guide shafts until the time of

testing. Subcutaneous saline and an analgesic (carprofen; 0.5 mg/kg) were administered at the beginning of the surgery. Mice were individually housed following surgery to prevent damage to head-mounts and allowed one week to recover prior to experimental testing.

Microinjection procedure

The procedure for bilateral microinjections was adapted from experiments in our lab using rats and mice (Gililland-Kaufman et al. 2008; Mark et al. 2006). Briefly, microinjectors were constructed from fused silica glass tubing (150 µm od X 75 µm id) protruding 0.5 or 2.5 mm from the end of a 28-gauge stainless steel guide to reach the target nuclei of the inferior colliculus (IC) or the LDT, respectively. The shorter microinjector was used in the locomotor experiment in order to target the IC. Bilateral microinjections into the IC were made as an anatomical control to assess the potential effect of OXO diffusing up the injector tract on locomotor activity. Microinjections were delivered over 60 s at a rate of 1.67 nl/s for a total volume of 100 nl per side. Injectors remained in place for 30 s following the injection.

Locomotor Apparatus

Locomotor activity was assessed in a rectangular (30.5 cm x 15.2 cm) clear Plexiglas chamber with a paper floor housed in a sound attenuating cabinet. An infrared activity camera (Coulbourn Instruments, H24-61) mounted 18 cm above the arena recorded all activity during the trial. Horizontal activity data were recorded in the dark as total distance (cm) moved using Ethovision (Noldus, Tacoma, WA) tracking software.

Microdialysis Apparatus

Mice were housed in a cylindrical clear polycarbonate chamber (38 cm x 27 cm; Instech Laboratories, Plymouth Meeting, PA) in a sound-attenuated enclosure during *in vivo* microdialysis. A fiber optic lamp on the lowest setting was positioned 30 cm above the polycarbonate chamber and provided illumination during the experiment. A counter-balanced arm held a single channel swivel extended over the middle of the chamber. A Hamilton syringe (Glenco, 2.5 cc gastight; Sigma, St. Louis, MO) was fitted onto a pump (Razel A99; Braintree Inc, Stamford, CT), which delivered buffered dialysis ringer (142 mM NaCl, 3.9 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.35 mM Na_2HPO_4, 0.3 mM NaH_2PO_4; pH 7.3) via PE20 tubing through the swivel to the microdialysis probe at a rate of 1 µl/min. In experiments 1 and 3 the AChE inhibitor neostigmine (0.3 µM neostigmine·Br) was added to the dialysis ringer to delay the breakdown of ACh before it could diffuse across the dialysis membrane and be collected.

Locomotor Activity Procedure

Locomotor activity and measurements of ACh and DA were performed in separate experiments because previous experiments in our lab have found that timed collection of dialysis samples can alter an animal's behavior. In addition, we have noted that ACh levels within the VTA are extremely sensitive to an animal's wakefulness and activity level. Therefore, since measuring ACh and DA could affect an animal's activity (which, in turn, would artificially alter transmitter levels) we decided to perform these measures separately.

Locomotor activity was tested over 3 consecutive days. Day 1 served as habituation to handling, injection procedures and the testing apparatus and also provided a measure of locomotion in a novel environment. Day 2 established baseline locomotor activity for all animals. On the first 2 days the microinjection and IP injection

administered were aCSF and saline, respectively. Day 3 was the test day, in which mice were administered an OXO microinjection (0.1 µl of 0, 1 or 10 nM per side) followed by an IP METH injection (0 or 2 mg/kg).

Each day, animals were brought into the testing room, weighed and their stylets were removed. Mice were then returned to their home cage to acclimate for 45 min prior to testing. After acclimation, each animal was lightly restrained, given a bilateral microinjection immediately followed by an IP injection and placed into the locomotor arena. Activity was recorded for 30 min. At the end of the session, the animals were removed from the testing chamber and returned to their home cage.

In vivo Microdialysis Procedure

Approximately 14 h before testing, a 7.5 mm long microdialysis probe was inserted into the guide shaft that extended 2.5 mm into the VTA or NAc. This allowed time for tissue trauma to dissipate and for the mouse to habituate to the testing chamber. Microdialysis probes were constructed as described previously (Dobbs and Mark 2008) and had a 0.75 or 1.0 mm long active membrane tip for use in the VTA or NAc, respectively. The microdialysis procedure for measuring DA and ACh was performed as previously described (Dobbs and Mark 2008), with the exception that neostigmine was not added to the dialysis ringer when collecting dialysates for DA. Neostigmine was removed for the DA analysis since it is only necessary to prevent the breakdown of ACh and may artificially inflate DA levels (You et al. 2008).

Baseline samples were collected every 20 min for 1 h and analyzed for ACh or DA content using HPLC or liquid-chromatography tandem mass spectrometry (LC-MS/MS). Following baseline sampling, each mouse was given a bilateral microinjection

into the LDT or PPT, immediately followed by an IP saline injection (0.1 ml/25 g). Samples were taken every 20 min for 80 min total. Mice were then administered a second bilateral microinjection into the LDT or PPT followed by an IP METH injection (3.5 mg/kg). Samples were taken every 20 min for up to 4 h until ACh or DA levels returned to baseline.

Experiment 1: Locomotor activity following methamphetamine and laterodorsal tegmental/inferior colliculus microinjection

The purpose of this experiment was to assess the role of LDT ACh on METH-induced locomotor activity. Each mouse experienced two bouts of the 3-day locomotor testing procedure, for a total of 6 testing days. During one testing bout the bilateral microinjection was administered into the LDT, while during the other bout the bilateral microinjection was administered into a dorsal control site, the IC. Thus, each mouse served as its own control for the dorsal diffusion of the drug. There was a 2 week wash-out period between the testing bouts. Mice were counterbalanced for microinjection site, so that half of the mice received the IC microinjection in the first testing bout. On the test day (Day 3) each mouse only received one OXO concentration (0.1 µl of 0, 1, or 10 nM per side) and one METH dose (0 or 2 mg/kg). This dose was the same for each testing bout.

Experiment 2: Intra-ventral tegmental in vivo microdialysis for acetylcholine following intra-laterodorsal tegmental oxotremorine

The goal of this experiment was to determine whether the LDT was the source of the METH-induced increases in intra-VTA ACh. *In vivo* microdialysis was performed within the VTA and dialysates were collected and assessed for ACh content.

After establishing a stable baseline of ACh levels each mouse received a control injection. This consisted of a bilateral intra-LDT microinjection (0.1 µl per side) of aCSF or OXO (1, 5, or 10 nM) immediately followed by an IP saline injection. The control injection served to assess the effect of intra-LDT OXO on basal VTA ACh levels and the effect of handling and injection on ACh levels. A total of 80 min after the control injection each mouse received a test injection, thus each mouse served as its own control. This consisted of a bilateral intra-LDT microinjection immediately followed by IP METH (3.5 mg/kg). Each mouse only received one dose of OXO and this dose was the same during the control and test injections.

Experiment 3: Intra-ventral tegmental in vivo microdialysis for acetylcholine following intra-pedunculopontine tegmental oxotremorine

The purpose of this experiment was to determine whether the PPT was involved in the METH-induced increases in intra-VTA ACh. *In vivo* microdialysis was performed within the VTA just as in Experiment 2. Each mouse received a control injection after baseline ACh levels were established. The control injection consisted of a bilateral intra-PPT microinjection (0.1 µl per side) of aCSF or OXO (1 or 10 nM) immediately followed by an IP saline injection. A total of 80 min after the control injection each mouse received a test injection, which included a bilateral intra-PPT microinjection immediately followed by IP METH (3.5 mg/kg). Just as in Experiment 2, each mouse only received one dose of OXO and this dose was the same during the control and test injections.

Experiment 4: Intra-nucleus accumbens in vivo microdialysis for dopamine following intra-laterodorsal tegmental oxotremorine

This experiment examined whether LDT-derived ACh influenced METH-induced increases in DA levels within the NAc. *In vivo* microdialysis was performed within the

NAc and dialysate samples were collected and analyzed for DA content. Each mouse received a control injection after baseline DA levels were established, which consisted of a bilateral intra-LDT microinjection (0.1 µl per side) of aCSF or OXO (1 or 10 nM per side) immediately followed by an IP saline injection. Eighty min following the control injection each mouse received a test injection. This consisted of a bilateral intra-LDT microinjection immediately followed by IP METH (3.5 mg/kg). Just as in Experiments 2 and 3, each mouse only received one dose of OXO and this dose was the same during the control and test injections.

Experiment 5: Intra-nucleus accumbens in vivo microdialysis for dopamine following intra-pedunculopontine oxotremorine

In this experiment, we examined whether PPT-derived ACh played a role METH-induced increases in DA levels within the NAc. *In vivo* microdialysis was performed within the NAc just as in Experiment 4. Each mouse received a control injection after baseline DA levels were established. This control injection included a bilateral intra-PPT microinjection (0.1 µl per side) of aCSF or OXO (1 or 10 nM) immediately followed by an IP saline injection. Each mouse received a test injection 80 min after the control injection. This consisted of a bilateral intra-PPT microinjection immediately followed by IP METH (3.5 mg/kg). Just as in Experiments 2 through 4, each mouse only received one concentration of OXO and this concentration was the same during the control and test injections.

Sample and Standard Preparation and Analysis

DA and ACh samples and their corresponding standard curves that were analyzed using HPLC with electrochemical detection were prepared as described previously (Dobbs and Mark 2008). Approximately two-thirds the way through the

dialysis experiment, we were not able to obtain immobilized enzyme reactors with suitable sensitivity for the electrochemical detection of ACh using HPLC. As a result, a different analytical method, LC-MS/MS, was used for the last third of subjects. The total number of samples in each OXO treatment group that were analyzed using LC-MS/MS was: 62 for aCSF, 24 for 1 nM OXO and 69 for 5 nM OXO. The total number of samples per OXO treatment group that were analyzed using HPLC was: 76 for aCSF, 104 for 1 nM OXO, 37 for 5 nM OXO and 128 for 10 nM OXO.

ACh dialysate samples that were analyzed with LC-MS/MS were spiked with 180 µl of an internal standard of deuterated ACh (d4 ACh; CDN Isotopes, Quebec, Canada). The internal standard was prepared in 75% acetonitrile, 25% methanol and 0.2% formic acid (v/v/v) to a final concentration of 0.1 pg/µl. After addition of the internal standard each sample was centrifuged at 10 000 rpm for 5 min at room temperature through a 22 µm filter (Fisher Scientific, Pittsburgh, PA). A standard curve was prepared using a range of ACh concentrations (Sigma, St. Louis, MO), with each containing 180 µl of d4 ACh. Aliquots of 20 µl were injected onto a polyhydroxylethyl column (50 x 2.1, 3µm, 100 Å; Poly LC Inc., Columbia, MD) with guard column for analysis.

Sample analysis

The procedure for samples analyzed for ACh or DA content using HPLC has been described previously (Dobbs and Mark 2008).

Analysis of ACh content using the LC-MS/MS system consisted of a quadrupole ion trap (4000 Q trap, Applied Biosystems/MDX SCIEX) equipped with electron spray ionization, a Prominence UFLC-XR autosampler, system controller, column oven and UV/VIS detector for liquid chromatography (Shimadzu Corp., Kyoto, Japan). Positive

ioninzation was attained using single reaction monitoring (SRM). The transition detected for ACh was *m/*z = 146/87 and for d4 ACh was *m/*z = 150/91.1. The ion spray voltage was 3 kV and maintained at 600 °C. Nitrogen was used for the curtain gas (50 psi) and for the two ion source gasses (40 and 60 psi). Collision fragmentation was attained using nitrogen. Mobile phase for this system consisted of 0.1% formic acid, 20 mM ammonium formate, acetonitrile and methanol and was maintained at a flow rate of 3 ml/min. ACh and d4 ACh analytes were identified by retention time and their respective fragment *m/z*. The amount of ACh was quantified by peak area ratio of d4 ACh versus ACh within each sample using Analyst version 1.5 (Applied Biosystems).

Table 1 shows the picograms of ACh per microliter of dialysate at baseline or after saline injection for samples analyzed with the HPLC and LC-MS/MS analytical methods. Analysis with Welch's t-test (to correct for unequal variance) showed no significant difference in the amount of ACh at baseline (t_{24} = 0.43, p = .668) or after saline injection (t_{45} = 1.17, p = .247) between the two analytical methods. In addition, there was no significant change in the amount of ACh when comparing baseline and post-saline injection using either the HPLC method (t_{119} = 0.17, p = 0.868) or the LC-MS/MS method (t_{58} = 0.32, p = 0.753).

Histology

Mice were deeply anesthetized before a thionin (300 nl) microinjection was administered into the LDT or PPT at the end of each experiment. Microdialysis probe placement was confirmed by intercalating a thionin pulse into the inlet of the probe that perfused thionin through the VTA. Mice were then decapitated and their brains flash-frozen in isopentane on dry ice. Frozen brains were stored at -80° C until they were sectioned (40 µm) on a Leica cryostat. Data were excluded if the subjects' microdialysis

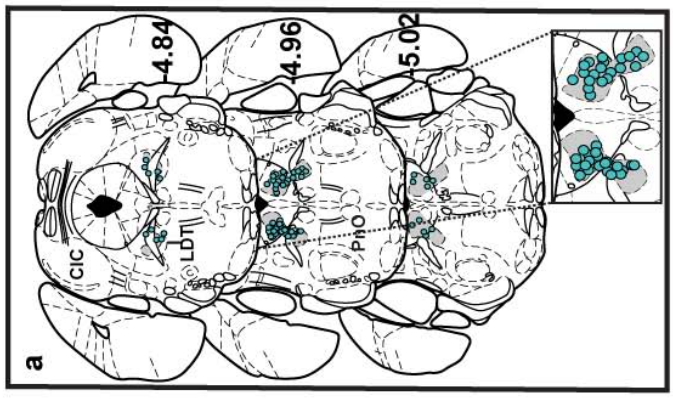
fiber intruded on the SN, when targeting the VTA, or if at least half of the fiber intruded on the caudate putamen, when targeting the NAc. Subjects were excluded if one or both microinjections were located outside of the target region. The number of mice excluded was 14, 23, 6, 14 and 5 for experiments 1 through 5, respectively. Figure 8 shows the range of microdialysis probe and microinjection locations (with target areas shaded) overlaid on plates from the atlas of Paxinos and Franklin (Paxinos and Franklin 2001) for mice included in the analyses of each experiment. Microinjection locations from Experiment 1 are shown in panel a. Panels b – e show the microdialysis probe placements (top) and microinjection locations (bottom) for Experiments 2 – 5, respectively.

Table 1. Extracellular levels of VTA ACh at baseline and following a control saline injection analyzed with HPLC and LC-MS/MS.

	HPLC[a]	LC-MS/MS[a]
Baseline	0.85 ± 0.07 (N = 58)	0.98 ± 0.29 (N = 23)
Post-Saline	0.83 ± 0.07 (N = 63)	1.09 ± 0.20 (N = 37)

[a] data expressed as pg ACh / µl dialysate

There was no difference in extracellular ACh between the two analytical methods, HPLC (left column) or LC-MS/MS (right column) using a Welch's corrected t-test at baseline (p = 0.668) or post-saline (p = 0.247). There was also no significant change in extracellular ACh levels between baseline and post-saline for the HPLC (p = 0.868) or LC-MS/MS method (p = 0.753).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LDT
PnO
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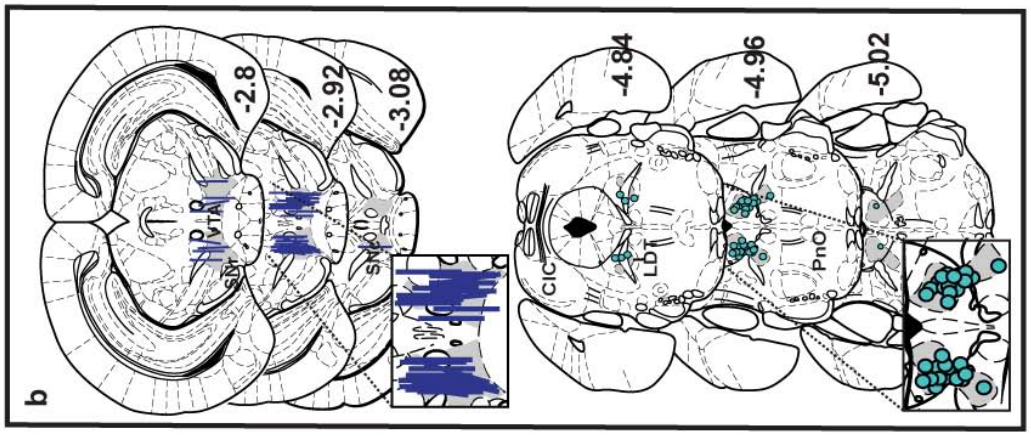
-4.96
-5.02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SN
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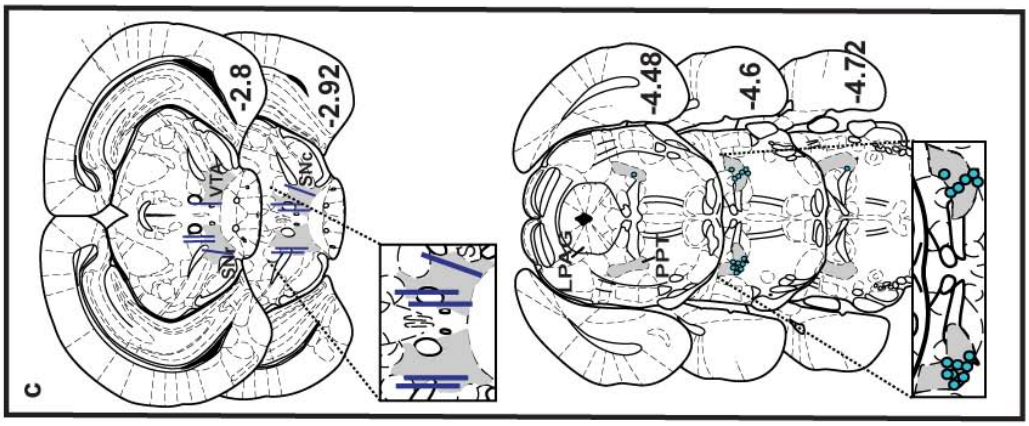
CIC
LDT
PnO
-4.84
-4.96
-5.02


c
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SN
SNc
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LPAG
LPPT
-4.48
-4.6
-4.72

Figure 8

Figure 8 (continued)

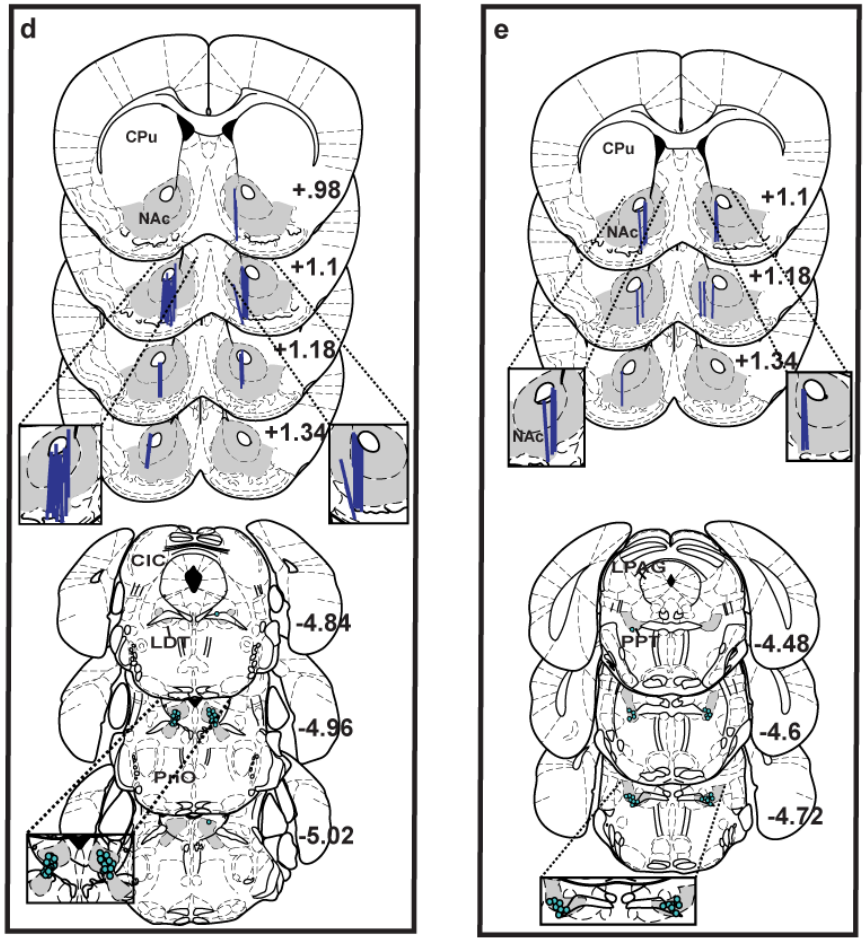


Figure 8. Microdialysis probe placements in the VTA and NAc and bilateral microinjection locations in the LDT and PPT are shaded and shown overlaid on plates taken from the atlas of Paxinos and Franklin (2001). Successful LDT microinjection targets for Experiment 1 are shown in panel (**a**). Panels (**b**) and (**c**) show successful intra-VTA microdialysis probe placements for Experiments 2 and 3 (top) with the corresponding LDT (**b**, bottom) and PPT (**c**, bottom) microinjection locations. Panels (**d**) and (**e**) show successful intra-NAc microdialysis probe placements for Experiments 4 and 5 (top) with the corresponding LDT (**d**, bottom) and PPT (**e**, bottom) microinjection locations. Probe placements were counterbalanced for side and plates are labeled as mm from bregma. CIC: central nucleus of inferior colliculus; CPu: caudate putamen; LDT: laterodorsal tegmental nucleus; LPAG: lateral periaqueductal grey; NAc: nucleus accumbens; PnO: pontine reticular nucleus (oral part); PPT: pedunculopontine tegmental nucleus; SNc: substantia nigra pars compacta; SNr: substantia nigra pars reticulata; VTA: ventral tegmental area.

Statistics

Locomotor data were analyzed using a three-way repeated measure ANOVA with Day as the within subjects factor and IP treatment (IP) and Microinjection Pretreatment (PreTx) as the between subjects factors. Greenhouse-Geisser was used to correct for violations to sphericity. Dialysis data were analyzed using 2-way repeated measure ANOVA with microinjection treatment as the between subjects factor (OXO; 4 levels for Experiment 2, 2 levels for Experiments 3 – 5) and time as the within subjects factor. For the within subject factor, time was collapsed into 2 time bins, post-saline and post-METH. For Experiments 2, 4 and 5 the post-saline time bin consisted of collapsing the 80 min of data following the saline injection. For Experiment 3 the last 60 min of post-saline injection data were collapsed. These time bins were chosen to create an equal baseline between OXO groups. For Experiments 2 and 3, data in the first 80 min following METH injection were collapsed to create the post-METH time bin. For Experiments 4 and 5, data in the first 40 min following METH injection were collapsed to create the post-METH time bin. Since some subjects had a sample missing or excluded following METH administration, a repeated measure ANOVA would have required eliminating these subjects from the analysis. Therefore, we collapsed data into one time bin following METH administration in order to include all subjects. Missing samples were due to problems such as the probe coming disconnected during dialysate collection, technical problems with sample injection onto the HPLC column, or HPLC/LC-MS/MS detection problems. A sample was considered below detection limit and excluded from the analysis if (1) the amount of ACh detected fell outside the range of the standard curve, (2) no sample peak was detected by the HPLC or LC-MS/MS, or (3) the signal-to-noise ratio was not at least 2:1. The total number of samples across all microdialysis experiments that were excluded from analysis was 80 out of 760 (10.5%). Post-METH time bins were selected that best represented the peak of the response following METH

injection. Significant two-way interactions were followed up with simple main effects analyses and post-hoc tests. Effects were considered significant at an alpha of 0.05.

RESULTS

Experiment 1

Locomotor activity following laterodorsal tegmental microinjection pretreatment

Data from a total of 39 mice were used in this analysis. A summary of activity (cm ± SEM) on baseline and test day for each treatment group is presented in Table 2. A significant Day x IP interaction indicated that IP injection of saline or METH resulted in a significant change in locomotor activity on test day compared to baseline ($F_{1,33} = 31.7$, $p < 0.001$; Figure 9a). Additionally, the significant Day x PreTx interaction indicated that administration of OXO into the LDT differentially affected test day locomotor activity compared to baseline ($F_{2,34} = 8.17$, $p = 0.001$; Figure 9a). There were also significant main effects of Day ($F_{1,33} = 7.94$, $p = 0.008$) and IP ($F_{1,33} = 23.4$, $p < 0.001$). The main effect of PreTx, IP x PreTx interaction and Day x IP x PreTx interaction were not significant.

Follow up analyses of the Day x IP interaction were performed collapsed across pretreatment dose (0, 1 or 10 nM OXO). Mice that received IP administration of saline on the test day showed significantly lower activity compared to baseline (from 3941 $\pm$ 1328 to 2053 $\pm$ 412) ($F_{1,18} = 10.04$, $p = 0.005$), while IP injection of 2 mg/kg METH produced a significant increase in locomotor activity (from 3759 $\pm$ 371 to 8235 $\pm$ 1075) ($F_{1,19} = 17.49$, $p = 0.001$). Since these comparisons were performed collapsed across pretreatment dose, the significant decrease in locomotor activity seen in the saline group

Figure 9

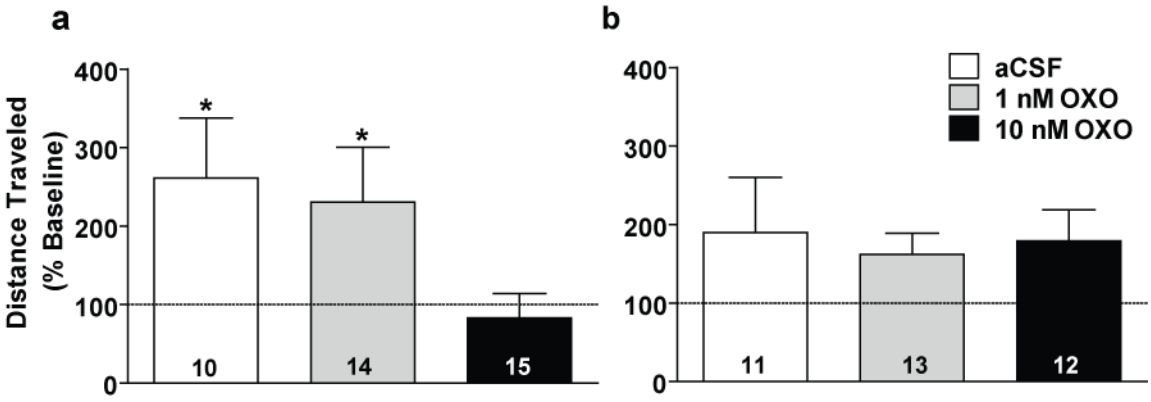


Figure 9. Effect of bilateral OXO microinjections into the LDT (**a**) or a dorsal control site, the IC (**b**). Mice received IP saline or METH immediately following the microinjection. Activity data from the test day are shown depicted as a percent of baseline (represented by the dotted line) and collapsed on IP treatment with OXO group sample sizes on their respective bar. Mice pretreated with a bilateral microinjection into the LDT of aCSF (white bar) or 1 nM OXO (grey bar) immediately followed by IP saline or METH (2 mg/kg) showed a significant increase in locomotor activity on test day compared to baseline (**a**). Mice pretreated with 10 nM OXO (black bar) immediately followed by IP saline or METH (2 mg/kg) showed no significant change in locomotor activity on test day compared to baseline. Bilateral OXO pretreatment into the IC had no effect on locomotor activity on test day compared to baseline (**b**). * $p < 0.05$ vs. baseline activity.

might have been driven by the ability of the high concentration of OXO to inhibit basal locomotor activity (Table 2).

Follow up analyses of the Day x PreTx interaction were performed collapsed across IP treatment (saline or METH). Mice that received intra-LDT aCSF ($F_{1,9} = 8.51$, $p = 0.017$) or 1 nM OXO ($F_{1,13} = 6.45$, $p = 0.025$) showed significantly higher activity on test day, regardless of the IP drug administered. However, animals pretreated with 10 nM OXO did not show an increase in activity on test day. These data suggest that 10 nM OXO inhibited saline and METH activity, while 1 nM and aCSF did not, regardless of IP drug administered.

Table 2. Mean distance traveled in 30 min on baseline and test day following a microinjection pretreatment in the LDT or IC and IP injection of saline or METH.

Microinjection Site	IP	Microinjection Pretreatment	Baseline Activity*	Test Day Activity*, †	N
LDT	Saline	aCSF	2547 (720)	2968 (1435)	5
		1 nM OXO	3415 (657)	2336 (1326)	6
		10 nM OXO	5207 (569)	1269 (1148)	8
	2 mg/kg METH	aCSF	3535 (720)	10114 (1453)	5
		1 nM OXO	3736 (569)	9890 (1148)	8
		10 nM OXO	3946 (609)	5004 (1228)	7
IC	Saline	aCSF	3920 (713)	3234 (985)	7
		1 nM OXO	3691 (713)	3662 (985)	7
		10 nM OXO	5084 (843)	3647 (1165)	5
	2 mg/kg METH	aCSF	3950 (943)	9149 (1303)	4
		1 nM OXO	4216 (770)	9811 (1064)	6
		10 nM OXO	3800 (713)	8146 (985)	7

***** data expressed as distance traveled in 30 min (cm ± SEM)

† In mice that received microinjection pretreatment into the LDT, simple main effects analysis of a significant Day x IP interaction revealed test activity was significantly different from baseline in mice that received IP saline ($p = 0.001$) or METH ($p = 0.001$).

Locomotor activity following inferior colliculus microinjection pretreatment

Data from a total of 36 mice were used for this analysis. Table 2 lists the mean activity by all treatment groups on the baseline and test day (cm ± SEM). Figure 9b shows locomotor activity in mice that received intra-IC OXO. For the purpose of comparison to Figure 9a, these data are shown collapsed across IP treatment. The significant Day x IP interaction indicated that administration of saline or METH resulted in a difference in activity on test day compared to baseline ($F_{1,30} = 55.86$, $p < 0.0005$).

There was also a significant main effect of Day ($F_{1,30} = 31.51$, $p < 0.0005$) and IP ($F_{1,30} = 15.41$, $p < 0.0005$), but no other main effects or interactions were significant.

Further exploration of the Day x IP interaction revealed that 2 mg/kg METH significantly increased locomotor activity on test day irrespective of intra-IC pretreatment ($F_{1,16} = 52.39$, $p < 0.0005$). In addition, there was a trend for saline-treated mice to have higher locomotor activity on baseline day ($F_{1,18} = 3.96$, $p = 0.062$). Importantly, none of the data analyses suggested there was an effect of OXO administered into this dorsal control site.

Experiment 2

Intra-ventral tegmental in vivo microdialysis for acetylcholine following intra-laterodorsal tegmental oxotremorine

Data from a total of 30 mice were used in these analyses. Bilateral OXO microinjections in the LDT dose-dependently attenuated intra-VTA ACh levels following IP METH, but not IP saline (significant Time x OXO interaction: $F_{3,26} = 3.44$, $p = 0.031$). In addition, there was a significant main effect of Time ($F_{1,26} = 12.61$, $p = 0.001$). The main effect of OXO was not significant. Figure 10a shows the time course of ACh responding in relation to OXO microinjections and IP (saline and METH) injections. The lack of an OXO effect on extracellular ACh in the VTA after saline injection may have been due to a floor effect. Simple main effects analyses found no differences in the amount of extracellular ACh between OXO dose groups at baseline, but did reveal an effect of OXO at the Post-METH time bin ($F_{3,27} = 4.26$, $p = 0.013$). Following IP METH, the aCSF-pretreated mice had significantly higher levels of ACh compared to 5 nM OXO-pretreated mice ($p = 0.019$).

Mice pretreated with 5 or 10 nM OXO showed no significant increase in extracellular ACh levels following IP METH compared to baseline (i.e., Post-Saline). Thus, the higher concentrations of OXO attenuated the METH-induced increase in extracellular ACh levels. Additionally, mice pretreated with aCSF ($F_{1,7}$ = 10.12, p = 0.015) or 1 nM OXO ($F_{1,7}$ = 10.8, p = 0.013) showed significantly increased extracellular ACh levels in the 80 min Post-METH injection compared to the Post-saline injection (Figure 10b).

Experiment 3

Intra-ventral tegmental in vivo microdialysis for acetylcholine following intra-pedunculopontine oxotremorine

Data from a total of 10 mice were used in these analyses. Figure 11a shows the intra-VTA ACh levels in 20 min time bins in response to OXO or aCSF pretreatment followed by IP saline and METH. Analysis of the collapsed data indicated that METH induced an increase in the levels of extracellular ACh regardless of intra-PPT pretreatment (main effect of Time; $F_{1,8}$ = 27.83, p = 0.001) (Figure 11b). The main effect of OXO and Time x OXO interaction were not significant. Microinjections of 10 nM OXO into the PPT had no effect on extracellular levels of ACh after saline or METH injection. When analyzing the data it appeared that the 10 nM OXO-treated group showed a shift in the peak ACh response compared to aCSF-treated animals. Therefore, we performed a repeated measure ANOVA comparing the collapsed control time bin with the individual post-METH 20 min time bins, which required the removal of 3 subjects due to missing samples (1, aCSF and 2, 10 nM). This analysis showed no significant main effects of Time or OXO and no significant Time x OXO interaction (data not shown).

Figure 10

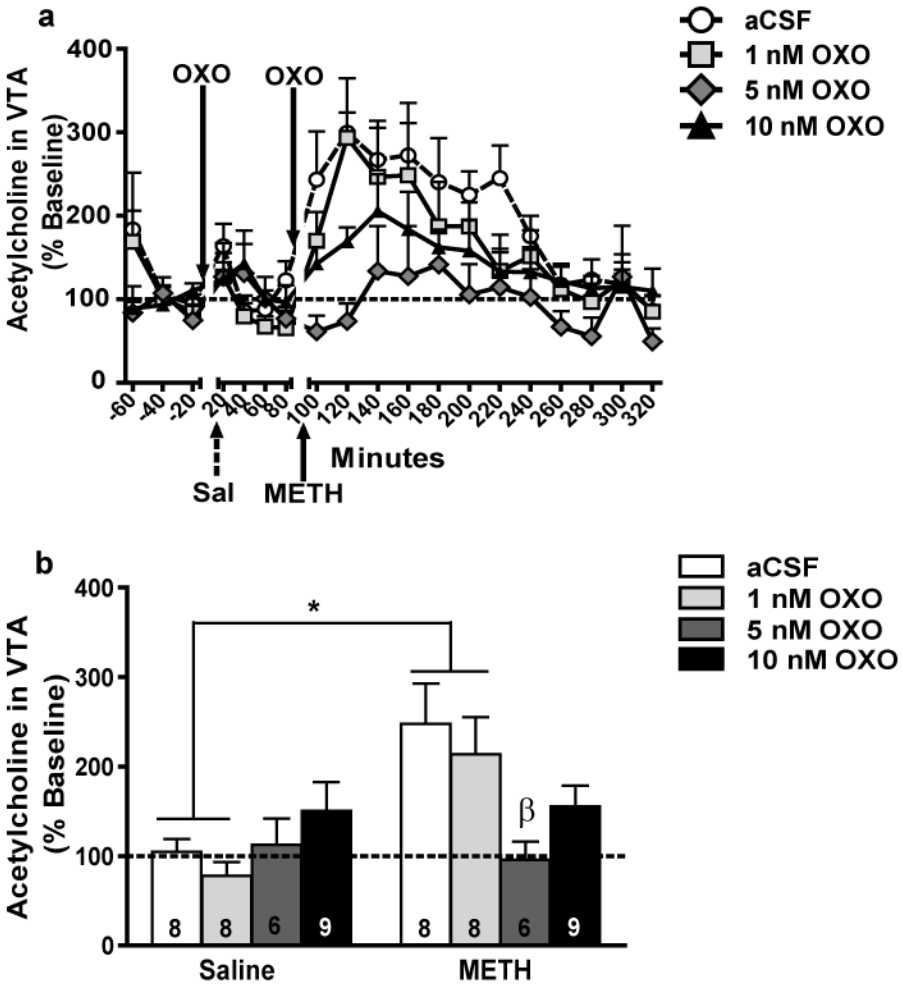


Figure 10. Effect of bilateral OXO microinjections into the LDT on ACh levels in the VTA. Data are depicted as a percent of baseline (represented by the dotted line). The ACh time course in response to aCSF (white circles), 1 nM (light grey squares), 5 nM (dark grey diamonds), or 10 nM (black triangles) OXO microinjection and IP saline (Sal; broken arrow) or METH (3.5 mg/kg; solid arrow) is shown in 20 min bins (**a**). ACh samples were collapsed for each mouse after saline injection (Saline) and in the first 80 min after METH injection (3.5 mg/kg; METH) for analysis (**b**). There was a significant increase in ACh levels in mice pretreated with aCSF (white bar) or 1 nM OXO (light grey bar) following IP METH compared to saline. Compared to aCSF, microinjection pretreatment with 5 nM OXO significantly decreased METH-induced ACh levels. Treatment group sample sizes are shown on their respective bar. * $p <$ 0.05 METH vs. saline; β $p < 0.05$ vs. aCSF METH group

Figure 11

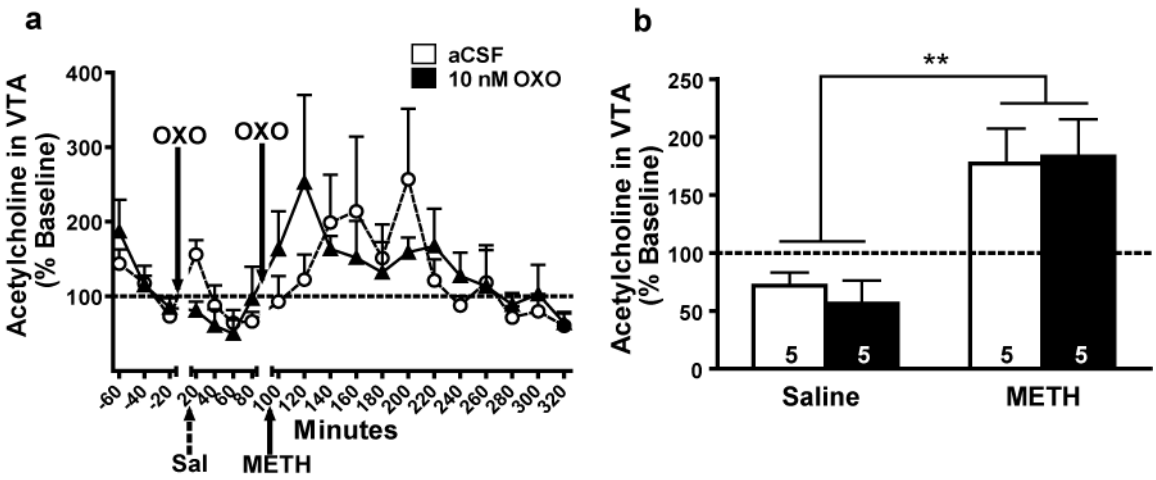


Figure 11. Effect of bilateral OXO microinjections into the PPT on ACh levels in the VTA. Data are expressed as a percent of baseline (represented by the dotted line). The time course of ACh in response to intra-PPT OXO (10 nM; black triangles) or aCSF (white circles) microinjection and IP injection of saline (Sal; broken arrow) or METH (3.5 mg/kg; solid arrow) is shown in 20 min bins (**a**). ACh samples were collapsed for each mouse after saline injection (Saline) and in the first 80 min after METH injection (3.5 mg/kg; METH) in each OXO treatment group for analysis (**b**). There was a significant increase in ACh levels following IP METH in both 10 nM OXO- (black bar) and aCSF-pretreated (white bar) mice. Treatment group sample sizes are shown on their respective bar. ** $p = 0.001$ vs. IP saline

Experiment 4

Intra-nucleus accumbens in vivo microdialysis for dopamine following intra-laterodorsal tegmental oxotremorine

Data from a total of 16 mice were used in the DA analyses. The time course of the DA and DA metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), response is shown in Figures 12a and 13a, respectively. A main effect of Time showed a significant increase in NAc DA levels in the first 40 min after METH injection compared to after saline injection ($F_{1,14} = 18.48$, $p = 0.001$) (Figure 12b). The main effect of OXO and Time x OXO interaction were not significant.

Figure 12

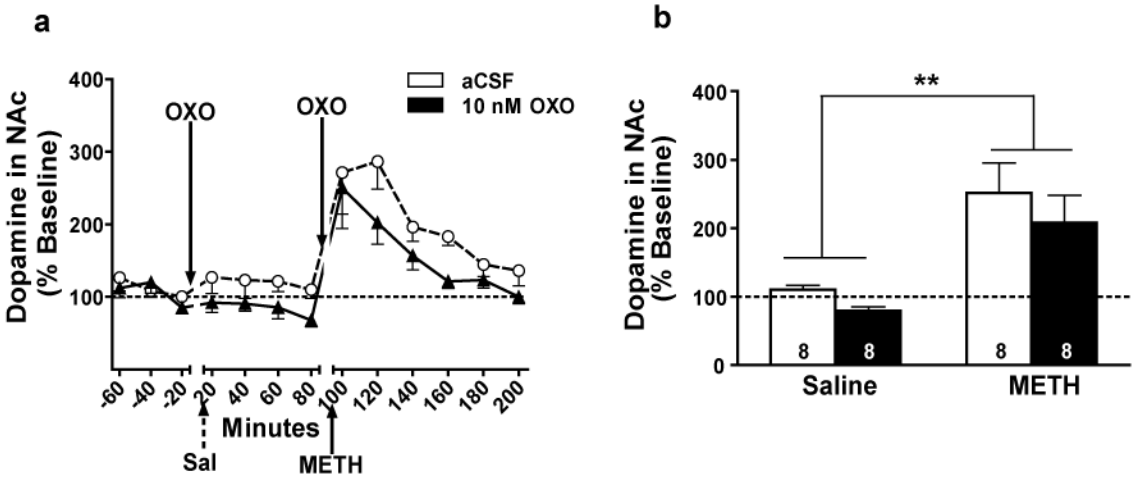


Figure 12. Effect of bilateral OXO microinjections into the LDT on DA levels in the NAc. Data are expressed as a percent of baseline (represented by the dotted line). The time course of DA levels in response to intra-LDT OXO (10 nM; black triangles) or aCSF (white circles) microinjection and IP injection of saline (Sal; broken arrow) or METH (3.5 mg/kg; solid arrow) is shown in 20 min bins (**a**). DA samples were collapsed for each mouse after saline injection (Saline) and in the first 40 min after METH injection (3.5 mg/kg; METH) in each OXO treatment group for analysis (**b**). Microinjection of 10 nM OXO (black bar) into the LDT had no effect on the METH-induced increase in DA levels. Treatment group sample sizes are shown on their respective bar. ** $p = 0.001$ vs. IP saline

As a measure of DA metabolism, levels of DOPAC were measured post-saline injection and compared to the first 40 min post-METH injection (Figure 13a). Data from a total of 15 mice were used in the DOPAC analyses. A main effect of time showed a decrease in extracellular DOPAC levels after METH injection compared to after saline injection ($F_{1,13} = 90.65$, $p < 0.0005$) (Figure 13b). This sustained decrease in DOPAC levels is most likely due to a METH-induced reversal of the DAT, which would inhibit DA re-uptake and metabolism into DOPAC. The main effect of OXO and Time x OXO interaction were not significant.

Experiment 5

Figure 13

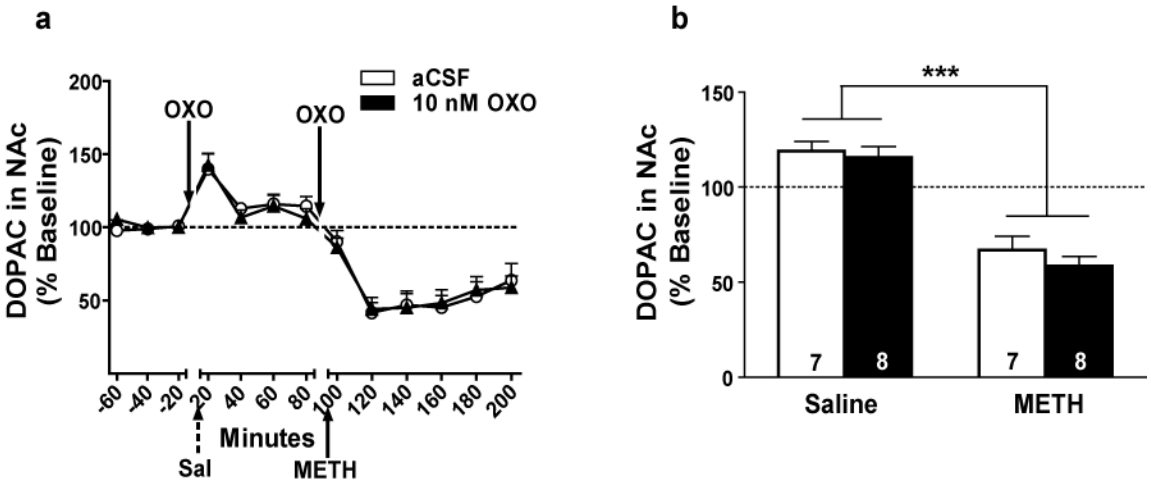


Figure 13. Effect of bilateral OXO microinjections into the LDT on levels of the DA metabolite, DOPAC, in the NAc. Data are expressed as a percent of baseline (represented by the dotted line). The time course of DOPAC levels in response to intra-LDT OXO (black triangles) or aCSF (white circles) microinjection and IP injections of saline (Sal; broken arrow) or METH (3.5 mg/kg; solid arrow) is shown in 20 min bins (**a**). Data were collapsed after saline injection (Saline) and the first 40 min after METH injection (METH) in each OXO treatment group for analysis (**b**). There was a significant decrease in DOPAC levels after IP METH in both 10 nM OXO- (black bar) and aCSF-pretreated (white bar) mice. Treatment group sample sizes are shown on their respective bar. *** $p < 0.0005$ vs. IP saline

Intra-nucleus accumbens in vivo microdialysis for dopamine following intra-pedunculopontine oxotremorine

Data from 12 mice were used in the DA analyses. The time course of the DA and DOPAC response is shown in Figures 14a and 15a, respectively. DA levels increased significantly after the METH injection compared to after the saline injection (main effect of Time; $F_{1,10} = 6.06$, $p = 0.034$), irrespective of intra-PPT OXO pretreatment (Figure 14b). The main effect of OXO and Time x OXO interaction were not significant.

Data from 13 mice were used in the DOPAC analysis. Similar to when OXO was microinjected into the LDT, NAc levels of the DA metabolite, DOPAC, in the first 40 min

Figure 14

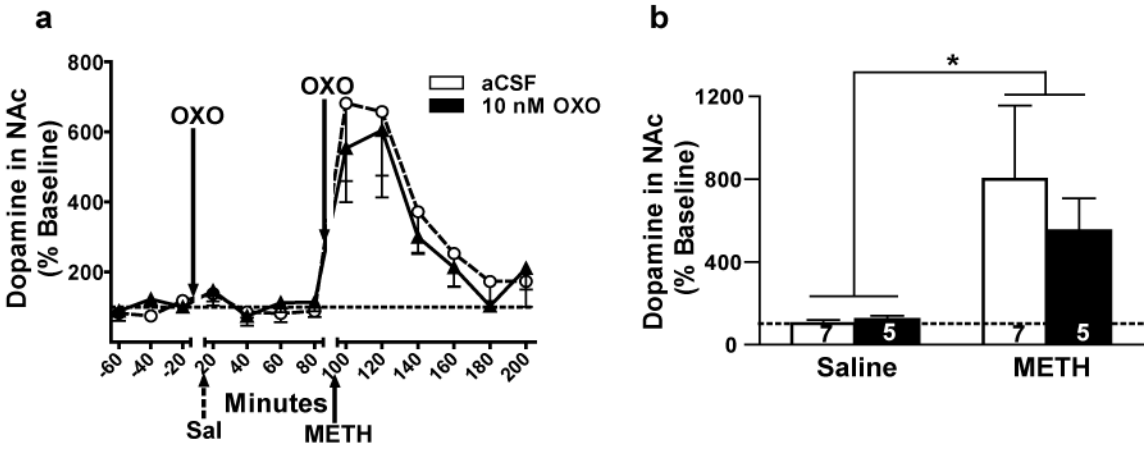


Figure 14. Effect of bilateral OXO microinjections into the PPT on DA levels in the NAc. Data are expressed as a percent of baseline (represented by the dotted line). The time course of DA levels in response to OXO (black triangles) or aCSF (white circles) and IP saline (Sal; broken arrow) or METH (3.5 mg/kg; solid arrow) is shown in 20 min bins (**a**). Data were collapsed after saline injection (Saline) and the first 40 min after METH injection (METH) in each OXO treatment group for analysis (**b**). There was a significant increase in DA levels following IP METH compared to saline in both 10 nM OXO- (black bar) and aCSF-pretreated (white bar) mice. Treatment group sample sizes are shown on their respective bar. * $p < 0.05$ vs. IP saline

after METH injection were significantly lower than after the saline injection (main effect of Time; $F_{1,11} = 79.49$, $p < 0.0005$, Figure 15b). The main effect of OXO and Time x OXO interaction were not significant.

DISCUSSION

ACh has been implicated in mediating DA neuron burst firing, locomotor activity and reward processing within the DA mesolimbic circuit. The experiments presented in this paper sought to examine the origin of the cholinergic tone within the VTA, namely the LDT and PPT within the mesopontine region, and determine their contribution to METH-induced changes in locomotor activity and ACh and DA levels within the mesolimbic circuit. The results support the proposal that while METH activates LDT

Figure 15

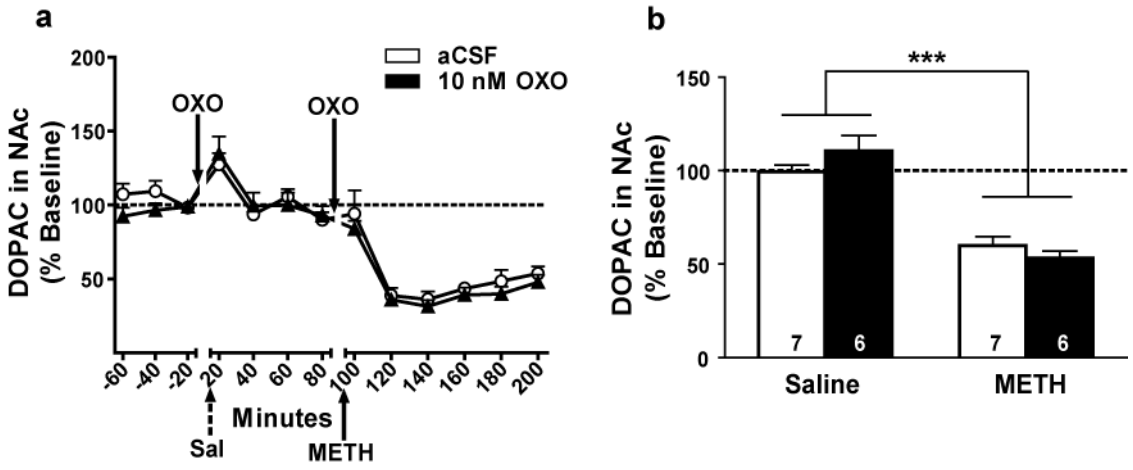


Figure 15. Effect of bilateral OXO microinjections into the PPT on levels of the DA metabolite, DOPAC, in the NAc. Data are expressed as a percent of baseline (represented by the dotted line). The time course of DOPAC levels in response to OXO (black triangles) or aCSF (white circles) and IP saline (Sal; broken arrow) or METH (3.5 mg/kg; solid arrow) is shown in 20 min bins (**a**). Data were collapsed after saline injection (Saline) and the first 40 min after METH injection (METH) in each OXO treatment group for analysis (**b**). There was a significant decrease in DOPAC levels following IP METH compared to saline in 10 nM OXO- (black bars) and aCSF-pretreated (white bars) mice. *** $p < 0.0005$ vs. IP saline

cholinergic neurons that project to the VTA, leading to increased ACh levels in the VTA, this is not critical for METH-induced increases in NAc DA levels. In addition, PPT cholinergic neurons do not seem to be recruited for basal or METH-induced increases in ACh levels within the VTA. These data also suggest that LDT cholinergic neurons are involved in locomotor activity after saline or METH injection.

Basal versus methamphetamine-induced acetylcholine responses

While intra-LDT OXO dose-dependently attenuated the METH-induced increase in intra-VTA ACh, basal levels of ACh were unaffected. Previous studies applying the mixed nicotinic/muscarinic ACh agonist carbachol to the mesopontine tegmentum suggest there may be separate groups or sub-types of cholinergic neurons within the

LDT and PPT (Kodama and Honda; Koyama and Sakai). These groups may be differentially responsive to METH and OXO or have a different expression of the M2 ACh receptor, resulting in differential effects on basal and METH-induced levels of ACh.

Investigations of the mesopontine cholinergic contribution to the ponto-geniculo-occipital waves associated with REM sleep have revealed two electrophysiologically different sub-types of cholinergic neurons within the LDT and PPT. Low-threshold bursting (LTB) neurons are suspected to maintain high levels of ACh in terminal regions through the ability to produce burst firing while non-LTB neurons, which are incapable of burst-firing and are thought to maintain tonic levels of ACh in terminal regions (Luebke et al. 1993; Wilcox et al. 1989). In cats, cholinergic agonists applied to the mesopontine tegmentum decreased burst firing-induced ACh levels in the lateral geniculate nucleus, likely through an action on M2 autoreceptors (Kodama and Honda 1996). These sub-types of cholinergic neurons within the LDT and PPT are heterogeneously distributed among other types of cells (principally GABAergic and glutamatergic neurons), making them difficult to target individually. It is not clear whether non-LTB cholinergic neurons possess the inhibitory M2 autoreceptor. One speculation is that cholinergic neurons on which METH and OXO act possess the inhibitory M2-type AChR, while cholinergic neurons responsible for maintaining basal tone in terminal regions do not express these receptors (at least to the same degree or sensitivity). METH would activate these cholinergic neurons indirectly. For instance, METH could increase mPFC DA levels and in turn stimulate mPFC glutamate projections (via DA receptors located on the glutamate neuron soma) to the LDT. OXO, on the other hand, could affect the LDT ACh neurons directly by binding to the M2-type mAChR. This is, of course, only one hypothesis for the method of action of these drugs and future studies are needed to clarify whether

these sub-types of cholinergic neurons differentially express M2 (or other) ACh receptors.

Mesopontine acetylcholine in methamphetamine-induced locomotor activity and dopamine levels

These experiments are the first to show that METH specifically activates LDT, but not PPT, ACh neurons and that inhibition of the LDT-to-VTA cholinergic projection is related to a non-selective impairment of locomotor activation (METH-induced and spontaneous). Furthermore, these data show that neither inhibition of LDT- nor PPT-derived cholinergic tone in the VTA affected METH-induced increases in DA levels within the NAc.

Although previous studies clearly support the role of mesopontine ACh in the modulation of DA burst firing and release (Chapman et al. 1997; Floresco et al. 2003; Lodge and Grace 2006), the current findings are not necessarily incompatible with the literature. METH is capable of inducing DA release in the absence of neuronal stimulation, thus cholinergic enhancement of DA activity may be insignificant compared to METH's affects at DA terminals. Several mechanisms have been reported to underlie the ability of amphetamine compounds to induce stimulation independent DA release. These include, but are not limited to, the weak base hypothesis and actions at the VMAT-2 and DAT (for an in depth review the reader is referred to Sulzer et al. 2005). The METH-induced increase in ACh, which can increase DA neuron excitability (Lacey et al. 1990; Mereu et al. 1987), is likely masked by the action of METH on DA terminals. The current data also suggest that mesopontine-derived cholinergic tone within the VTA is not necessary for *basal* DA levels in the NAc. Interestingly, the METH-induced increase in extracellular DA levels was more short-lived compared to the sustained

decrease in DOPAC levels. Amphetamines (including METH) are known to reverse the DAT and inhibit MAO, thereby inhibiting DA re-uptake and metabolism into DOPAC (for a review see Sulzer et al. 2005). Thus, this prolonged effect on DOPAC levels suggests METH was still present in the synapse 1.5 h after administration even though DA levels had returned to baseline.

Microinjection pretreatment of 10 nM OXO into the LDT selectively decreased METH-induced (but not basal) ACh levels in the dialysis experiment, but was related to a non-selective inhibition of locomotor activity (METH-induced and spontaneous). While we cannot establish a causal link between the neurochemical response and locomotor activity measured in these two experiments, previous experiments in our lab enable us to make some inferences about the current data. We previously reported that 2 mg/kg METH significantly increases VTA ACh levels (Dobbs and Mark 2008). Since 10 nM OXO inhibited the 3.5 mg/kg METH-induced increase in ACh levels in the current study, it is likely that it was able to inhibit the 2.0 mg/kg METH-induced increase in ACh in the locomotor experiment. Previous studies also suggest that neostigmine may affect the local pharmacologic milieu and ultimately have effects on behavior (De Boer and Abercrombie 1996; Ikemoto and Wise 2002; You et al. 2008). While we cannot rule out an effect of neostigmine on our cholinergic microdialysis data, we feel that a significant effect is unlikely given the low concentration (0.3 µM) used in the current experiments. Thus, these data suggest that inhibition of the LDT-to-VTA cholinergic pathway does not alter METH-*specific* locomotor activity.

There are several possible explanations for why OXO affected locomotion after saline injection independently of DA or ACh levels. First, OXO may have diffused into a neighboring region to exert its effects on activity independently of DA or ACh levels in

the NAc and VTA, respectively. To address the site specificity of OXO's effects on locomotor activity injections were made into the IC, a control site located 1 mm dorsal to the LDT. The most likely diffusion route would be in a dorsal direction via the injector tract; however, microinjections into the IC had no effect on basal or METH-induced locomotor activation. Although less likely, it is possible that OXO diffused in a lateral and anterior direction to affect the PPT. While we did not asses the contribution of PPT-derived ACh on METH-induced locomotor activity, evidence suggests that this region is *not essential* for locomotor activation (Maskos 2008; Winn 2006; 2008). The cholinergic neurons of the anterior PPT (aPPT) project mainly to the SN, while the cholinergic neurons located in the posterior PPT (pPPT), which is closer to our LDT injection target, project to DA neurons in the VTA. If diffusion occurred in a lateral and anterior direction it is most likely that the pPPT cholinergic neurons were affected, which have the same primary target DA neurons as the LDT cholinergic neurons (Mena-Segovia et al. 2008; Winn 2008). Thus, any limited diffusion of OXO to the pPPT might result in an outcome similar to an LDT microinjection.

Second, it is also possible that a decrease in ACh or DA levels after OXO and saline injection was not detected due to a floor effect. Although a non-significant decrease in picograms of ACh was observed across OXO treatment groups when comparing levels between baseline and after saline injection, it is possible that a further decrease in ACh could have occurred but was not detected. ACh levels at baseline and after saline injection in the VTA were low (Table 1) and following the high concentration of intra-LDT OXO some ACh levels fell below detection limits. When this occurred, samples were excluded from the analysis.

Third, the different contexts and handling procedures associated with the locomotor activity and microdialysis experiments could have contributed to the different pattern of results between the two experiments. The locomotor and microdialysis experiments were performed separately and in different testing chambers. It is possible that the different chambers and basic differences in handling used in these two experiments could have contributed to the lack of an observed relationship between locomotor activity and neurotransmitter levels following OXO and saline treatment. Ideally, we would like to be able to measure ACh and DA during locomotor activity; however there are significant problems with performing these experiments simultaneously. Previous experiments in our lab have found that the timed collection of dialysis samples disrupts the animal's behavior and that behavioral disruptions can artificially affect transmitter levels (unpublished observations). In both experiments mice were allowed to habituate to their chambers the day before data was collected. However, in the locomotor experiment mice were also habituated to handling and injection the day before collecting baseline activity data, while samples analyzed for ACh and DA were collected with no previous habituation to injection. This may have resulted in a stress response in the microdialysis experiment and prevented a significant decrease in ACh or DA levels following IP saline. Conversely, habituation to handling and injection in the locomotor experiment may have attenuated any stress response, thereby allowing us to see an effect of OXO on activity following IP saline. Locomotor activity did tend to be higher on the first day compared to the second day of testing (data not shown). Although, it is unknown how much novelty and handling stress each contributed to the difference in activity between day one and two.

Despite the procedural differences between the dialysis and locomotor experiments, these data provide several significant and novel findings that enhance our

understanding of METH's neurochemical and behavioral effects. Although inhibition of LDT-derived ACh did not affect locomotor activity in a METH-specific fashion, this does not preclude the possibility that LDT ACh could underlie other METH-related (and non-METH-related) behaviors. Indeed, there are multiple neurochemical and behavioral changes that occur following METH and/or OXO administration, which were not measured in these experiments.

Finally, the difference in the pattern of results following OXO administration could be due to the distribution of M2 ACh receptors, the receptor subtype to which OXO preferentially binds. This receptor subtype may be located on non-cholinergic cells, such as glutamatergic neurons, so that activation of these receptors may mediate basal locomotor activity independent of an effect on cholinergic neurons. Previous research has confirmed that the M2 ACh receptor is located on cholinergic neurons within the LDT and PPT and that activation of this receptor results in a TTX-insensitive hyperpolarization and subsequent neuronal inactivation (Buckley et al. 1988; Li et al. 1991; Vilaro et al. 1991; Vilaro et al. 1992). It is not clear, however, if the M2 receptor is expressed on non-cholinergic neurons, such as glutamate or GABA, within the LDT and PPT. One study found more restricted labeling of ChAT mRNA, a biomarker for ACh neurons, than the ACh M2 receptor mRNA (Vilaro et al. 1992). Further, bath application of carbachol induced a hyperpolarization *in vitro* regardless of neuronal phenotype, although 79% of neurons tested were cholinergic (Luebke et al. 1993). These studies suggest that non-cholinergic neurons may express M2 receptors. There are also known glutamate projections from the LDT to the VTA (Cornwall et al. 1990; Semba and Fibiger 1992) and glutamate tone in the VTA mediates nicotine-induced NAc DA levels (Schilstrom et al. 1998). Furthermore, application of glutamatergic agonists in the VTA enhanced, while antagonists inhibited, locomotor activity and DA levels in terminal

regions (Kalivas and Duffy 1989; Swanson and Kalivas 2000). Thus, it is conceivable that the locomotor attenuation seen in the current data may in part be due to effects via the glutamate projection to the VTA. Further investigation is needed to clarify if the M2 ACh receptor is expressed on non-cholinergic neurons within the LDT and PPT.

Conclusions

The present findings suggest that attenuation of LDT-derived ACh in the VTA, but not NAc DA, is related to an inhibition in locomotor activity in C57BL/6J mice, which is consistent with previous research implicating the LDT in spontaneous and drug-induced activity (Alderson et al. 2005; Nelson et al. 2007). In addition, although METH stimulated LDT cholinergic neurons to induce an increase in VTA ACh, this did not appear to play a significant role in the METH-induced increase in DA within the NAc, nor was this specific to METH-induced locomotor activity. While the present findings significantly contribute to our understanding of METH's neurochemical and behavioral effects, additional studies are needed to clarify if the LDT-to-VTA cholinergic pathway is involved in other METH-related behaviors.

The LDT and PPT are situated in a key area to receive sensory information and mediate locomotor output. This neurochemically diverse brainstem region is important for interpreting sensory information to guide appropriate motor behaviors. Given the mesopontine region's extensive and reciprocal functional connections with the mesolimbic reward circuit it is reasonable to hypothesize a role of the PPT or LDT in learning about reward-associated cues.

Chapter 4

THE ROLE OF THE LATERODORSAL TEGMENTAL NUCLEUS IN METHAMPHETAMINE CONDITIONED PLACE PREFERENCE AND LOCOMOTOR ACTIVITY

This chapter was adapted from:

Dobbs L.K. & Cunningham, C. L., The role of laterodorsal tegmental nucleus in methamphetamine conditioned place preference and locomotor activity. Submitted to *Psychopharmacology* April, 2012.

ABSTRACT

METH indirectly stimulates the LDT ACh neurons to increase ACh within the VTA, while LDT ACh inhibition inhibits METH and saline locomotor activity. The aim of these experiments was to determine whether LDT ACh contributes to METH CPP.

Mice received a bilateral electrolytic or sham lesion of the LDT. After recovery, mice received alternating pairings of METH (0.25 or 0.5 mg/kg) and saline with distinct tactile floor cues over 8 days. During preference tests, mice were given access to both floor types and time spent on each was recorded. Mice in the 0.5 mg/kg METH group were tested again after exposure to both extinction and reconditioning trials. Brains were then processed for ChAT immunohistochemistry to label LDT ACh neurons. Lesioned mice had significantly fewer LDT ACh neurons and showed increased saline and METH (0.25 and 0.5 mg/kg) locomotor activity during conditioning trials and during a drug-free preference test (0.25 mg/kg group only) compared to sham mice. Locomotor activity (saline and METH) was negatively correlated with ACh neuron loss. Lesioned and sham mice showed similar CPP at both METH doses. There was no effect of lesion on the extinction or reconditioning of METH CPP. LDT ACh neurons are not necessary for METH reward as indexed by CPP, but may be important for basal and METH-induced locomotor activity.

INTRODUCTION

Midbrain DA neurons in the VTA are well-known for their role in reward prediction (Schultz 2010), energizing goal-directed behaviors and creating Pavlovian associations between conditioned stimuli and natural or drug rewards (US) (Di Chiara 2002). Administration of ACh agonists in the VTA increases DA neuron firing (Lacey et al. 1990; Mereu et al. 1987) and DA release in terminal fields (Miller and Blaha 2005; Westerink et al. 1998; Westerink et al. 1996). In addition, rats will self-administer neostigmine, an indirect ACh agonist into the VTA (Ikemoto and Wise 2002). The VTA receives its cholinergic input from the dense cholinergic fields of the LDT and the posterior portion of a neighboring region known as the PPT (Bolam et al. 1991; Geisler and Zahm 2005; Omelchenko and Sesack 2005). Electrical stimulation of the LDT increases intra-VTA ACh, which activates DA neurons and stimulates DA output in the mesocorticolimbic pathway (Forster and Blaha 2000; Forster et al. 2001; Lodge and Grace 2006). Thus, this cholinergic input may have implications for reward-related behaviors.

METH is a highly addictive psychostimulant that can induce DA release from terminals within the reward pathway independent of neuronal stimulation (Sulzer et al. 2005). Recent evidence shows that METH selectively stimulates the LDT to increase ACh levels within the VTA (Dobbs and Mark 2008; 2012). Furthermore, reversible inhibition of LDT ACh neurons attenuates basal and METH-induced locomotor activity in mice (Dobbs and Mark 2012) and cocaine and food self-administration in rats (Shabani et al. 2010). Given these data, it is possible that LDT ACh may mediate METH reward as well.

Cholinergic neurons in the LDT receive sensory input from regions such as the superior colliculus and are ideally situated to convey sensory information (such as cues

and context) to midbrain DA neurons (Koyama et al. 1994; Satoh and Fibiger 1986; Semba and Fibiger 1992). Further, while exposure to METH induces a prolonged increase in extracellular ACh within the VTA, exposure to a previously cocaine-paired cue alone, such as a stimulus light, can induce cocaine-seeking behavior and VTA ACh levels (You et al. 2008). Taken together, these data suggest that the LDT-to-VTA cholinergic projection may be important in forming associations between drug-paired cues and the appetitive aspects of psychostimulants.

CPP is a form of Pavlovian learning and a reliable method to assess the rewarding or aversive properties of psychoactive drugs (Cunningham et al. 2006; Cunningham et al. 2011). For instance, a subject will approach and spend more time in the environment previously paired with a rewarding drug. This method allows the experimenter to easily manipulate variables before or during conditioning and measure their effect on the formation of the CS-US association and preference in a drug-free state. Previous research indicates that DBA/2J and C57BL/6J mice find METH rewarding using a CPP paradigm (Cunningham and Noble 1992; Takamatsu et al. 2006a).

In previous experiments, we reversibly inhibited LDT cholinergic neurons using intra-LDT microinjections of the M2 subtype-preferring cholinergic agonist, OXO. However, unpublished findings in our lab suggest that the acquisition of a place preference in mice is extremely sensitive to the handling involved in repeated microinjections, irrespective of the drug being microinjected. Since previous literature suggests that METH conditions a moderate level of preference (a preference less robust than seen with ethanol CPP in DBA/2J mice), we decided to first test the effect of a pre-

conditioning lesion on the acquisition of METH CPP before performing the more neurochemically selective microinjection manipulation.

In the current set of experiments we performed bilateral electrolytic LDT lesions in male C57BL/6J mice prior to METH CPP and confirmed cholinergic cell loss using ChAT immunohistochemistry (IHC). We hypothesized that bilateral LDT lesion would attenuate the acquisition of METH CPP.

METHODS

Subjects

A total of 96 male, C57BL/6J mice (9 weeks old at surgery) were used for the two experiments (n = 48 per experiment). Mice were group housed, four to a cage in a climate and humidity controlled vivarium on a 12h light cycle (lights on a 0700). Food and water was available *ad libitum*. All procedures were carried out in accordance with the National Research Council of the National Academies (Academies 2003) and approved by the Institutional Animal Care and Use Committee at Oregon Health and Science University.

Drugs

Methamphetamine hydrochloride was dissolved in 0.9% sterile saline to a final dose of 0.25 or 0.5 mg/kg and administered IP in a volume of 10 ml/kg. The 0.5 mg/kg dose was selected based on its ability to condition a METH preference in previous studies performed in DBA/2J mice (Cunningham and Noble 1992) and mice selectively bred for a METH drinking phenotype (Wheeler et al. 2009). The lower dose (0.25 mg/kg) was selected to condition a less robust METH preference, which I hypothesized to be more susceptible to the loss of LDT cholinergic projections to the VTA.

Surgical Procedure

Mice were anesthetized using 2% isoflurane in oxygen for the stereotaxic surgery. The LDT was targeted relative to bregma (AP: -5.02, ML: ±0.65, DV: -3.5) (Paxinos and Franklin 2001) and an electrode was lowered into one side of the LDT at a time to make bilateral electrolytic or sham lesions. Mice in the lesion group (n = 24/dose) received 0.5 mA current for 2 s. Mice in the sham group (n = 24/dose) had electrodes lowered into the LDT but no current was passed. Mice were allowed 3-5 days to recover before experimental procedures began.

Conditioned Place Preference Apparatus

Conditioning chambers (30 cm x 15 cm x 15 cm) were constructed of clear acrylic walls and lid with aluminum end panels. Each chamber was contained in a sound- and light-attenuating enclosure (Coulbourn Instruments E10-20; 56.1 cm x 46.0 cm x 39.4 cm) equipped with a ventilation fan. The enclosure was not illuminated during conditioning trials or testing. The subject's activity and side position (left or right) were detected with 6 sets of infrared photobeam detectors positioned 2.2 cm above the floor and 5 cm apart. Photobeam breaks were recorded every minute by a computer with a 10 ms resolution. Each of the interchangeable tactile floor cues served as half of the chamber floor. The grid floor was constructed of 2.3 mm stainless steel rods mounted 6.4 mm apart in an acrylic frame. The hole floor was constructed of 16 gauge stainless steel perforated with 6.4 mm diameter holes staggered 9.5 mm apart mounted on an acrylic frame. A clear acrylic panel divided the chamber into two equal sections during conditioning trials (two-compartment procedure). This barrier was removed for the test day when both floor types were present.

Conditioned Place Preference Procedure

All mice were exposed to an unbiased place conditioning procedure using an unbiased apparatus (Cunningham et al. 2003). Each METH dose (0.25 or 0.5 mg/kg) was tested in a separate experiment that compared lesion to sham control mice. Mice were exposed to one habituation session, eight conditioning sessions (4 METH and 4 saline) and two preference tests. The first preference test was given after two conditioning trials of each type and the second preference test occurred after the fourth conditioning trial of each type. Mice trained with the 0.5 mg/kg dose were also exposed to both extinction and reconditioning sessions followed by additional tests.

Habituation

During habituation, mice were removed from their home cage, injected IP with saline (10 ml/kg) and immediately placed into the conditioning apparatus on a white paper floor; no divider was present during this session. Mice were allowed to explore the apparatus for 5 min before being returned to their home cage.

Conditioning

After recovery from surgery, mice within each treatment group were randomly assigned to one of two conditioning subgroups (GRID+ or GRID-) counterbalanced for days of post-operative recovery. For mice in the GRID+ subgroup, METH (0.25 or 0.5 mg/kg) was injected immediately before placement on the grid floor whereas saline was injected on alternate days before placement on the hole floor. These contingencies were reversed for mice in the GRID- conditioning subgroup. During conditioning, each trial alternated between mice receiving METH paired with one specific floor type (CS+) and saline paired with the other floor type (CS-); order of exposure to METH and saline

was counterbalanced. Activity was measured for 30 min and then mice were returned to their home cage.

Preference Tests

On test days, the center divider was removed so that the mouse had access to both floor cue types. Mice were given an IP saline injection and placed in the center of the apparatus. Activity and time on each floor type were measured for 30 min. Mice were returned to their home cage after the session.

Extinction

During extinction, saline was paired with the CS+ and CS- floor types. Mice in the 0.5 mg/kg group were exposed to both trial types (CS+ and CS-) every day, in the same counterbalanced order they received during conditioning. The two 30-min trials were separated by 4 h and mice were returned to the home cage at the end of each trial. Preference tests were administered every 2 days and mice received a total of 6 extinction trials of each type across 6 days.

Reconditioning

On the day after the last post-extinction preference test, mice in the 0.5 mg/kg group began reconditioning the METH CPP. Mice were administered 0.5 mg/kg METH or saline and placed back onto their original CS+ or CS- floor type, respectively. Mice received one CS+ and one CS- trial (30 min) over 2 days in the same counterbalanced order they received during conditioning. A final preference test was administered the day after the last reconditioning trial.

Immunohistochemistry and Histology

Following the last preference test, mice were euthanized with pentobarbital (25 mg/ml) and brains were extracted and post-fixed in 2% paraformaldehyde in PBS. Following overnight cryoprotection in 20% then 30% sucrose in PBS, brains were sectioned (40 µm) on a Leica cryostat. The LDT was sectioned through its entire anterior-posterior extent and alternating sections were collected for ChAT IHC or thionin staining. For the ChAT IHC two sections each were selected to represent a rostral (-5.0 mm), medial (-5.2 mm), and caudal (-5.4 mm) target of the LDT, relative to bregma. Sections were stored in PBS with 0.1% sodium azide at 4° C until free-floating IHC processing. Peroxidase activity was quenched with 0.3% H_2O_2 in PBS and sections were blocked using 4.5% normal Horse (Vector Labs) in PBS and 0.3% Triton-X 100 (Sigma-Aldrich). Sections were treated with a 1:5000 dilution of the primary antibody directed at ChAT (Millipore) in PBS/Triton with 0.1% bovine serum albumin overnight at room temperature. Detection of the primary antibody was accomplished with 0.5% biotinylated anti-goat (raised in horse; Vector Labs) in PBS/Triton-X. The immunoreaction was detected using a Vectastain ABC kit (Vector Labs) in PBS/Triton-X and developed with a DAB kit (Thermo Scientific). Sections were mounted on slides and coverslipped using Cytoseal 60 (Richard-Allan Scientific). ChAT positive cells were counted manually using an Olympus BX51 microscope equipped with an Olympus Q Color 3 camera by an individual blind to surgical group. Images were collected using QCapture (QImaging v2.8.1) under the following settings: magnification of 20x, exposure time of 95.5 ms, offset of 78, and gain of 0.665. A final single ChAT cell count was obtained for each mouse by adding the averaged bilateral cell count for the rostral, medial, and caudal target regions.

Sections used for thionin staining were mounted on slides and dehydrated before staining with 1% thionin in distilled water. Slides were dehydrated again, soaked in

xylene for 12 min and coverslipped with Cytoseal 60. Sections throughout the entire extent of the LDT were examined for the presence of a lesion using an Olympus BX51 microscope equipped with an Olympus Q Color 3 camera.

Statistical Analyses

Subject's data were removed if there was less than 50% ChAT cell loss in the lesioned subjects compared to the average number of ChAT cells in sham control mice (n = 18). Nine subjects were excluded from the 0.25 mg/kg lesion group due to insufficient lesion. Nine subjects were also excluded from the 0.5 mg/kg lesion group due to insufficient lesion and three subjects were excluded due to poor ChAT staining. One subject from the 0.5 mg/kg sham group was excluded from all analyses due to a conditioning error and one subject was excluded from the extinction and reconditioning phases due to a wound inflicted by a cage-mate. In order to perform the repeated measure analysis, some subject's data were excluded from the analysis of the conditioning activity due to apparatus error for the 0.25 mg/kg experiment (sham = 20; lesion = 9). The number of mice per group used in the final analyses for each experiment is provided in the figure legends.

Conditioning and extinction activity were analyzed using a three-way repeated measure ANOVA with trial (8 or 12 trials for conditioning or extinction, respectively) and trial type (METH or Saline) as the within subjects factors and surgical group (Sham or Lesion) as the between subjects factor. Preference was expressed as the time spent on the grid floor for subjects that had METH paired with the grid (GRID+) or hole (GRID-) floor. A significant difference between these subgroups provides evidence of place conditioning (Cunningham et al. 2003). Grid time data were analyzed using two-way ANOVA with conditioning subgroup (GRID+ or GRID-) and surgical group (Sham or

Lesion) as between subjects factors. The amount of preference following extinction was compared to that following conditioning and reconditioning, expressed as the time spent on the grid floor, using a two-way repeated measure ANOVA with test (Post-extinction and Post-reconditioning) as the within subject factor and surgical group (Sham or Lesion) as the between subjects factor. Violations to sphericity in the repeated measure ANOVA were corrected using Greenhouse-Geisser. Interactions and main effects were followed up with simple main effects and Bonferroni corrected post-hoc tests when appropriate. Results were considered significant at an alpha of 0.05. Data are expressed as the mean ± standard error.

Correlations were evaluated between the number of ChAT stained cells and several dependent variables: 1) locomotor activity following METH and saline on conditioning trials 1 and 4 and the single reconditioning trial; 2) locomotor activity on the CS+ and CS- floors on extinction days 1 and 6; 3) locomotor activity during the post-conditioning, post-extinction and post-reconditioning preference tests; and 4) percent time on the drug floor during the post-conditioning, post-extinction and post-reconditioning preference tests. Data from mice trained with 0.25 mg/kg were not included in analyses involving either the post-extinction or post-reconditioning tests. All subjects were included in these correlational analyses, regardless of the extent of cell loss following the lesion, except those excluded for other reasons (see above). Significance was set at a p-value of 0.05.

RESULTS

ChAT Labeling

Figure 16

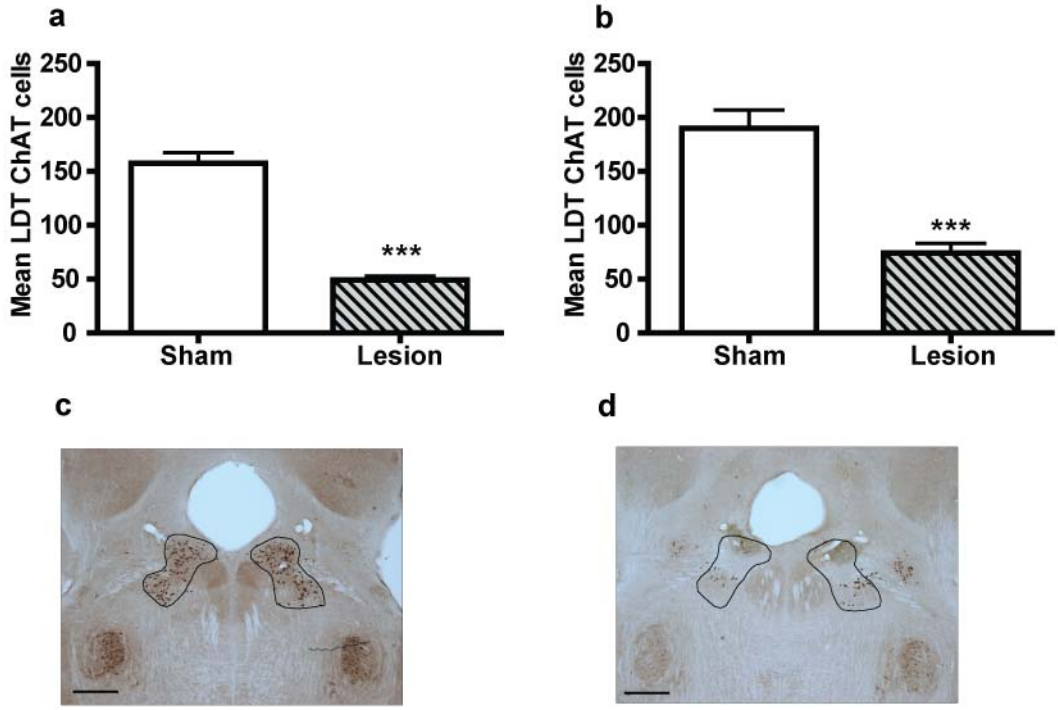


Figure 16. There was a significant reduction in the mean number of choline acetyltransferase (ChAT) labeled cells within the LDT following a bilateral LDT lesion compared to sham operated mice for the 0.25 mg/kg METH experiment (lesion n = 15, sham n = 24) (**a**) and for the 0.5 mg/kg METH experiment (lesion n = 13, sham n = 17) (**b**). A representative section of a sham-operated (**c**) and lesioned (**d**) subject is shown at 4x magnification with the boundaries of the LDT outlined in black. Scale bar: 500 µm. *** $p < 0.0005$

Figures 16a and 16b show that lesioned mice had significantly fewer ChAT stained cells in the LDT compared to sham-operated mice for the 0.25 mg/kg ($F_{1,37} = 72.89$, $p < 0.0005$) and 0.5 mg/kg ($F_{1,26} = 33.55$, $p < 0.0005$) groups, respectively.

Conditioning

Figures 17a and 17b show that sham-operated and LDT lesioned mice had similar levels of METH place preference for the 0.25 mg/kg (main effect of conditioning;

Figure 17

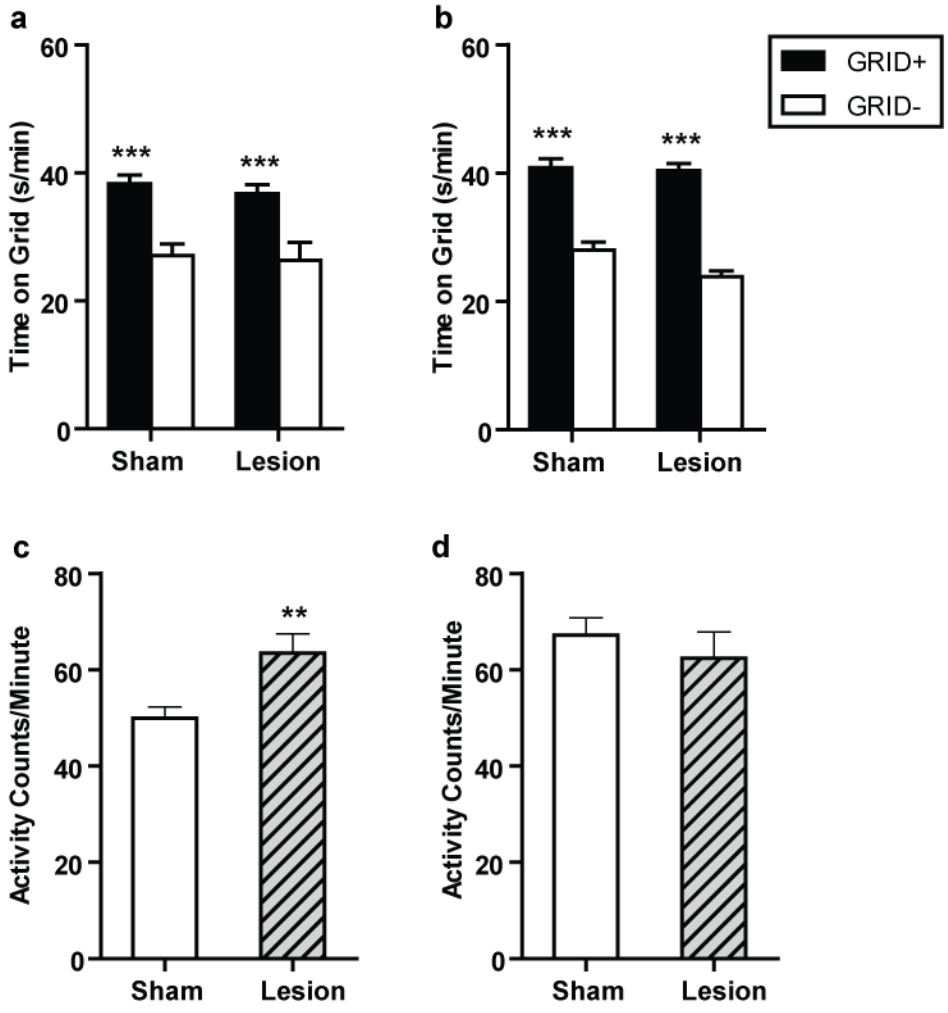


Figure 17. METH conditioned place preference after conditioning with 0.25 mg/kg (**a**) or 0.5 mg/kg (**b**) is shown for subjects that had METH paired with the grid floor (GRID+; black bars) or with the hole floor (GRID-; white bars). In the 0.25 mg/kg experiment, sham-operated (n = 24) and LDT lesioned mice (n = 15) showed similar METH preference, but LDT lesioned mice had significantly higher locomotor activity compared to sham mice (**c**). In the 0.5 mg/kg experiment, sham-operated (n = 23) and LDT lesioned (n = 12) mice showed similar METH preference and locomotor activity during the preference test (**d**). ** $p < 0.01$; *** $p < 0.0005$ GRID+ vs. GRID-, collapsed across surgical group.

$F_{1,33} = 31.80$, $p < 0.0005$) and 0.5 mg/kg dose (main effect of conditioning; $F_{1,31} = 87.80$, $p < 0.0005$). There was no main effect of surgical group or interaction of surgical group and conditioning for either METH dose. In addition, for the 0.25 mg/kg group, lesioned mice were more active than sham mice, as indicated by a significant main effect of surgical group ($F_{1,35} = 10.03$, $p = 0.003$) (Figure 17c). However, in the 0.5 mg/kg METH group, sham and lesioned mice exhibited similar levels of test activity (Figure 17d).

Locomotor activity during the conditioning trials for the 0.25 mg/kg and 0.5 mg/kg METH experiments are shown in Figure 18. Analysis of the conditioning activity for the 0.25 mg/kg group (Figure 18a) revealed a significant three-way interaction of trial x trial type x surgical group ($F_{7,189} = 23.05$, $p < 0.0005$). There were also significant main effects of trial ($F_{7,189} = 488.46$, $p < 0.0005$), trial type ($F_{1,27} = 9.78$, $p = 0.004$) and surgical group ($F_{1,27} = 32.75$, $p < 0.0005$). In addition, the trial by surgical group ($F_{7,189} = 20.93$, $p < 0.0005$), trial type by surgical group ($F_{1,27} = 25.25$, $p < 0.0005$), and trial by trial type ($F_{7,189} = 26.63$, $p < 0.0005$) interactions were significant. Follow-up analyses showed that lesioned mice exhibited significantly higher locomotor activation compared to sham mice following METH-prime on conditioning trials 1, 2 and 3 ($p < 0.0005$) and saline-prime on conditioning trials 1, 2, 3 and 4 ($p < 0.0005$).

For the 0.5 mg/kg group (Figure 18b), analysis of the conditioning activity revealed a significant three-way interaction of trial by trial type by surgical group ($F_{7,231} = 4.01$, $p < 0.0005$). There were also significant main effects of trial ($F_{7,231} = 279.94$, $p < 0.0005$) and trial type ($F_{1,33} = 4.66$, $p = 0.038$), but not surgical group. The trial by surgical group ($F_{7,231} = 2.05$, $p = 0.049$) and trial by trial type ($F_{7,231} = 7.92$, $p < 0.0005$) interactions were also significant. Follow up analyses revealed that LDT lesioned mice

Figure 18

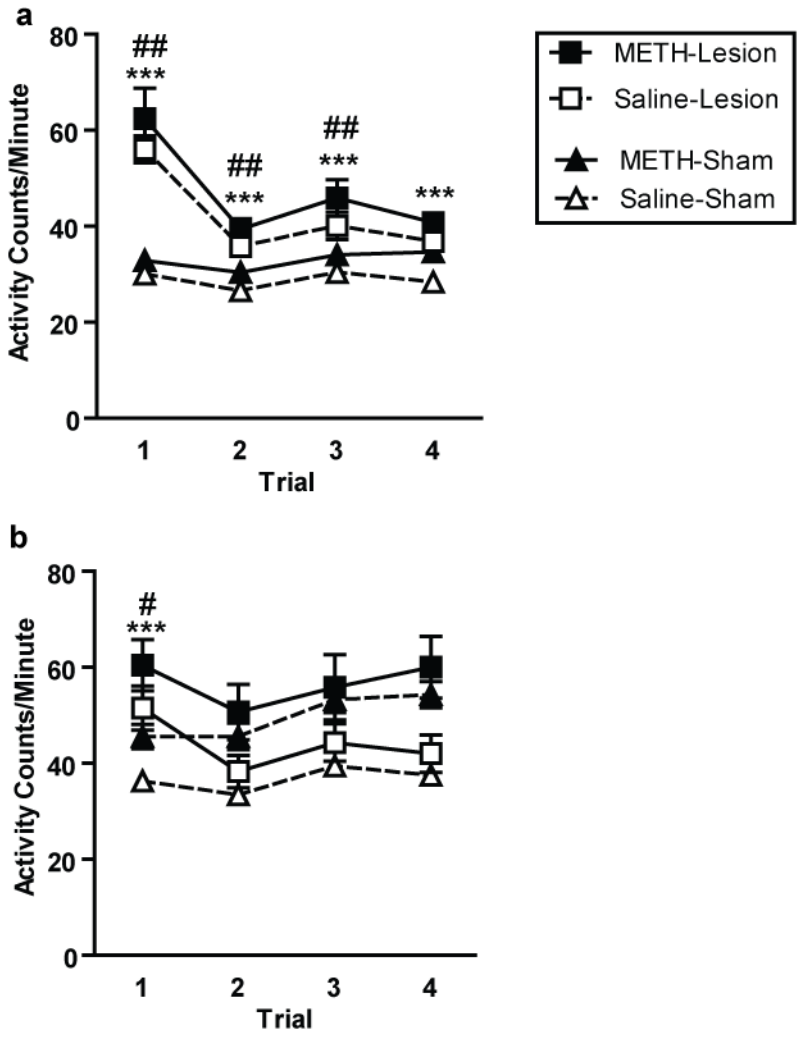


Figure 18. Locomotor activity during METH (black symbols) and saline (white symbols) conditioning trials is shown for the 0.25 mg/kg (**a**) and 0.5 mg/kg (**b**) METH experiments. In the 0.25 mg/kg experiment, LDT lesioned mice (n = 9; squares) showed significantly higher locomotor activation compared to sham-operated mice (n = 20; triangles) after METH or saline. In the 0.5 mg/kg experiment, LDT lesioned mice (n = 12; squares) showed greater locomotor activity compared to sham-operated mice (n = 23; triangles) following saline (white symbols) and METH (black symbols) in the first conditioning trial only. # $p < 0.01$ Lesion METH vs. Sham METH; ** $p < 0.01$ Lesion Saline vs. Sham Saline; ## $p < 0.0005$ Lesion METH vs. Sham METH; *** $p < 0.0005$ Lesion Saline vs. Sham Saline.

exhibited significantly higher locomotor activity following METH- ($p = 0.003$) and saline-prime ($p < 0.0005$) compared to sham mice on the first conditioning trial only.

Extinction

Sham-operated and LDT-lesioned mice showed a significant reduction in METH preference after extinction compared to the preference test after conditioning (main effect of test, $F_{1,32} = 16.36$, $p < 0.0005$). In addition, both sham and lesioned mice showed similar METH place preference (main effect of conditioning; $F_{1,30} = 24.04$, $p < 0.0005$), but the sham mice spent significantly more time on the grid floor across both the GRID+ and GRID- subgroups compared to lesioned mice ($F_{1,30} = 11.08$, $p = 0.002$) (Figure 19a). There was no difference in the amount of locomotor activity expressed during the preference test between groups (data not shown).

Analysis of locomotor activity during the extinction trials revealed a significant three-way interaction of trial type by trial by surgical group interactions ($F_{11,341} = 3.15$, $p < 0.0005$) (Figure 19b). There was also a significant main effect of trial ($F_{11,341} = 434.35$, $p < 0.0005$) and significant trial type by surgical group ($F_{1,31} = 5.25$, $p = 0.029$), trial by surgical group ($F1_{1,341} = 1.98$, $p = 0.03$), and trial type by trial ($F_{11,341} = 10.85$, $p < 0.0005$) interactions. The main effects of surgical group and trial type were not significant. Follow up analyses showed that LDT lesioned mice had significantly higher locomotor activation for the CS- trial type on trials 3 ($p = 0.032$), 5 ($p = 0.025$), and 6 ($p = 0.014$). Additionally, within each trial, the sham and lesion mice expressed similar locomotor activity for the CS+ and CS- floor types.

Reconditioning

Figure 19

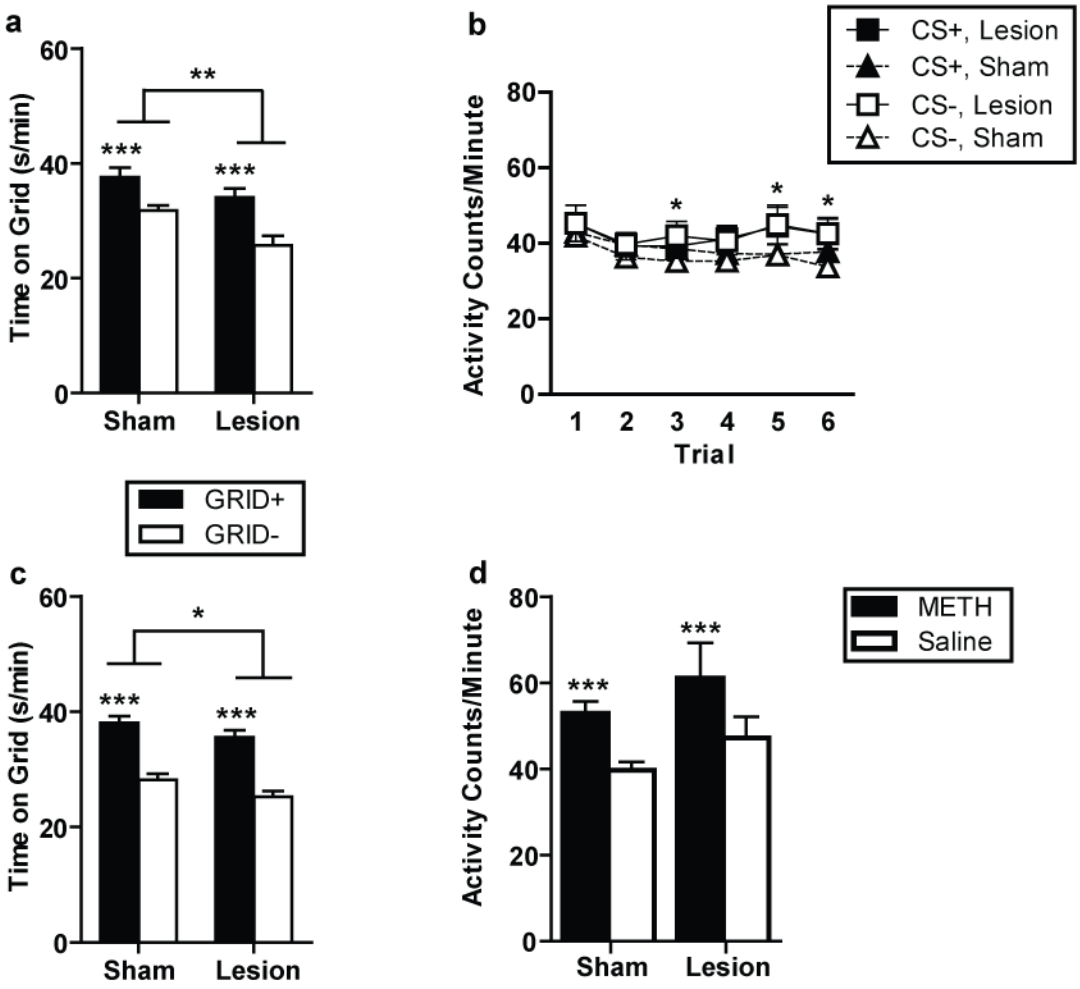


Figure 19. LDT lesioned and sham mice showed similar METH preference after extinction trials, but sham mice spent more time on the GRID floor, regardless of floor conditioning type (**a**). Activity on the first extinction trial was significantly higher than on trials 2, 3, 4 and 6 when collapsed across trial type (METH or saline) and surgical group (sham or lesion). Locomotor activity during saline-only extinction trials is shown for sham-operated (triangles; n = 22) and LDT lesioned (squares; n = 12) subjects (**b**). LDT lesioned and sham mice showed similar METH preference after re-conditioning, but sham mice spent more time on the GRID floor, regardless of floor conditioning type (**c**). Re-conditioning with METH (0.5 mg/kg) resulted in an increase in locomotor activity compared to the saline-treated trial for sham and LDT lesioned subjects (**d**). * $p < 0.05$ Sham vs. Lesion, CS- trial type only (**b**), or Sham vs. Lesion, collapsed on floor type (**c**); ** $p < 0.01$ Sham vs. Lesion, collapsed on floor type (**a**); *** $p < 0.0005$ GRID+ vs. GRID- (**a**) and (**c**), or METH vs. Saline, within each surgical group (**d**).

Figure 20

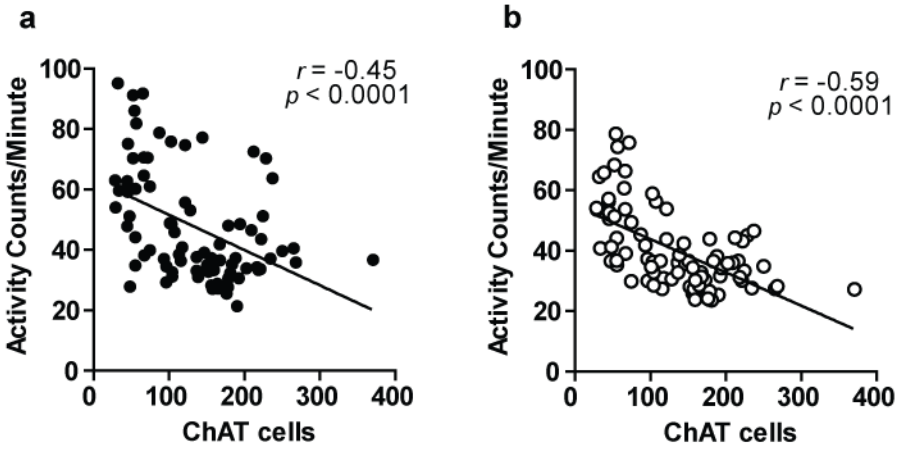


Figure 20. Correlations between the number of ChAT stained cells within the LDT (after sham or LDT lesion) and locomotor activity are shown. The number of ChAT cells was correlated with conditioning activity in conditioning trial 1 following METH injection (n = 82) (**a**) and saline injection (n = 84) (**b**). The Pearson correlation coefficient and corresponding p-value is displayed on each panel.

LDT lesioned and sham-operated mice expressed similar METH place preference (main effect of conditioning; $F_{1,30} = 64.62$, $p < 0.0005$) (Figure 19c); however, sham mice spent more time on the grid floor in the GRID+ and GRID- floor subtypes compared to lesioned mice (main effect of surgical group; $F_{1,30} = 4.49$, $p = 0.043$). There was no difference between the two surgical groups in the amount of activity during the preference test (sham: 57.23 ± 1.51; lesion: 58.70 ± 2.04). The analysis of locomotor activity during the reconditioning trial indicated main effects of trial ($F_{1,32} = 45.03$, $p < 0.0005$) and trial type ($F_{1,32} = 339.47$, $p < 0.0005$) and a trial by trial type interaction ($F_{1,32} = 33.63$, $p < 0.0005$). There were no other significant main effects or interactions. Figure 19d shows that sham and lesioned mice exhibited significant locomotor activation to 0.5 mg/kg METH during the single reconditioning trial ($p < 0.0005$).

Correlations

Figures 20a and 20b show that the number of ChAT stained cells was negatively correlated with locomotor activity following METH (r = -0.45, p < 0.0001, n = 82) or saline (r = -0.59, p < 0.001, n = 84) on conditioning trial 1. The number of ChAT stained cells was not correlated with trial 4 locomotor activity following METH or saline or with percent time spent on the drug paired floor. Additionally, locomotor activity was not correlated with activity during reconditioning or extinction trials or with time spent on the drug paired floor for the post-extinction or post-reconditioning tests.

DISCUSSION

These data show that a bilateral lesion of the LDT has no effect on the acquisition, expression, extinction or reconditioning of a METH CPP. Furthermore, cholinergic cell loss, measured by ChAT immunohistochemistry, was not correlated with the amount of METH preference following conditioning, extinction or reconditioning. However, the number of LDT cholinergic cells was negatively related to METH and saline locomotor activity during conditioning trial 1.

Reversible inhibition of LDT cholinergic neurons attenuated cocaine and food self-administration in rats (Shabani et al. 2010). Studies have reported that administration of muscarinic cholinergic agonists into the LDT produces a decrease in cholinergic activity and a depression in locomotor activity (Dobbs and Mark 2012), while intra-LDT mAChR antagonists induce hyperlocomotion (Laviolette et al. 2000). While Shabani et al. reported only a marginal stimulating effect of intra-LDT OXO microinjection on locomotor activity in previously food trained rats, it is possible that intra-LDT OXO may have affected cocaine-induced activity, which was not tested (Shabani et al. 2010). Therefore, it is possible that the effect of intra-LDT OXO microinjection on cocaine self-administration was driven by a general decrement in

locomotor activity. In addition, unlike METH, cocaine requires neuronal stimulation to increase DA levels within the terminal and then subsequently prolongs DA's time in the synapse by blocking the DAT. It is most likely that LDT ACh does not contribute to METH reward because METH can increase DA levels in the NAc independently of neuronal stimulation. Thus, any DA-potentiating effects of LDT ACh on METH reward in the current experiments were negligible.

The negative correlations of LDT ACh neurons with locomotor activity suggest that the LDT, and possibly ACh in particular, is important for mediating locomotor activity. In fact, the role of the cholinergic system in locomotor activity is well documented. Systemic, intra-PPT or intra-LDT administration of the muscarinic ACh antagonist scopolamine induced robust locomotor activation through the disinhibition of mesopontine cholinergic projection neurons to VTA DA neurons (Laviolette et al. 2000; Mathur et al. 1997). Furthermore, scopolamine-induced locomotor activity was not attenuated by the nicotinic antagonist dihydro-beta-erythroidine (DhβE), but mice lacking the M5 subtype muscarinic ACh receptor showed slightly *elevated* scopolamine locomotor activity (Chintoh et al. 2003). The M5 subtype muscarinic ACh receptor is the only muscarinic receptor that has been localized on VTA DA neurons (Reever et al. 1997; Vilaro et al. 1990; Weiner et al. 1990), and its activation increased DA release and reward (Forster et al. 2002b; Yeomans et al. 2001). This suggests that while nicotinic receptors are not involved, the M5 subtype muscarinic receptor may play a role in scopolamine-induced locomotor activity. Furthermore, the increase in locomotor activity seen in M5 knock-out mice suggests scopolamine-induced locomotor activity may function independently of VTA DA activity.

The role of the cholinergic system in psychostimulant-induced locomotor activation has also been investigated. Scopolamine pretreatment increased acute cocaine locomotor activity and time spent in stereotypy, and when administered during cocaine CPP trials scopolamine blocked conditioned hyperlocomotion (Heidbreder and Shippenberg 1996; Itzhak and Martin 2000). In addition, IP scopolamine or THP (a mAChR antagonist) enhanced METH and cocaine locomotor activity (Shimosato et al. 2003).

Previously, we showed that METH-induced DA levels in the NAc, but not METH or saline locomotor activity, does not depend on LDT ACh (Dobbs and Mark 2012). From our current data we have also determined that METH reward, but once again not locomotor activity, is independent of LDT ACh. The accumulation of these data suggests that METH induces locomotor activity similar to that of scopolamine. So, while METH co-ops the mesopontine cholinergic projections to the VTA to affect locomotor activity, this action does not affect METH DA responses and reward.

Reversible inhibition of LDT cholinergic neurons significantly inhibits saline and METH activity (Dobbs and Mark 2012); however, the current results showed that a lesion of the entire LDT induced a general locomotor activation. This discrepancy may suggest that other neurotransmitters in the LDT are involved in the balance of cholinergic locomotor activity. The GABA and glutamate projection neurons in the LDT were likely also significantly reduced as a result of the electrolytic lesion used to destroy the LDT in the current study. Previous research suggests that $GABA_A$ receptors are involved in amphetamine sensitization (Panhelainen et al. 2011) and that $GABA_B$ receptors in the VTA are involved in acute ethanol locomotor activation (Boehm et al. 2002). In addition,

plasticity of the LDT-to-VTA glutamate projections is involved in amphetamine sensitization (Nelson et al. 2007).

One limitation of these experiments is that we used a lesion, as opposed to a specific and/or reversible neurochemical manipulation, to disrupt the cholinergic neurons in the LDT. As mentioned above, it is likely that this lesion also damaged LDT GABA and glutamate projections to the VTA. Additionally, it is possible that the lesion also damaged fibers en passage, and as a result affected the integrity of the VTA. However, previous literature suggests the VTA is not necessary for the expression of cocaine CPP (Seip and Morrell 2009). We therefore feel that if there were significant damage to the VTA, it is unlikely that it affected METH CPP, especially given the null outcome of the lesion in the present experiments. However, it is possible that selective inhibition of the cholinergic neurons within the LDT could yield a different outcome than what was observed in the current METH CPP experiments.

In addition, cholinergic projections from the PPT may have compensated for the loss following the LDT lesion. That is, the bilateral LDT lesion may have triggered a compensatory response from the PPT to increase the strength of its connections to the VTA. A compensatory response from the PPT may explain the difference in locomotor activity between our previous study, which used acute, reversible ACh inhibition, and the current findings.

We conclude that the cholinergic neurons in the LDT are not necessary for METH reward, but are likely involved in METH and saline locomotor activity. METH's ability to act independently of DA neuron stimulation likely masks the effect of ACh to potentiate DA responses and reward. This is supported by previous research that

shows the inhibition of LDT ACh attenuates self-administration of cocaine, a drug which requires DA neuron stimulation to exert its rewarding effects.

Chapter 5

General Discussion

1. Summary of experimental results

The experiments presented in the previous chapters characterized the effect of METH on ACh and DA levels within the reward pathway and determined the role of mesopontine-derived ACh in METH reward. These experiments showed that systemic administration or direct perfusion of METH into the VTA induced an increase in somatodendritic DA levels within the VTA. In agreement with previous literature, systemic administration of METH also increased extracellular DA levels within the NAc. Somewhat unexpectedly, systemic administration of METH induced a prolonged increase in extracellular ACh levels within the VTA. However, when perfused directly into the VTA, METH failed to affect VTA ACh levels (**Chapter 2**). This suggested that when METH was administered systemically, it acted outside the VTA to induce the increase in ACh levels. Since the source of cholinergic tone in the VTA originates mainly in the LDT with a lesser contribution coming from the PPT (Oakman et al. 1995; Omelchenko and Sesack 2005), I hypothesized that the LDT cholinergic projection specifically was the source of the METH-induced ACh levels in the VTA. Specifically, I hypothesized that METH indirectly activates the LDT cholinergic neurons by increasing DA levels within the mPFC through direct action at DA terminals in the mPFC. This increase in DA levels could stimulate mPFC glutamate neurons (via DA receptors located on the soma) that project to the LDT. It might also be possible for METH to indirectly stimulate the mPFC by increasing DA levels within the VTA or NAc. For instance, following METH-induced increases in NAc DA levels, DA D2 receptor-mediated inhibition of GABA neurons could disinhibit the mPFC glutamatergic projections to the LDT. The experiments that followed showed that this was indeed the case. Reversible inhibition (via intra-LDT microinjection of OXO) of the LDT, but not PPT, cholinergic neurons attenuated the METH-induced increase in VTA ACh levels (**Chapter 3**).

The last set of experiments sought to characterize the role of LDT-derived ACh in the formation of METH reward. Since the acquisition of CPP can be very sensitive to the microinjection procedure in mice (Cunningham, unpublished observations), I decided to perform an electrolytic lesion of the LDT for these experiments. A pre-training bilateral LDT lesion had no effect on the acquisition, expression, extinction or reconditioning of METH CPP (**Chapter 4**). Furthermore, mice did not condition an aversion to METH using a higher dose (3.5 mg/kg) in the CPP procedure or using the same higher dose in a procedure specifically designed to induce place aversion. The details of this CPA experiment are presented in **Appendix I**.

These experimental findings contribute significant information to the field of drug abuse research by furthering our understanding of the cholinergic system in METH reward and locomotor activation. These studies were the first to (1) functionally dissociate the LDT from the PPT via intracranial microinjection in a mouse, (2) characterize the contribution of each region in METH's neurochemical effects in the mesolimbic pathway, and (3) characterize the role of LDT ACh in METH reward. The mechanisms whereby METH is thought to induce somatodendritic DA release (e.g., VMAT-2; DA stored in vesicle-like organelles) are well known (Fleckenstein et al. 2007; Nirenberg et al. 1996). The experiments in this dissertation support the literature by showing that METH induces increases in somatodendritic DA levels *in vivo* within the VTA.

Perhaps one of the most interesting findings of this dissertation came from the *in vivo* microdialysis experiments that showed METH induces a prolonged increase in VTA ACh levels. Moreover, 5 mg/kg METH not only induced a prolonged increase in ACh

levels, but also a protracted locomotor response (**Chapter 2**). Although these experiments indicate that LDT-derived ACh is not related to METH reward, they suggest that LDT ACh is involved in the protracted locomotor response to METH. Additionally, inhibition of LDT ACh neurons blocked METH and saline locomotor activity (**Chapter 3**) and the loss of cholinergic neurons following a bilateral LDT lesion was related to an increase in locomotor activity (**Chapter 4**). Thus, LDT ACh might regulate spontaneous (saline) as well as protracted METH locomotor activation. An intriguing hypothesis that arises from these behavioral and neurochemical findings is that METH's locomotor activating effects are independent from its rewarding effects. A future study could address this by administering intra-LDT OXO during METH conditioning trials, which should attenuate METH-induced locomotor activity. Failure of OXO pretreatment to inhibit the acquisition of METH CPP would support the hypothesis that the locomotor activating and rewarding effects of METH are mediated by distinct neural substrates. Additionally, the microdialysis experiments suggest that LDT ACh efferents to other brain areas may also have shown significant increases in extracellular ACh levels. The LDT cholinergic neurons have a variety of projection targets (Satoh and Fibiger 1986), and the activation of these areas may underlie other METH-related behaviors.

In **sections 2** and **3**, the role of the LDT cholinergic projection in METH reward and locomotor activity is discussed in more detail, respectively. The potential role of the LDT, PPT and ACh in other METH-related behaviors (e.g., aversion) is discussed in **section 4**. **Section 5** addresses various technical considerations of the experiments presented in this dissertation. Final conclusions drawn from these experiments are provided in **section 6**.

2. The role of laterodorsal tegmental acetylcholine in methamphetamine reward

The results of these experiments are in agreement with the anterograde and retrograde tracing studies that suggested the VTA receives its cholinergic input primarily from the LDT (Oakman et al. 1995; Omelchenko and Sesack 2006; Satoh and Fibiger 1986). The results of the *in vivo* microdialysis experiments (**Chapters 2** and **3**) led me to hypothesize that METH indirectly stimulates the mPFC via its actions on the DA terminals in the NAc and on DA neuron soma in the VTA. This hypothesis is supported by tracing studies that confirm LDT cholinergic neurons (primarily located in the laterodorsal region of the LDT) have reciprocal connections to the mPFC (Satoh and Fibiger 1986). Furthermore, it appears that LDT cholinergic afferents terminate in the same mPFC region as DA projections from the VTA (Lindvall et al. 1978). Indeed, the intra-LDT microinjection experiments (**Chapter 3**) showed that METH stimulates the LDT to induce increases in VTA ACh levels. This finding, in addition to a broad literature indicating that (1) the LDT receives input from sensory areas such as the superior colliculus (Satoh and Fibiger 1986) and responds to sensory stimuli (Koyama et al. 1994), (2) the LDT ACh neurons induce increases in DA burst firing and release (Forster and Blaha 2000; Lodge and Grace 2006), and (3) LDT ACh neurons might be involved in cocaine-seeking behavior (Shabani et al. 2010; You et al. 2008), led me to hypothesize that LDT ACh was involved in forming an association between METH related cues and METH reward.

The results of the first experiment, in which I tested the role of LDT ACh in the acquisition of METH CPP using a 0.5 mg/kg METH dose (**Chapter 4**), did not support this hypothesis. Since METH can induce increases in DA levels independently of DA neuronal stimulation, I speculated that a weaker METH CPP may be more sensitive to cholinergic modulation. Therefore, I hypothesized that a lower dose of METH (0.25 mg/kg) would condition a weaker CPP and “unmask” an effect of the LDT lesion.

Unfortunately, mice still exhibited a place preference to the 0.25 mg/kg METH dose. An even lower METH dose or fewer conditioning trials could potentially result in a reduced METH DA response and condition a weak METH CPP. Therefore, it still might be possible to see a modulatory effect of ACh on DA levels and METH reward with a weaker METH CPP.

Although the hypothesis that LDT ACh is involved in forming a METH CPP was not supported, this does not necessarily negate the previous research suggesting a role for LDT ACh in cocaine reward. While METH and cocaine are both psychostimulants, they have very different mechanisms of action. Cocaine inhibits the reuptake of DA by blocking the DAT, and thus is dependent on neuronal stimulation to induce increases in DA levels; however, METH acts independently of neuronal stimulation to release DA into the synapse. Neuronal activity-independent DA release likely makes any ACh potentiation of DA levels insignificant. Therefore, it appears that the LDT-to-VTA cholinergic projection is minimally involved in METH reward. The original schematic illustrating connections of the mesocorticolimbic pathway (Figure 2) has been redesigned with the information from the aforementioned experimental results (Figure 21). This new figure emphasizes METH's actions within the NAc and mPFC and reduces the contribution of the LDT ACh projection to reflect the minimal contribution of LDT ACh in METH reward. So, although the LDT cholinergic projections to the VTA are not involved in *METH* reward, they may still be important for magnifying DA levels in the NAc, thereby mediating *cocaine* reward. One limitation to consider, however, is that these experiments did not assess the role of the LDT cholinergic projection in other METH reward procedures, such as self-administration. This topic is addressed in **section 5**, Technical Considerations.

Figure 21

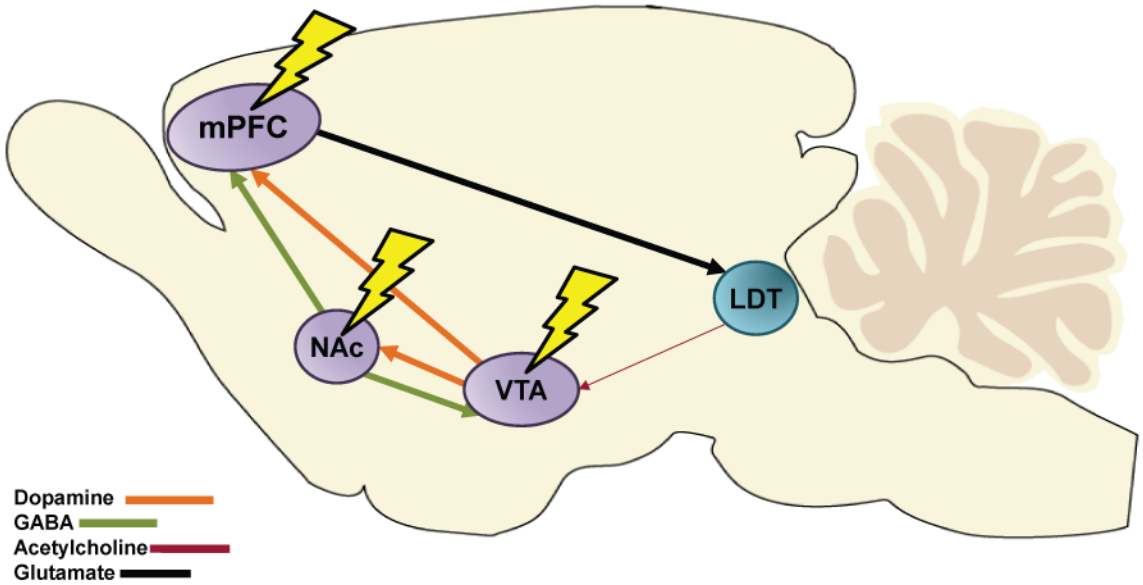


Figure 21. Hypothesized circuit underlying METH reward. METH (yellow lightning bolts) acts on midbrain DA terminals within the NAc and mPFC and on the DA soma and dendrites in the VTA. This stimulates dopamine projections (orange arrows) to the mPFC or DA terminals within the mPFC itself. GABA projections (green arrows) from the NAc to the mPFC and VTA may regulate DA firing during METH stimulation. The stimulated mPFC then sends glutamate projections (black arrows) to the LDT in the mesopontine tegmentum. These connections are hypothesized to underlie METH reward. Although stimulation of the LDT leads to an increase in VTA ACh levels, it is not involved in METH reward. Thus, the ACh projection (red arrow) is minimal and depicted as a thin arrow.

However, as mentioned earlier, LDT ACh might modulate a weak METH CPP. To identify whether LDT ACh modulates a weak METH CPP, future experiments should (1) measure METH CPP using a lower range of METH doses and/or fewer conditioning trials to establish a weaker CPP, (2) perform a bilateral LDT lesion and then use the optimal METH dose and/or conditioning trials to condition a weak CPP, and (3) perform ChAT IHC to confirm LDT cholinergic cell loss. Unfortunately, while these experiments would determine if LDT cholinergic neurons contribute to a weak METH CPP and further clarify the roles of ACh and DA in METH reward, the results of such experiments might

have little relevance to METH addiction, in which individuals commonly take METH for a prolonged time and use much higher doses (≥ 2000 mg/ day) (Cruickshank and Dyer 2009). Therefore, future research on the role of LDT cholinergic neurons in reward should focus on drugs such as cocaine (or other rewarding events, such as brain stimulation) that require VTA DA neuronal stimulation.

A logical question following the results of these experiments is, "What are the substrates of METH reward"? This is discussed in detail in the sub-section below. Additionally, despite the negative results from these experiments, it is clear that there is a functional cholinergic connection from the LDT to the VTA and that METH stimulates these cholinergic projections. The potential role(s) of METH-stimulated LDT cholinergic projections is discussed in **section 4**.

Other potential mediators of methamphetamine reward. Since the mesopontine cholinergic neurons are not involved in METH reward, an obvious question is what neurochemicals, receptors, or neuroanatomical sites are involved in mediating METH reward? Since METH releases DA independent of neuronal stimulation, most likely none of the DA-modulatory neurotransmitters within the VTA would have an effect on METH-reward. I believe the best general target to inhibit METH reward is the DA system. However since the DA system underlies a variety of behaviors, manipulation of it is likely to result in side effects and limit their practicality in treating a human population. As reviewed in the general introduction (**Chapter 1**), DA within the NAc is a key substrate for METH reward. For instance, Buproprion, a common treatment for depression and nicotine dependence, works by inhibiting the reuptake of DA (Julien 2001) and was recently investigated as a treatment for METH abuse. Buproprion treatment increased the number of weeks abstinent from METH in METH-dependent

individuals (Elkashef et al. 2008). METH has a variety of effects on the DA system in addition to increasing DA levels including, (1) stimulating DA release via actions at the DAT and VMAT-2 (Fleckenstein et al. 2007), (2) increasing TH levels and the synthesis of DA (Keller et al. 2011; Shepard et al. 2006), and (3) inhibiting the enzyme that metabolizes DA, MAO (Egashira et al. 1987; Seiden et al. 1993).

Targeting the cellular machinery on which METH acts to increase DA levels in the NAc may be a way to decrease METH reward. For instance, the VMAT-2 inhibitor lobeline decreased METH-induced DA release *in vitro* (Miller et al. 2001) and METH self-administration (Harrod et al. 2001a); however lobeline is also an antagonist at the nAChR (Dwoskin and Crooks 2002). A novel VMAT-2 inhibitor, GZ-793A also blocked METH-induced DA release (Horton et al. 2011) and attenuated METH reward in a variety of measures. GZ-793A dose-dependently decreased METH infusions in rats previously trained to self-administer METH but had no effect on operant food self-administration (in pre-food trained rats) (Beckmann et al. 2012). Furthermore, GZ-793A produced a downward shift in the METH dose-response curve, did not substitute for METH in operant self-administration and blocked the acquisition of METH CPP (Beckmann et al. 2012). In addition, repeated administration of GZ-793A did not induce tolerance to the METH administration suppressive effect (Beckmann et al. 2012). However, repeated administration of GZ-793A increased food self-administration and weight gain compared to vehicle controls (Beckmann et al. 2012). This investigation provides encouraging evidence that the VMAT-2 may be a potential therapeutic target for the treatment of METH abuse. It should be noted, however, that DA also regulates approach to non-drug rewards (Day and Carelli 2007; Wise 1982), feeding (Cannon and Palmiter 2003) and general motor control (Brooks 2001; Devos et al. 2010). Thus, treatment with drugs that assist in the uptake of DA into vesicles may have potential side

effects. This is evidenced in the study by Beckman and colleagues in that the VMAT-2 inhibitor GZ-793A altered food consumption and body weight.

METH also increases TH levels, thereby increasing DA synthesis and therefore targeting TH may be a potential route to decrease METH reward. Previous studies have reported that the TH-inhibitor α-methyl-para-tyrosine blocked METH-induced neurochemical changes associated with neurotoxicity (Commins and Seiden 1986; Schmidt et al. 1985), blocked METH-induced hyperthermia (Yuan et al. 2010) and attenuated METH-induced DA depletion (Wagner et al. 1983). A caveat of inhibitingTH, however, is that TH and DA are both precursors in the synthesis of NE. Therefore, altering the levels of TH and DA would subsequently have effects on the levels of NE and its downstream behavioral effects. The neurotransmitter NE is the main afferent of the LC and important in regulating stress and anxiety responses (Morilak et al. 2005) and cardiac function (Colucci 1998; Katzung 2007). Thus, altering the NE system could result in unwanted and perhaps dangerous side effects. In addition, it would not be clear if a treatment effect provided by altering TH levels was caused by changes in the DA or NE systems (or a combination of the two).

METH also inhibits MAO, the enzyme responsible for DA, NE and 5HT metabolism (Suzuki et al. 1980). A review of the MAO activity in METH's neurochemical and behavioral effects indicated several investigations that have tested the effect of the irreversible MAO inhibitor, clorgyline on METH CPP, stereotypy and locomotor behavior (Kitanaka et al. 2006a). Clorgyline decreased METH-induced hyperlocomotion in the mouse (Kitanaka et al. 2005), but had no effect on the acquisition of METH CPP in mice (Kitanaka et al. 2006b). MAO inhibitors have been used for decades to treat depression, but due to their poor side effect profile and the advent of new antidepressants with fewer

serious side effects, MAO inhibitors are not as commonly used today (Wimbiscus et al. 2010). However, METH is an MAO inhibitor, and therefore MAO *upregulation* may attenuate METH reward. To my knowledge there are no publications investigating the effect of MAO upregulation on METH-reward. This may be due to a lack of pharmacological substances available, that I am aware of, that increase MAO. Although since MAO also metabolizes catecholamines other than DA, and since DA is involved in a variety of reward-related behaviors, any treatment effect seen by targeting MAO is likely not METH specific and might carry the potential for serious side effects.

Another approach to inhibiting METH reward is to target DA receptors. Recent evidence suggests that the DA D3 receptor may be a potential target for the development of therapeutics for METH addiction. The D3 receptor is found in high concentration within the VTA and NAc (Sokoloff et al. 1990) and previous preclinical investigation found the D3 antagonists SB-277011A and NGB 2904 useful in attenuating METH-enhanced brain stimulation reward (Spiller et al. 2008) and in inhibiting the acquisition and expression of cocaine CPP (Vorel et al. 2002). However, the D3 antagonist SB-277011A has poor bioavailability and short half-life in the primate (Austin et al. 2001). A recent investigation with a new D3 antagonist, PG01037, revealed that it decreased the breakpoint for METH self-administration and decreased cue-induced reinstatement of METH seeking in rats (Higley et al. 2011a). However, PG01037 also decreased the breakpoint for sucrose administration, suggesting that the D3-mediated effects are not specific for METH reward. This is not surprising given the role of DA in natural, as well as drug, reward.

Although pontine cholinergic neurons are not involved in METH reward, it is possible that the cholinergic interneurons in the NAc may play a role in METH reward.

As mentioned in the general introduction (**Chapter 2**), METH induces increases in dorsal striatal ACh. NAc ACh levels appear to balance striatal DA-induced approach behaviors. That is, increased NAc ACh levels are associated with avoidance behavior, such as conditioned taste aversion, escape behaviors related to aversive brain stimulation and morphine withdrawal (for a review see Hoebel et al. 2007). This review suggests that perhaps upregulation of the NAc cholinergic responses may attenuate approach behaviors generated by drugs of abuse, such as METH.

3. The role of laterodorsal tegmental acetylcholine in locomotor activity

Although there was no relationship between LDT ACh and METH reward, these experiments repeatedly showed a relationship between LDT ACh and METH- and saline-induced locomotor activity. Reversible inhibition of LDT cholinergic neurons suppressed saline and METH activity (**Chapter 3**), and bilateral LDT lesion increased activity during CPP conditioning trials for saline or 0.25 mg/kg METH and transiently for 0.5 mg/kg METH (**Chapter 4**). Although the effect on locomotor activity is opposite between the experiments, it is highly probable that there was a substantial loss in glutamatergic and GABAergic cell loss following the bilateral LDT lesion. The loss of additional LDT neurochemical signaling could lead to a general increase in locomotor activity compared to inhibition of LDT ACh alone. In addition, the bilateral LDT lesion may have triggered compensation from the PPT cholinergic projections to the VTA, which could have contributed to the general increased locomotor activity in the CPP experiment. The PPT and the SN are components of the locomotor executing system and the muscle-tone inhibitory system. Activation of the PPT inhibits α motoneurons in muscles via signaling through the pontine reticular formation (PRF), medullary reticulospinal neuron and spinal interneurons (for a review see Takakusaki et al. 2004). Stimulation of the SNr stimulates GABA projections to the PPT, which inhibit the PPT-

mediated muscle-tone inhibition. Thus, SNr stimulation results in a general increase in locomotor activity. Therefore, in the absence of the LDT, it is possible that METH-induced activation of the SN stimulated the PPT, thus causing a general disinhibition of muscle tone. A hypothesized circuit underlying the initiation of METH-induced activity is shown in Figure 22.

The interaction of the basal ganglia and mesopontine tegmentum may also be involved in initiating METH-induced locomotor activity. Grillner and colleagues suggest that the GABA projection from the striatum to the globus pallidus is important in the stimulation of locomotor behavior (Grillner et al. 2005). This GABA projection inhibits the globus pallidus GABA projection, which results in the disinhibition of downstream areas, such as the mesencephalic locomotor region (MLR) and the mesopontine tegmentum, implicated in locomotor behavior (Takakusaki et al. 2004). Moreover, the striatal GABA neurons require a strong excitatory input, which is mediated by the D1 DA receptor. Thus, a bolus injection of METH might provide enough D1 activation in the striatum to stimulate GABA neurons and thus initiate locomotor behavior.

The MLR is a relatively ambiguous region in the brainstem that has historically been functionally, as opposed to anatomically, defined and has often been considered a part of the PPT (Jordan 1998; Whelan 1996). However, reviews by Winn and colleagues suggest that the PPT is not necessary for spontaneous locomotor activity, and is more involved in the selection of appropriate actions (Inglis and Winn 1995; Winn 2006). Thus, the PPT may in fact be separate from the MLR. The ventral and dorsal striatum projections to the PPT are thus thought to assist in the selection of motivational responses (Inglis and Winn 1995). A caveat to the conclusion that the PPT is not involved in spontaneous locomotor behavior, is that it does affect locomotor responses

Figure 22

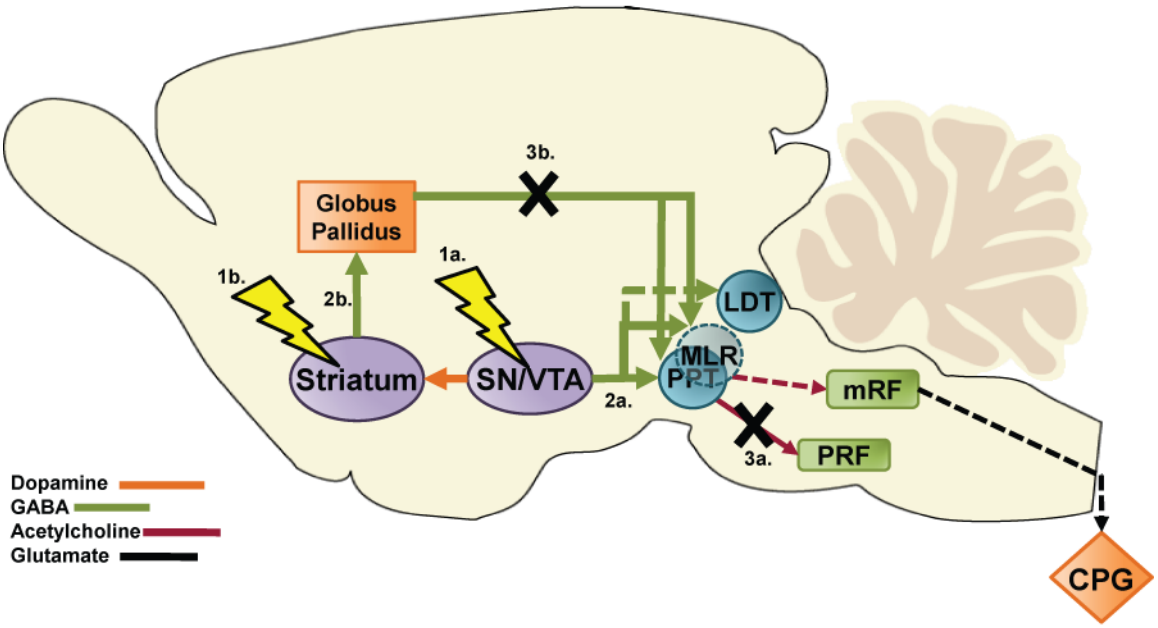


Figure 22. Hypothesized circuit underlying the initiation of METH locomotor activity. METH (yellow lightning bolts) activates the SN (**1a**), which stimulates inhibitory GABA projections (green arrows) to the PPT (**2a**). This inhibits ACh projections (red arrow) from the PPT to the PRF, which ultimately disinhibits the descending muscle tone inhibitory system (**3a**). Alternatively, METH can increase striatal DA levels, which then stimulate striatal DA D1 receptors (**1b**), thereby stimulating GABA projections to the globus pallidus (**2b**). This disinhibits regions implicated in the initiation of locomotor behavior, such as the mesenchephalic locomotor region (MLR) and PPT (**3b**). Hypothesized cholinergic projections from the MLR (red dashed arrow) are shown to the medial reticular formation (mRF). The mRF sends likely glutamatergic projections (black dashed arrow) to the spinal central pattern generator (CPG), thus initiating locomotor activity. Although it is unknown whether the LDT has locomotor-output similar to the PPT or MLR, it does receive projections from the SN and VTA, which may be GABAergic (dashed green arrow). The MLR is shown as semi-transparent and overlapping with the PPT to highlight the fact that the MLR is a functionally, not anatomically, defined area and has often been considered a part of the PPT, and may also include other anatomical areas.

to amphetamine (Alderson et al. 2003; Steiniger and Kretschmer 2004) and other drugs (Alderson et al. 2008; Laviolette et al. 2002). Therefore, the PPT tegmentum may indeed be involved in METH-induced locomotor responses.

Although the literature has typically emphasized the roles of the MLR and PPT in the initiation of locomotor activity, the LDT also receives innervation from the regions that project to the PPT and MLR, such as the SN, VTA and VP (Cornwall et al. 1990; Semba and Fibiger 1992). Therefore, the LDT is also positioned to mediate appetitive responding. However, the LDT, compared to the PPT, has fewer projections to the nigrostriatal pathway (i.e., SN → striatum), which provides major output and input to the basal ganglia. Thus, the LDT might not affect the output of the basal ganglia and subsequent locomotor activity as much as the PPT. This may explain why the LDT lesion did not consistently induce changes in locomotor activity (**Chapter 4**). Nevertheless, the data presented in this dissertation as well as previous research (Alderson et al. 2005; Nelson et al. 2007) suggest that the LDT, and in particular the cholinergic neurons, are involved in locomotor behavior. While it is possible that the cholinergic neurons of the LDT constitute a small portion of the MLR, it remains to be elucidated whether this effect is due to the connections of the LDT to the SN and basal ganglia structures.

There is, however, an important caveat to this hypothesized mechanism for METH-induced locomotor behavior. These circuits have been implicated in the initiation of locomotor behavior, but not necessarily in the maintenance of sustained locomotor activity (I am considering sustained activity separately from stereotypy, which is discussed in **section 4**). However, a prolonged increase in striatal DA tone might induce persistent activation of the basal ganglia and thus support protracted locomotor activity. Therefore, METH might induce sustained locomotor activation due to its direct and indirect actions on the striatum and other basal ganglia structures. Moreover, M5 subtype mAChRs in the VTA have been implicated in prolonged DA release and protracted physiological processes (e.g., salivation) (Forster et al. 2002b; Yeomans et al.

2001). This receptor is present on DA neurons in the VTA and SN (Vilaro et al. 1990), and is a potential mechanism through which mesopontine cholinergic projections might support protracted locomotor activity. Indeed, METH induced a protracted increase in VTA ACh levels (**Chapter 2**), which might have stimulated the M5 subtype receptor and assisted in protracted terminal DA release. Although terminal DA levels were not measured in this experiment, the experiments presented in **Chapter 3** showed that 3.5 mg/kg METH stimulates significant DA release 40 min post injection. It is unknown whether a higher METH dose (e.g., 5 mg/kg) would induce a more protracted DA release.

Since it is hypothesized that striatal DA D1 receptor activation initiates basal ganglia motor activity, future studies could use the following approaches to clarify the role of DA in protracted METH-induced locomotor activity: (1) administer DA D1 receptor antagonists into the striatum, (2) use DA D1 receptor knockouts, (3) measure GABA levels within the globus pallidus, or (4) use optogenetic techniques to inhibit or excite the DA D1 receptors on globus pallidus-projecting GABA neurons within the striatum. Additionally, the use of intra-VTA M5 subtype receptor knockouts would establish whether this receptor is necessary for METH-induced protracted DA release in the terminal. Thus, future investigations have several avenues to better characterize the role of the M5 subtype receptor and striatal DA D1 receptor activation in protracted METH locomotor activation.

4. Hypothesized role of mesopontine tegmental acetylcholine in other methamphetamine-related behaviors

Since METH stimulated the LDT to increase VTA ACh levels, it is reasonable to hypothesize that levels of ACh were also increased in other brain areas that receive LDT

cholinergic projections. The LDT, and in particular ACh in the LDT, has been implicated in several behaviors and physiological events, such as REM sleep, psychosis and aversion. Thus, while METH-induced ACh in the VTA may merely have been an epiphenomenon (i.e., not underlying a specific METH behavior), different LDT (or even PPT) cholinergic projections may underlie other non-reward associated METH behaviors, such as METH-related aversion, insomnia and psychosis. The following sub-sections will discuss how cholinergic projections from the LDT and PPT could potentially underlie additional METH-induced behaviors.

Role of mesopontine acetylcholine in methamphetamine aversion. Following the null outcome of LDT lesion on METH reward, I began to consider the possibility that METH-induced increases in VTA ACh levels were related to an aversive state rather than reward. Studies in humans (for a review see Cruickshank and Dyer 2009) and mice (Shabani et al. 2012b) suggest that METH has aversive qualities, especially at higher doses.

LDT ACh has also been associated with aversive-like states. For instance, when a startle-inducing air puff is delivered, rats produce a 22 kHz alarm call. Rats that produce this alarm call show significant c-Fos expression in the cholinergic cells of the LDT, but not of the PPT (Brudzynski et al. 2011). In addition, cholinergic activation of the lateral septum, a projection target of LDT ACh neurons, or glutamatergic activation of the LDT elicited the 22 kHz alarm call in rats (Bihari et al. 2003). These effects were blocked by microinjection of intra-LDT scopolamine (Bihari et al. 2003). Additionally, when investigators exposed naïve rats to alarm pheromones collected from stressed rats, the naïve rats displayed a stress response (Kiyokawa et al. 2004) and an increase

in c-Fos expression in a number of brain areas, including the LDT (Kiyokawa et al. 2005).

The METH doses that elicited CPP (0.25 and 0.5 mg/kg) were much lower than the doses used in the microdialysis experiments, which elicited the prolonged VTA ACh response (3.5 and 5.0 mg/kg). Therefore, it is conceivable that higher METH doses, like those used in the microdialysis experiments, elicit an aversion. However, unpublished data from the lab suggested that the C57BL/6J mouse is fairly resistant to developing a METH aversion. In fact, the data indicated that the C57BL/6J mouse showed METH CPP up to a dose of 16 mg/kg METH (Cunningham, unpublished observations). Thus, before testing an effect of LDT lesion on METH CPA, I decided to test if C57BL/6J mice would even acquire a METH CPA. The details of this experiment are presented in **Appendix I**. The results of this experiment indicated that C57BL/6J mice do not condition an aversion to a higher dose of METH (3.5 mg/kg) using a standard CPP procedure. In fact, the mice conditioned using the standard CPP procedure showed a preference for the chamber paired with 3.5 mg/kg METH. Additionally, although mice conditioned using a procedure specifically designed to induce drug aversion showed a small CPA after 2 conditioning trials, this effect was no longer significant following a total of 4 conditioning trials. The latter experiment might suggest that the mice developed tolerance to conditioned METH aversion. However, since the initial METH aversion was mild, transient and only observed in the CPA-specific procedure, it is unlikely that the 3.5 mg/kg acute METH dose used in the microdialysis experiments (**Chapter 3**) induced aversion. Therefore, I feel it is highly unlikely that LDT ACh is involved in METH aversion in the C57BL/6J mouse. However, it is important to note that other inbred mouse strains do show METH CPA (Cunningham and Noble 1992), but since I only tested one inbred strain and one METH dose I cannot generalize the role of LDT ACh in

METH aversion (this issue is addressed in more detail in **section 5**, technical considerations). A future experiment could test higher METH doses (e.g., 5 mg/kg or higher) for the development of METH CPA in C57BL/6J mice; however given the previous unpublished data from our lab I doubt higher METH doses would successfully condition a METH place aversion in this strain. A better approach may be to test the contribution of the LDT cholinergic neurons in METH aversion by using a strain of mice known to develop METH CPA (e.g. DBA/2J mice). These experiments could further characterize the role of LDT ACh in METH aversion and expand these conclusions beyond the C57BL/6J mouse.

Role of mesopontine acetylcholine in rapid eye movement sleep. The mesopontine cholinergic neurons are an important part of the ascending arousal system. Cholinergic projections from the PPT and LDT and parallel glutamate projections stemming from the PRF relay through thalamocoritcal cells to activate the cortex during REM sleep and wakefulness (Jones 2005). ACh in the thalamus acts through nAChRs and M1-type mAChRs to stimulate thalamocortical relay cells. The result is a desynchronization of these thalamocortical relay cells and an induction of ponto-geniculo-occipital waves characteristic of wakefulness and REM sleep (Jones 1991; Kayama and Koyama 2003). In addition, muscle atonia associated with REM sleep is induced through descending mesopontine cholinergic projections to the PRF (Jones 1991). To maintain wakefulness, ACh works in combination with NE to stimulate cortical arousal and muscle tone. In the absence of NE, though, high levels of mesopontine ACh induce the loss of muscle tone (Jones 2005).

LDT cholinergic neurons burst fire in order to release the large amount of ACh in the lateral geniculate nucleus of the thalamus required to induce REM sleep (Kodama

and Honda 1996). Furthermore, there are 2 populations of LDT presumed cholinergic neurons that exhibit differential firing patterns during different sleep stages: (1) those that display Ca^{++} dependent, low-threshold, slow, rhythmic burst firing (termed LTB neurons), and (2) those that do not burst fire (termed non-LTB neurons) (Koyama and Sakai 2000; Luebke et al. 1993). In addition, other neurotransmitters, including GABA, NE, glutamate, and histamine, in the LDT modulate ACh firing to affect REM sleep and wakefulness (Kohlmeier and Kristiansen 2010; Kohlmeier and Reiner 1999; Koyama and Sakai 2000).

METH is a potent stimulant that has wake-potentiating effects, with users often staying awake up to 4 days on a METH binge (Cruickshank and Dyer 2009; Watson et al. 1972). Upon METH withdrawal, METH-dependent subjects (Watson et al. 1972), non-dependent rats (Martins et al. 2008) and non-dependent C57BL/6, C57BR, and BALB/c mice (Kitahama and Valatx 1979) showed a significant increase in the amount of time spent sleeping, a decreased latency to enter REM sleep and an increase in time spent in REM sleep. It is likely that the two important structures that mediate wakefulness, the mesopontine cholinergic and LC NE, projections are dysregulated during METH intoxication, which induces persistent insomnia. It also appears that the dysregulation in mesopontine cholinergic tone persists after METH withdrawal, as suggested by the presence of REM rebound. Thus, the ascending LDT and PPT cholinergic projections likely play a role in METH wakefulness and arousal.

Role of mesopontine acetylcholine in methamphetamine psychosis. High doses of cholinergic muscarinic receptor antagonists, such as scopolamine, have historically been known to produce a form of psychosis in humans (Yeomans 1995). The general effects of high dose scopolamine are represented by the adage, “hot as a hare, dry as a bone,

blind as a bat, red as a beet, mad as a hatter." This statement refers to the ability of the mAChRs to mediate a wide range of physiological processes including: sweating and salivation, pupillary constriction, cutaneous vasodilation, and psychosis (Julien 2001; Pappano 2007). Auditory and visual hallucinations have been reported with antimuscarinic psychosis and the elderly or patients with dementia seem to be particularly vulnerable to experiencing hallucinations (Gulsun et al. 2006; Hori et al. 2011; Tsao and Heilman 2003; Vallersnes et al. 2009). Amphetamines are also known to induce hallucinations (Cruickshank and Dyer 2009) and METH psychosis has been treated in part by administration of the M1 subtype mAChR antagonist, biperiden (Yamamoto et al. 2007).

There is also an association between schizophrenia and the mesopontine cholinergic system. Post mortem analysis of brain tissue from schizophrenics indicated an increase in cholinergic cells within the LDT and PPT. While this may seem to contradict the mechanism of antimuscarinic psychosis (i.e., cholinergic antagonism), it is hypothesized that the M2 subtype of mACh receptors, which is found in high concentration on LDT and PPT ACh neurons, is the site of action for antimuscarinic-induced psychosis (Fendt et al. 2001; Yeomans 1995). In fact, M2 and M4 subtype mAChR binding was associated with delusions and hallucinations in patients with dementia associated with Lewy bodies (Teaktong et al. 2005). Thus, an increase in mesopontine ACh neurons is in agreement with the antimuscarinic model of psychosis because administration of mAChR antagonists would inhibit the M2 type autoreceptor, thus disinhibiting the ACh neuron resulting in an overall increase in cholinergic tone in terminal regions (Li et al. 1991; Vilaro et al. 1992). Furthermore, administration of non-selective antimuscarinics aggravates symptoms of schizophrenia (Yeomans 1995).

Interestingly, individuals with schizophrenia have a shortened latency to entering REM sleep and shortened slow-wave sleep (Kyung Lee and Douglass 2010; Yeomans 1995). This further supports the role of cholinergics in the ascending arousal system and psychosis; processes that are disturbed during METH intoxication and withdrawal. Therefore, METH may be activating the mesopontine cholinergic system to potentiate psychotic symptoms and insomnia.

Role of mesopontine acetylcholine in methamphetamine stereotypy. A common trait of psychostimulants is the ability to elicit locomotor activation and patterns of stereotyped behavior (termed stereotypy) at high doses. In rodents, METH-induced stereotypy is often exhibited as continuous circling, sniffing, head bobbing, grooming and biting behaviors (Tatsuta et al. 2005; 2006).

Interestingly, a theoretical framework recently proposed that amphetamine stereotypy be considered a problem of response selection and inhibition (Wolgin 2011). Behavioral response selection is commonly thought to be mediated by the basal ganglia (Grillner et al. 2005; Mink 1996; Redgrave et al. 1999). Indeed, this proposed framework is supported by a variety of studies showing that amphetamine and METH stereotypy involve dopaminergic responses within basal ganglia structures. For instance, repeated METH administration induced sensitization of stereotypy, which was related to the sensitization of the METH-induced DA response in the striatum of rats (Suzuki et al. 1997). In addition, intra-striatal amphetamine microinjection induced oral stereotypies (Baker et al. 1998) and systemic administration of amphetamine induced stereotypy and c-Fos expression in the striatum (Graybiel et al. 1990).

The cholinergic system, perhaps through its modulatory actions on the DA system, also appears to play a role in stereotypy. For instance, the mixed VMAT-2 inhibitor/nAChR antagonist lobeline dose-dependently attenuated stereotypy induced by a single high-dose (10 mg/kg) systemic METH injection (Tatsuta et al. 2006). In addition, systemic administration of the mAChR antagonist scopolamine increased stereotypy (Laviolette et al. 2000), while administration of the mixed nAChR/mAChR agonist carbachol blocked scopolamine-induced stereotypy (Mathur et al. 1997). However, while administration of scopolamine enhanced METH stereotypy in naïve rats, chronic treatment with a combination of scopolamine and METH blocked sensitization of METH-induced stereotypy (Ohmori et al. 1995). In combination with my findings that METH induces increases in ACh within the midbrain DA region, these studies support the hypothesis that increases in cholinergic tone in naïve animals induce stereotypies. However, the mesopontine cholinergic system may attenuate stereotypy after *chronic* METH exposure.

The sole source of ACh to the midbrain DA neurons within the midbrain originates in the LDT and PPT (Oakman et al. 1995; Semba and Fibiger 1992). However, LDT and PPT lesion experiments contradict the hypothesis that enhanced cholinergic tone within the midbrain DA region potentiates stereotyped behaviors. Bilateral excitotoxic lesion of the LDT (Forster et al. 2002a) or PPT (Allen and Winn 1995; Inglis et al. 1994a; Miller et al. 2002) enhanced amphetamine oral stereotypies. Furthermore, mesopontine cholinergic cell loss has been found in patients with Parkinson's disease (Hirsch et al. 1987; Jellinger 1988). This suggests that the mesopontine region as a whole normally inhibits stereotypic behaviors.

It is important to note that these previous pharmacological experiments did not selectively activate or inhibit mesopontine ACh neurons and that the previous lesion experiments did not selectively destroy cholinergic neurons. Thus, it is difficult to draw a definitive conclusion for the role of mesopontine cholinergic projections in psychostimulant stereotypy. It may be that the entire mesopontine region, including the glutamatergic, GABAergic and cholinergic projections, ultimately functions to inhibit stereotyped behaviors, but that potentiation of solely the cholinergic projection exacerbates stereotypy.

Future experiments should specifically target the cholinergic neurons within the PPT or LDT to determine whether these neurons inhibit or potentiate METH stereotypy. Since the experiments presented in **Chapters 2** and **3** determined that METH stimulates ACh levels in the VTA, a simple experiment would be to reversibly inhibit these projections with intra-LDT or intra-PPT microinjection of OXO and measure stereotypic behavior. However, I did not measure METH-induced ACh levels in the SN and would need to confirm that METH increases SN ACh levels before testing the contribution of the PPT-to-SN projection on stereotypy. I hypothesize that METH would increase SN ACh levels via action on the PPT. This would be tested using the same methods from my experiments presented in **Chapter 3**. The proposed experiment would (1) determine if METH also enhances cholinergic levels in the SN and (2) if METH indirectly activates the PPT to stimulate ACh levels in the SN. Following the results of this experiment I would use intra-PPT OXO microinjection to test whether the PPT ACh neurons enhance or inhibit METH stereotypy. In contrast to the experiments in **Chapter 3**, I would likely need to use a higher METH dose to induce robust METH stereotypy under control conditions.

5. Technical Considerations

Electrolytic lesion versus pharmacological reversible inhibition. Although I specifically and reversibly inhibited LDT or PPT cholinergic neurons in the microdialysis experiments, I performed general electrolytic lesions in the CPP experiments. Thus, all cell types within the LDT were likely damaged following the lesion for the CPP experiments. Previous data from ethanol CPP experiments suggest that even vehicle administration using a microinjection procedure can attenuate the acquisition of ethanol CPP in DBA/2J mice (Cunningham, unpublished observations). Thus, use of microinjections during acquisition risks not being able to detect a preference in the control group, and also makes interpretation of an inhibition of preference in the experimental group difficult.

However, since a general lesion was used in the CPP experiments, the potential loss of non-cholinergic neurons must be taken into consideration in the interpretation of the results. For instance, while the LDT lesion had no effect on the acquisition or expression of METH CPP, this may have been due to compensation from the cholinergic projections of the PPT. Although the projections are sparser from the PPT to the VTA, it is possible that the PPT influence on the VTA becomes significant in the absence of LDT ACh neurons.

In the analyses of the CPP data I only included subjects that showed at least a 50% cholinergic cell loss; however, all subjects were included in the correlations between the number of ChAT stained cells and several CPP outcome variables. These correlations revealed no relationship between the number of cholinergic neurons in the LDT and METH preference. Thus, this finding further supported the ANOVA results which indicated no effect of LDT lesion on the acquisition or expression of METH CPP.

Nevertheless, it may be that the bilateral lesions did not ablate enough of the LDT to see an effect on METH CPP. While it would be possible to make a larger, more encompassing lesion of the LDT, a larger lesion would make it very difficult, if not impossible, to avoid damaging the PPT. The PPT is located more rostral and lateral relative to the LDT. However, at about -4.96 mm from bregma, where the LDT is quite prominent, the PPT is located only 0.25 mm laterally from the LDT. The LDT spans approximately 0.68 mm rostrocaudally, and where it is most prominent (-4.96 - -5.20 mm from bregma) it spans approximately 1 mm dorsoventrally and 0.63 mm mediolaterally. However, at its most rostral and caudal extents it is much smaller, and at its most rostral extent the LDT is quite close to the PPT. Thus, merely making a larger lesion from one focus point would likely cause a large lesion that spreads outward in all directions and damages surrounding areas, including the PPT. In order to make a more complete LDT lesion, it may be necessary to perform 2 small bilateral lesions, for a total of 4 lesions: 1 bilateral lesion in the rostral LDT and 1 bilateral lesion in the caudal LDT. However, this many lesions would likely greatly increase the number of misses and as a result increase the sample size and time needed to perform such an experiment.

It is also possible that GABAergic and glutamatergic cells were lesioned in addition to the cholinergic cells. Thus, this combination of cell loss may have differentially affected METH CPP compared to if cholinergic cells alone were ablated. Although the 192 IgG-saporin toxin has been used to reliably lesion the cholinergic neurons in the basal forebrain, this toxin is ineffective in the mesopontine region since the mesopontine ACh neurons do not express the p75 neurotrophic growth factor receptor protein (Mesulam et al. 1989; Woolf et al. 1989). Recent reports indicate that the PPT and LDT cholinergic neurons of the CD-1 mouse selectively express the urotensin II receptor (UR-II) (Clark et al. 2005). Furthermore, excitation the UR-II

receptor excited LDT cholinergic neurons and increased DA output in the NAc (Clark et al. 2005). Thus, the UR-II provided an ideal target for the development of a selective mesopontine cholinergic neurotoxin. The diphtheria toxin was fused with the UR-II protein to create a neurotoxin that selectively ablates ACh neurons in the PPT of the rat (Clark et al. 2007). However, it is unclear whether this neurotoxin would effectively ablate LDT cholinergic neurons or whether it would be effective within either structure in the mouse. Future experiments should first characterize whether this neurotoxin can ablate cholinergic cells within the LDT or PPT of the mouse. A selective lesion would be a favorable outcome and encourage future use of the neurotoxin to selectively lesion the LDT ACh neurons and test their contribution in METH CPP or other METH related behaviors (see **section 4** of **Chapter 6**).

Selection of conditioned place preference over other measures commonly used to index drug reward. Despite my data suggesting that the LDT is not required for the formation of a METH-cue association, as indexed by CPP, this structure, and ACh in particular, might be involved in other aspects of drug reward and reinforcement learning. Detection of an effect of the LDT on other aspects of reward learning or reinforcement could be achieved by using a different technique, such as the ones described in **Chapter 1, section 5.** For example, a bilateral LDT lesion might affect the learning or performance of an operant behavior associated with METH self-administration. Previous research showed that reversible inhibition of the LDT ACh neurons (using intra-LDT OXO microinjection) attenuated cocaine and food self-administration in rats (Shabani et al. 2010). However, this study also found that intra-LDT OXO induced a mild increase in locomotor activity, which is contradictory to the literature on mesopontine cholinergic behavioral pharmacology (Alderson et al. 2005; Laviolette et al. 2000; Mathur et al. 1997). It is important to note that cocaine has a very different pharmacodynamic profile

compared to METH. Since METH can induce DA release irrespective of neuronal activation, cholinergic modulation from the LDT may not affect METH self-administration. In a separate study, cues previously paired with the delivery of cocaine increased VTA ACh levels and VTA ACh levels were associated with drug-seeking behavior in the absence of cocaine (You et al. 2008). Therefore, a reasonable extension of these data would be to characterize the role of the LDT cholinergic projection in cue-induced METH-seeking behavior.

Additionally, in regards to the self-administration procedure, future studies need to establish a method that reliably supports intravenous METH self-administration in the mouse. Ideally, subjects will not receive food training prior to training on METH self-administration. At the very least, initial food training should be minimal and use a different discriminative stimulus than what it is used for signaling METH delivery. Once a method is successfully established and a reliable dose-response curve obtained, it will be possible to test the role of LDT cholinergic neurons in the acquisition of METH self-administration or in the cue-induced reinstatement of METH seeking. An important consideration in the interpretation of operant behavior will be the effect of LDT modulation on locomotor activity. The data presented in this dissertation indicate that the LDT cholinergic neurons modulate locomotor activity. Therefore, if reversible inhibition of the LDT cholinergic neurons is used in self-administration experiments, attenuation of activity may be misinterpreted as an inhibition of reinforcement.

The LDT-to-VTA ACh projection might also be involved in conditioned reinforcement, that is, the ability of a Pavlovian CS to enhance the acquisition of an instrumental response. Glutamatergic projections from the basolateral amygdala, central nucleus of the amygdala and subiculum of the hippocampus to the NAc have

been implicated in mediating different aspects of conditioned reinforcement through the potentiation of NAc DA responses (Everitt et al. 2001). Additionally, a few studies in rats have evaluated drugs of abuse as the US in conditioned reinforcer experiments. These studies found that when cocaine or heroin administration were paired with a CS, subsequent presentation of the CS facilitated a new lever-press response (Di Ciano et al. 2007; Di Ciano and Everitt 2004; Di Ciano et al. 2008). The responding maintained by the previously cocaine-paired conditioned reinforcer was attenuated by inactivation of the dorsal striatum or NAc shell (Di Ciano et al. 2008). Additionally, the acquisition, but not the maintenance, of a cocaine-paired conditioned reinforcer was impaired by mPFC inactivation (Di Ciano et al. 2007). Cholinergic tone in the VTA, however, can also alter DA responding and thus might affect the learning of or performance for a conditioned reinforcer. Furthermore, the LDT receives input from brain areas involved in sensory detection and respond to sensory stimuli (Koyama et al. 1994; Satoh and Fibiger 1986). The role of VTA cholinergic tone in conditioned reinforcement is relatively unknown; however, one study reported that systemic nicotine pretreatment enhanced acquisition of a conditioned reinforcer in rats (Olausson et al. 2004). A possible future study could lesion the LDT after the Pavlovian association is established (or perform intra-LDT OXO microinjections during the conditioned reinforcer training sessions) to test the role of the LDT (or LDT ACh) in the acquisition of a conditioned reinforcer.

Selection of species and strain. The C57BL/6J mouse is a very commonly used strain in the investigation of the rewarding aspects of drugs likely for several reasons. C57BL/6J mice show sensitivity to the rewarding aspects of drugs of abuse including alcohol (Fidler et al. 2011; Grahame and Cunningham 1997), morphine (Elmer et al. 2010), amphetamines (Meliska et al. 1995; Orsini et al. 2004) and cocaine (Griffin and

Middaugh 2003; Orsini et al. 2005). In addition, the C57BL/6J mouse is often used as the background strain in the generation of genetically altered mice.

Selecting an inbred strain that is sensitive to your experimental manipulation can be quite useful. Moreover, using an inbred strain reduces the amount of unwanted error variance in a study produced by genetic variation. However, lack of genetic variability severely limits the generalizability of experimental findings. For example, experiments in this dissertation found that METH reliably induced increases in ACh levels within the VTA; however, since these experiments were all performed in the C57BL/6J mouse, it is not clear whether this effect would be observed in other inbred or outbred mouse strains. Indeed, different inbred mouse strains can show a wide variance on a single phenotype. For instance, in contrast to the C57BL/6J mouse, the DBA/2J mouse shows less reward sensitivity to morphine, amphetamines or cocaine (Elmer et al. 2010; Meliska et al. 1995; Orsini et al. 2005; Orsini et al. 2004), but they are more sensitive to ethanol reward (Cunningham et al. 1992). Furthermore, it is not clear if these METH-induced effects would be observed in other rodent models or within a primate.

An interesting future experiment could be to evaluate the mouse lines that were selectively bred for high (MAHDR) or low (MALDR) drinking of METH. Several behavioral experiments suggest that the MAHDR and MALDR lines are differentially sensitive to the rewarding and aversive aspects of METH (Shabani et al. 2012a; Shabani et al. 2012b; Shabani et al. 2011; Wheeler et al. 2009). However, no experiments have examined these lines for potential differences in basal or METH-induced neurochemical responses within the reward pathway. A potential future experiment could be to test these lines for METH-induced increases in ACh levels within the VTA. Additionally, there are a range of additional METH-related behaviors to which the mesopontine

cholinergic response could be related. It may be interesting to test whether these selected lines, which robustly differ in their sensitivity to METH reward, also differ in other METH phenotypes, such as METH-induced insomnia, psychosis and stereotypy.

6. Final Conclusions

The mesopontine cholinergic neurons send diffuse ascending projections to a wide range of structures and have been implicated in a variety of behaviors and physiological processes. The findings of this dissertation support the anatomical tracing studies that indicate a strong cholinergic projection from the LDT to the VTA. Furthermore, this dissertation indicates that METH stimulates LDT ACh neurons, most likely through indirect activation of the reciprocal projections from the mPFC to the LDT. While temporary inhibition of LDT cholinergic neurons inhibited locomotor activity, permanent ablation of the cholinergic neurons induced locomotor activation in some of the CPP experiments. These data suggest that LDT ACh neurons can modulate locomotor activity. However, the lack of a robust and replicable effect on activity suggests that these cholinergic neurons alone are not a strong influence on locomotor activity. Previous research suggests that anticholinergic drugs induce locomotor activation, most likely through the disinhibition of the mesopontine cholinergic neurons (Chintoh et al. 2003; Laviolette et al. 2000; Mathur et al. 1997). Therefore, I believe it is likely that the cholinergic neurons within the PPT work in tandem with LDT cholinergic neurons to modulate locomotor behavior.

Despite my findings that LDT ACh modulates (although moderately) locomotor behavior, these cholinergic neurons are not involved in METH reward as indexed by CPP. This outcome is most likely due to the ability of METH to induce DA release independently of neuronal stimulation. In other words, the ability of ACh to increase DA

levels and reward through its actions at the DA neuron soma is likely overshadowed by METH's direct effects on the DA terminal. Indeed, blockade of mAChRs in the VTA or temporary inhibition of LDT ACh neurons affects self-administration of cocaine, a drug that requires neuronal stimulation to exert its rewarding effects (Shabani et al. 2010; You et al. 2008). I conclude that METH induces increased cholinergic tone in the VTA through indirect stimulation of the LDT. However, the LDT cholinergic projections to the VTA are not necessary for METH reward or aversion in the C57BL/6J mouse, but may be involved in other METH-related behaviors, such as METH-induced psychosis and insomnia.

Appendix I

ASSESSMENT OF METHAMPHETAMINE CONDITIONED PLACE AVERSION IN C57BL/6J MICE

Dobbs, L.K. & Cunningham, C.L.

ABSTRACT

METH has been reported to induce aversion in humans and selectively bred mouse lines, but the neurochemical substrates of METH aversion are unknown. In the C57BL/6J mouse, METH induces an increase in VTA ACh levels that is unrelated to METH reward, but may underlie METH aversion. However, previous research suggests that the C57BL/6J mouse is resistant to developing METH aversion. The aim of these experiments was to determine if the C57BL/6J mouse can acquire a METH place aversion using a METH dose that elicits increased VTA ACh levels. Over 8 days, mice received alternating pairings of 3.5 mg/kg METH and saline with distinct tactile floor cues. Mice were tested using a CPP or CPA procedure. In the CPP procedure, mice received the METH or saline injection immediately before being placed onto the distinct floor cue for 30 min. In the CPA procedure, mice received the METH or saline injection after the 30-min session, immediately before being returned to their home cage. During preference tests, mice were given access to both floor types and time spent on each was recorded. Mice in the CPP procedure showed significant METH place preference after 2 and 4 conditioning trials. Mice in the CPA procedure showed an initial place aversion after 2 conditioning trials, but showed neither preference nor aversion after 4 conditioning trials. Mice in the CPP procedure showed greater locomotor activity after METH injection during the conditioning trials and during the drug-free preference tests compared to mice in the CPA procedure. Given the absence of METH aversion in the CPP procedure and the mild and transient nature of the METH aversion in the CPA procedure, we conclude that it is unlikely that VTA ACh levels induced by this METH dose underlie METH aversion in C57BL/6J mice.

INTRODUCTION

METH is a psychostimulant with rewarding and reinforcing qualities in humans (Hart et al. 2001; Sevak et al. 2009) and rodent models (Roth and Carroll 2004; Shabani et al. 2012a; Yan et al. 2007a). However, METH has also been reported to elicit aversion, especially at higher doses, in humans (Cruickshank and Dyer 2009), and condition aversion in the rat (Harrod et al. 2010), in selectively bred mouse lines (Shabani et al. 2012b; Shabani et al. 2011) and in the DBA/2J inbred strain (Cunningham and Noble 1992). In humans, METH aversion is often characterized by dysphoria, nervousness, and headache (Cruickshank and Dyer 2009). Mice selectively bred for high (MAHDR) or low (MALDR) drinking of METH show disparate sensitivity to developing METH place and taste aversion. While the MAHDR mice exhibited robust METH preference, MALDR mice showed enhanced sensitivity to the aversive effects of METH, as indexed by CPA (Shabani et al. 2012b; Shabani et al. 2011) and conditioned taste aversion (CTA) (Shabani et al. 2012b).

Previous research showed that systemic injection of 3.5 mg/kg and 5.0 mg/kg METH stimulates the LDT to induce a prolonged increase in VTA ACh levels (Dobbs and Mark, 2008, 2012). Although reversible inhibition of the LDT ACh neurons attenuated ACh levels and locomotor activity (Dobbs and Mark, 2012), lesion of the LDT had no effect on METH reward, as indexed by CPP. Moreover, quantification of LDT cholinergic neurons showed that the loss of LDT ACh neurons was negatively correlated with locomotor activity during the first conditioning trial, but not related to the amount of METH preference expressed following conditioning with 0.25 or 0.5 mg/kg METH. Therefore, it appears that the LDT cholinergic neurons do not underlie METH reward. However, the prolonged METH-induced increase in ACh was stimulated following moderate/high doses of METH (3.5 and 5.0 mg/kg) and it is unknown whether the lower

doses of METH used in the CPP experiments (0.25 and 0.5 mg/kg) induce a similar cholinergic response. Since METH can induce aversion at higher doses, it is possible that the higher doses used in our previous microdialysis experiments induced an aversive state in our previous CPP experiment. Therefore, the METH-induced increase in VTA ACh levels may underlie METH aversion, as opposed to METH reward. However, unpublished data from our lab suggests that the C57BL/6J mouse is relatively insensitive to developing a METH aversion, and in fact shows preference up to a dose of 16 mg/kg METH (Cunningham, unpublished observations). Therefore, in the current study we tested whether the C57BL/6J mouse would condition a METH place aversion using a CPP or a CPA procedure.

The CPP procedure is a reliable method to assess the rewarding or aversive properties of psychoactive drugs (Cunningham et al. 2006; Cunningham et al. 2011). In our previous METH CPP experiments we have conditioned reliable METH preference in the C57BL/6J mouse through alternate pairings of METH and saline with distinct tactile floor cues. In this procedure, the injection is administered immediately prior to exposure to the floor cue. In contrast, the CPA procedure conditions robust aversion by administering the drug injection immediately after removal from the distinct tactile floor cue (i.e., at the end of the conditioning trial and immediately before the subject is returned to their home cage). The CPA procedure is not METH-specific, and has been shown to condition robust aversion to amphetamine and nicotine (Fudala and Iwamoto 1987; 1990).

In the current set of experiments we administered 3.5mg/kg METH and saline either immediately before (CPP procedure) or after (CPA procedure) the conditioning

trials. We hypothesized that C57BL/6J mice would acquire a METH aversion under these conditions.

METHODS

Subjects

A total of 48 male, C57BL/6J mice (10 weeks old at the start of conditioning) were used for this experiment. Mice were group housed, four to a cage in a climate and humidity controlled vivarium on a 12h light cycle (lights on a 0700). Food and water were available *ad libitum*. All procedures were carried out in accordance with the National Research Council of the National Academies (Academies 2003) and approved by the Institutional Animal Care and Use Committee at Oregon Health and Science University.

Drugs

METH hydrochloride was dissolved in isotonic saline to a final dose of 3.5 mg/kg and administered IP in a volume of 10 ml/kg.

Conditioned Place Preference Apparatus

Conditioning chambers (30 cm x 15 cm x 15 cm) were constructed of clear acrylic walls and lid with aluminum end panels. Each chamber was housed in a sound- and light-attenuating enclosure (Coulbourn Instruments E10-20; 56.1 cm x 46.0 cm x 39.4 cm). The enclosure was equipped with a ventilation fan and not illuminated during conditioning trials or testing. Activity and side position (left or right) were detected with 6 sets of infrared photobeam detectors positioned 2.2 cm above the floor and 5 cm apart. Photobeam breaks were recorded every minute by a computer with a 10 ms resolution. Each of the interchangeable tactile floor cues served as half of the chamber floor. The

grid floor was constructed of 2.3 mm stainless steel rods, 6.4 mm apart in an acrylic frame. The hole floor was constructed of 16 gauge stainless steel perforated with 6.4 mm diameter holes staggered 9.5 mm apart mounted on an acrylic frame. A clear acrylic panel divided the chamber into two equal sections during conditioning trials (two-compartment procedure) so that the subject only had access to one floor type during conditioning. For the test day, this barrier was removed and the subject had access to both floor types.

Conditioned Place Preference and Aversion Procedures

An unbiased place conditioning procedure and an unbiased apparatus was used for this experiment (Cunningham et al. 2003). Mice received one habituation session, eight conditioning sessions (4 METH and 4 saline) and two preference tests. The first preference test was given after two conditioning trials of each type and the second preference test occurred after the fourth conditioning trial of each type. During the conditioning trials, half the mice either received the METH and saline injections immediately prior to placement in the conditioning apparatus (CPP procedure, or "Pre" group) or immediately after removal from the conditioning apparatus (CPA procedure, or "Post" group).

Habituation

During habituation, the conditioning apparatus had a white paper floor and no divider was present. Mice in the Pre group received an IP saline injection (10 ml/kg) immediately before being placed into the conditioning apparatus, while mice in the Post group received the saline injection immediately after removal from the apparatus. Mice in both groups were allowed to explore the apparatus for 5 min before being returned to their home cage.

Conditioning

Mice were randomly assigned to one of two conditioning subgroups (GRID+ or GRID-). Mice in the GRID+ subgroup received 3.5 mg/kg METH immediately before placement on (CPP procedure; Pre group) or after removal from (CPA procedure; Post group) the grid floor, whereas saline was injected on alternate days and paired with the hole floor. These contingencies were reversed for mice in the GRID- conditioning subgroup. Conditioning trials alternated between mice receiving METH paired with one specific floor type (CS+) and saline paired with the other floor type (CS-) and the order of exposure to METH and saline was counterbalanced. During the conditioning trials, activity was measured for 30 min and then mice were returned to their home cage.

Preference Tests

On test days, the center divider was removed so that the mouse had access to both floor cue types. For the CPP procedure, mice were administered a saline injection and placed in the center of the apparatus. Mice in the CPA procedure received no saline injection either before or after the test. Activity and time on each floor type were measured for 30 min. Mice were returned to their home cage after the session.

Statistical Analyses

Two subjects from the CPP procedure group were excluded from the analyses due to receiving incorrect METH-floor pairings. Additionally, 1 subject was euthanized after sustaining a large wound inflicted by a cage-mate. This subject's data were included in the analysis of the first preference test, but not in the analysis of conditioning activity or the last preference test. Activity during the 30 min conditioning trials was analyzed using a two-way repeated measure ANOVA with Trial (8 trials) and Trial Type

(METH or saline) as the within subjects factors and the timing of the METH/saline injection during conditioning (Time; Pre or Post) as the between subjects factor. The sample sizes for the analysis of the first preference test was n = 22 and n = 24 for the Pre and Post groups, respectively. The sample sizes for the analyses of conditioning activity and the second preference test was n = 21 and n = 24 for the Pre and Post groups, respectively. The amount of time spent on the grid floor for subjects that had METH paired with the grid (GRID+) or hole (GRID-) floor during the preference test was used as the measure of METH preference. A significant difference between these subgroups provides evidence of place conditioning (Cunningham et al. 2003). Grid time data were analyzed using two-way ANOVA with conditioning subgroup (Cond; GRID+ or GRID-) and timing of METH/saline injection during conditioning (Time; Pre or Post) as the between subjects factors. Significant interactions were followed up with simple main effects tests. All analyses were considered significant at an alpha of 0.05. Data are expressed as the mean ± SEM.

RESULTS

The first preference test was performed following 2 conditioning trials of each type. Two-way ANOVA showed a significant main effect of conditioning group ($F_{1,42}$ = 15.54, $p < 0.0005$) and a significant Cond x Time interaction ($F_{1,42}$ = 50.5, $p < 0.0005$). The main effect of Time was not significant. Figure 23a shows that mice in the Pre group exhibited a significant METH CPP ($F_{1,20}$ = 51.06, $p < 0.0005$) and mice in the Post group showed a significant METH CPA ($F_{1,22}$ = 6.03, $p = 0.022$). Figure 23b shows that during the preference test, mice in the Pre group showed greater locomotor activity compared to mice in the Post group ($F_{1,44}$ = 8.51, $p = 0.006$).

Figure 23

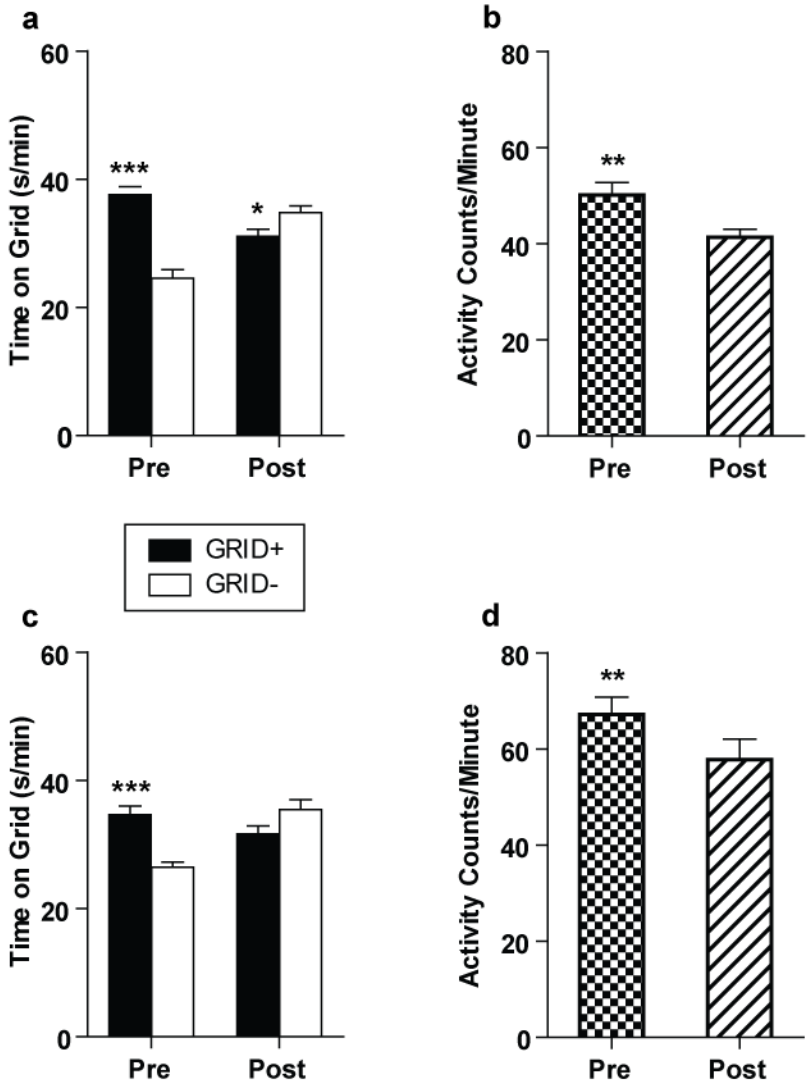


Figure 23. METH place preference or aversion is shown for subjects that received 3.5 mg/kg METH immediately before (Pre, left bars) or after (Post, right bars) a total of 2 (**a**) or 4 (**c**) conditioning trials. Subjects had METH paired with the grid floor (GRID+, black bars) or the hole floor (GRID-, white bars). Panels (**c**) and (**d**) show locomotor activity during the corresponding preference test for subjects that received METH prior to (Pre, checked bar on left) or following (Post, striped bar on right) conditioning trials. The Pre group showed significant METH place preference after 2 and 4 conditoning trials, while the Post subjects showed METH place aversion after 2 conditioning trials, but not after a total of 4 conditioning trials. The Pre subjects showed greater conditioned activity compared to the Post subjects during both preference tests. * $p < 0.05$ GRID+ vs. GRID-; ** $p < 0.01$ Pre vs. Post; *** $p < 0.0005$ GRID+ vs. GRID-.

After two more conditioning trials of each type (4 total of each), there was a significant main effect of Time ($F_{1,41} = 5.22$, $p = 0.028$) and a significant Cond x Time interaction ($F_{1,41} = 21.17$, $p < 0.0005$). The main effect of conditioning group was not significant. Simple main effects showed that mice in the Pre group still showed a METH preference ($F_{1,19} = 25.53$, $p < 0.0005$), but mice in the Post group showed neither a preference or aversion to METH ($F_{1,22} = 3.68$, $p = 0.068$) (Figure 23c). Figure 23d shows that during the second preference test, mice in the Pre group showed greater locomotor activity compared to mice in the Post group ($F_{1,43} = 8.58$, $p = 0.005$).

Figure 24 shows the locomotor activity during conditioning. Analysis of locomotor activity during conditioning trials revealed significant main effects of Trial Type ($F_{1,43} = 1903.07$, $p < 0.0005$) and Time ($F_{1,43} = 317.06$, $p < 0.0005$) and a significant Trial Type x Time interaction ($F_{1,43} = 317.06$, $p < 0.0005$). All other main effects and interactions were non-significant. Follow up analyses were performed collapsed across trials within each trial type (METH or saline). Mice that received METH ($F_{1,43} = 382.15$, $p < 0.0005$) or saline ($F_{1,43} = 165.77$, $p < 0.0005$) prior to the conditioning trials (Pre group) showed greater locomotor activity compared to mice that received the same type of injection after the conditioning trials (Post group). In addition, comparisons were made between Trial Type (METH or saline) within each Time group (Pre or Post) collapsed across trials. In mice that received injections prior to conditioning trials, METH treatment resulted in greater locomotor activity compared to saline treatment ($F_{1,20} = 320.66$, $p < 0.0005$). There was no effect of trial type on locomotor activity in mice that received injections after the conditioning trials.

DISCUSSION

Figure 24

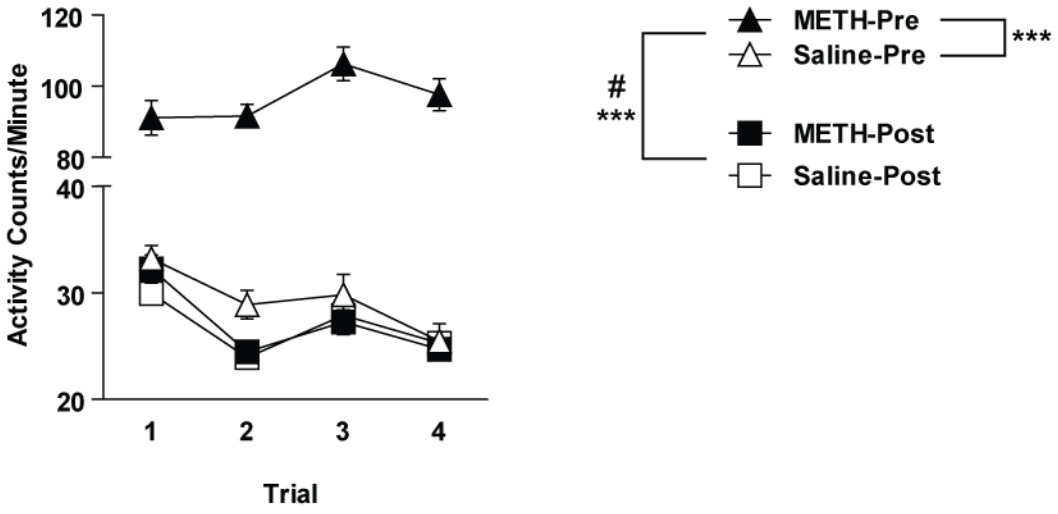


Figure 24. Locomotor activity during 3.5 mg/kg METH (black symbols) and saline (white symbols) conditioning trials is shown for subjects that received drug injections immediately prior to (triangles) or after (squares) each conditioning trial. METH induced greater locomotor activity compared to saline in subjects that received injections before the conditioning trials. Additionally, subjects that received METH or saline injections prior to conditioning trials showed greater locomotor activation compared to subjects that received METH or saline injections, respectively, after the conditioning trials. *** $p < 0.0005$ METH-Pre vs. Saline-Pre, or Saline-Pre vs. Saline-Post; # $p < 0.0005$ METH-Pre vs. METH-Post.

In agreement with previous unpublished findings from our lab, these data indicate that the C57BL/6J mouse is resistant to developing a METH place aversion using a standard CPP procedure. Although C57BL/6J mice conditioned using the CPA procedure initially expressed a weak METH CPA after 2 conditioning trials, the CPA was not observed following 2 additional conditioning trials. The unpublished data previously collected in our lab indicate that C57BL/6J showed a preference with METH doses up to 16 mg/kg when tested in a drug-free state. Furthermore, these mice did not show a METH aversion (or preference) when given a preference test following an injection of 4, 8 or 16

mg/kg METH. Thus, the accumulation of these data suggests this inbred strain is insensitive to the aversive qualities of METH as indexed by a standard CPP procedure. Further, the transient nature of the METH aversion indexed using the CPA procedure might suggest that these mice developed a tolerance to the aversive effects with continued conditioning trials.

The MAHDR and MALDR mice were selectively bred from a starting population of an F2 cross of the C57BL/6J and DBA/2J strains. The MAHDR mice readily acquired a CPP for METH with 0.5, 2.0 and 4.0 mg/kg METH in a drug-free preference test (Shabani et al. 2011). Although, the replicate line, MAHDR-2, showed place aversion at a 4 mg/kg METH dose, this line did not show a METH CTA to 2 or 4 mg/kg METH (Shabani et al. 2012b). These data suggest a strong genetic component underlies the sensitivity to the rewarding aspects of METH and the insensitivity to METH's aversive qualities. Furthermore, given the data from our lab, it might be that genes from the C57BL/6J strain, as opposed to the DBA/2J, are contributing to the MAHDR's insensitivity to METH aversion.

Based on these data and data previously collected in our lab, it is unlikely that the 3.5 or 5.0 mg/kg METH dose used in the microdialysis experiments (**Chapters 2** and **3**) (Dobbs and Mark, 2008, 2012) elicited a METH aversion in the C57BL/6J mouse. Therefore, it is doubtful that the METH-stimulated increase in VTA ACh levels was associated with a state of METH aversion. Given the similar insensitivity to the development of METH place aversion in the MAHDR line, it would be interesting to measure whether this selectively bred line (and its counterpart, the MALDR line) also exhibits a prolonged METH-induced increase in VTA ACh levels. While it is doubtful that

this neurochemical effect underlies METH aversion in the C57BL/6J mouse, it might play a role in METH aversion in prone strains or lines (i.e., the MALDR line or DBA/2J strain).

Appendix II

CUE- BUT NOT DRUG INDUCED REINSTATEMENT OF METHAMPHETAMINE-REINFORCED LEVER-PRESSING IN MICE

Dobbs, L.K. & Cunningham, C.L.

ABSTRACT

Reinstatement of METH seeking following exposure to drug related cues or small amounts of METH itself has not been well characterized using a behavioral mouse model. The current study used a mouse model of intravenous METH self-administration to test METH-primed and cue-induced reinstatement of METH seeking behavior. Nineteen male C57BL/6J mice were initially trained in a 2-lever operant procedure for food pellets before switching to METH self-administration. Mice self-administered METH (0.05 mg/kg/infusion) on an FR5 schedule in daily 30 min sessions for 5 days before undergoing extinction. Mice were then tested for METH-primed (0.25 and 1.0 mg/kg) and cue-induced reinstatement of METH–seeking. Pretreatment with 0.25 mg/kg METH did not affect active lever pressing, but pretreatment with 1.0 mg/kg METH significantly reduced active and inactive lever pressing compared to extinction levels. Presentation of the cue-light previously associated with food and METH delivery significantly increased active lever pressing on the reinstatement day and this enhancement continued on the following extinction day. These data suggest that higher METH doses may inhibit METH reinstatement through its motor activating properties. More generally, these data support previous work in mice showing a lack of drug-primed reinstatement after IP injection of psychostimulants. In contrast, conditioned cues previously presented during the delivery of food and METH exert strong and persistent control over reinstatement of operant behavior in mice.

INTRODUCTION

METH is a highly addictive psychostimulant with an estimated 1.2 million Americans over age 12 having tried it at least once in 2009 (SAMHSA 2010b). Addicted individuals are particularly vulnerable to relapse during a period of abstinence when the individual is exposed to the drug or stimuli (cues) previously associated with drug use. During abstinence, drug related cues or small doses of the drug itself are known to produce increased self-reported drug craving and reinstatement of drug taking (for reviews see de Wit 1996; Shaham et al. 2003). Although several different laboratories have successfully demonstrated METH-primed reinstatement to drug-seeking in rats (Moffett and Goeders 2007; Reichel and See 2010; Shepard et al. 2004), only one lab has studied this phenomenon in mice and the findings are mixed. In their first study, C57BL/6J mice failed to show drug-primed reinstatement at 0.5 or 1.0 mg/kg (Yan et al. 2006). In contrast, two later studies revealed reinstatement after priming with 1.0 mg/kg, but not at lower or higher doses (Yan et al. 2006; Yan et al. 2007a). However, interpretation of the latter studies is complicated by the fact that reinstatement was tested with ascending METH doses within subjects on consecutive days with no intervening extinction sessions, raising the possibility that the effects observed at higher doses depended on consecutive days of exposure to METH priming at lower doses. It is therefore important to better characterize METH-primed reinstatement in mice using a procedure that avoids this potential confound.

Previous experiments on METH reinstatement were conducted in the C57BL/6J mouse and this strain has also historically shown sensitivity to the rewarding aspects of drugs of abuse (Griffin and Middaugh 2003; Meliska et al. 1995; Orsini et al. 2005; Orsini et al. 2004). Therefore, in the current study, we trained male C57BL/6J mice to self-administer intravenous METH then extinguished this behavior and tested cue-induced

and METH-primed reinstatement of drug-seeking behavior. We predicted that a METH prime and cue presentation would reinstate active lever responding.

METHODS

Subjects

Adult, male C57BL/6J mice (n = 19) from Jackson Laboratories (Sacramento, CA) were housed in a temperature and humidity controlled vivarium on a 12 h reverse light-dark cycle. All behavioral testing was conducted during the dark cycle. Food and water were available *ad libitum*, except during test sessions. All procedures were approved by the Oregon Health & Science University Institutional Animal Care and Use Committee.

Drugs

METH hydrochloride was provided by the National Institutes on Drug Abuse drug supply program (Research Triangle Park, NC). METH was dissolved in saline and self-administered intravenously at a unit dose of 0.05 mg/kg/infusion in a volume of 10 µl. For the reinstatement tests, METH was administered IP at 0.25 or 1.0 mg/kg (10 ml/kg).

Surgery

Mice under isoflurance anesthesia were surgically implanted with an intra-jugular catheter constructed of silastic tubing (0.012" id; Braintree Scientific) connected to a 26-gauge threaded pedestal (Plastics One) housed in dental acrylic (Lang Dental). Catheters were assembled and implanted as described previously (Thomsen and Caine 2005). Mice were individually housed post-operatively and allowed 5 days recovery before beginning the experiment. Catheters were flushed daily with 0.02 ml heparin-saline (30 USP U/ml).

Apparatus

Acrylic and aluminum operant chambers (Med Associates, St. Albans, VT; 12.5 cm x 22 cm x 18.75 cm) were individually housed in melamine cabinets (Med Associates). METH was delivered from a 2.5 ml gastight Hamilton syringe mounted on a pump controlled by a computer (.27 ml/min). The syringe was connected via PE20 tubing to a single channel fluid swivel (Instech, Plymouth Meeting, PA) mounted above the center of each chamber. Additional PE20 tubing connected the swivel to the animal's catheter. Each chamber had two fixed levers, 2 cm above the floor and 7 cm apart with a yellow cue light (5 mm dia, 10W) 2 cm above each lever. A food hopper was centered between the levers. The house light was positioned 10 cm above the floor in the center of the opposite wall and illumination signaled the beginning of each training session.

Behavioral Procedure

Food Training

Mice were food restricted to 2 g chow/day for the first day of food training. This restriction was lifted as the animal acquired food self-administration. Each mouse was trained up to a FR 3 schedule of reinforcement to self-administer food pellets and on *ad libitum* food in their home cage before transitioning to METH administration. Completion of the operant on the active lever resulted in delivery of a 20 mg food pellet concurrent with illumination of the cue light and darkening of the house light (18 sec). Mice were implanted with catheters when: (a) the number of active lever presses exceeded the number of inactive lever presses, and (b) they received $\geq$ 10 pellets in a 30 min session for 2 consecutive days. The mean number of food training days before surgery was 8.4 $\pm$ 0.3.

Methamphetamine Self-Administration

Following post-operative recovery, mice were placed in the operant chamber, tethered to the infusion line and allowed to lever press for food pellets. Mice began METH self-administration the day after re-achieving the food training acquisition criteria. METH was available on a FR 5 schedule. The maximum number of METH infusions per session was limited to 5, 10 and 10 during the first 3 sessions, respectively, to avoid over dosing during the transition from food to drug. For each 0.05 mg/kg METH infusion, the cue light was illuminated and the house light was darkened (18 sec). Mice received 5 consecutive days of 30 min METH self-administration sessions. Reponses on the inactive lever were counted, but had no scheduled consequence.

Extinction

During 30 min extinction sessions, lever presses were recorded but did not result in cue light illumination or reinforcer delivery. Extinction sessions continued for a maximum of 7 days. Within that 7-day period, mice that had 2 consecutive days with active lever presses $\leq$ 33% of the mean of the last 2 days of METH responding were considered to have extinguished and were tested for reinstatement on the following day (n = 8). After 7 extinction days, mice with $\leq$ 50% of METH responding (n = 8) were also considered to have extinguished and were tested for reinstatement. The remaining mice (n = 3) were still responding $\geq$ 65% of their METH responding and were not included in reinstatement testing.

Reinstatement

Mice were tested for METH-primed and cue-induced reinstatement of active lever pressing. For the METH-primed tests, mice were injected (0.25 or 1.0 mg/kg METH IP)

in their home cage 15 min before the 30 min reinstatement test, which was performed under extinction conditions. METH was administered as an IP prime because by this stage of the experiment, catheter patency was beginning to fail. Cue-induced reinstatement consisted of three non-contingent cue light presentations 2.5 sec long, 5 sec apart at the beginning of the session. After that, the cue light was presented under a FR 5 schedule. Each mouse received all reinstatement tests (with additional extinction sessions between each test until responding met extinction criteria) in the following order: 0.25 mg/kg, 1.0 mg/kg, cue light.

Data Analysis

Lever responses over the last 2 days of METH self-administration were averaged to reflect the mean asymptotic responding for METH. Mean METH responding and responses on the first and last day of extinction were analyzed with a two-way repeated measure ANOVA. Responses during reinstatement tests were compared to responding on the previous day's extinction session and the day immediately following the reinstatement test using a two-way repeated measure ANOVA. Violations to sphericity were corrected using Greenhouse-Geisser. Baselines for each reinstatement test were compared using a two-way repeated measure ANOVA. The within subjects factors for all analyses were Lever (2 levels) and Day (3 levels). A Pearson correlation coefficient matrix was calculated using the following variables: mean active lever responses during 0.25 mg/kg, 1.0 mg/kg and cue reinstatement tests, mean METH and food intake (calculated over the last 2 days for each phase), total METH and food intake, and days to reach extinction and food training criteria. All significant interactions were followed up with simple main effects and Bonferroni corrected post-hoc tests. Results were considered significant at an alpha of 0.05.

RESULTS

Methamphetamine Acquisition & Extinction

Mice self-administered a mean of 0.52 mg/kg METH during the last two 30 min sessions (51.9 ± 3.3 active lever responses; Fig. 25a). Comparison of METH responding with responding on the first and last day of extinction (Fig. 25b) revealed significant main effects of Lever ($F_{1, 15} = 146.32$, $p < 0.005$) and Day ($F_{1.2, 17.3} = 35.15$, $p < 0.005$) and a significant Day x Lever interaction ($F_{1.3, 20.2} = 31.31$, $p < 0.0005$). Follow up analyses indicated that mice exhibited an "extinction burst", characterized by significantly more active lever responses on the first day of extinction compared to METH responding ($p = 0.037$). Responding on the active lever on the last day of extinction was significantly lower than for METH and the first day of extinction ($p < 0.0005$). There were no differences in responding on the inactive lever. In addition, mean active lever pressing for METH was correlated with the total ($r = 0.60$, $p = 0.014$) and mean ($r = 0.56$, $p = 0.024$) food pellet intake as well as the total ($r = 0.99$, $p < 0.0001$) and mean ($r = 0.99$, $p < 0.0001$) METH intake.

Although we pooled mice from 2 extinction groups ($\leq$ 33% or $\leq$ 50%) to complete our analyses, we also analyzed the data with extinction group as a factor. While the magnitude of lever responding sometimes differed, the pattern of lever responding was the same between the groups during METH administration and the reinstatement tests. The number of days to reach the extinction criteria was not correlated with any other variable (data not shown).

Methamphetamine-Primed Reinstatement

A METH-prime of 0.25 mg/kg failed to reinstate active lever pressing behavior (Fig. 26a). Neither the main effect of Day ($p = 0.064$) nor the Day x Lever interaction

Figure 25

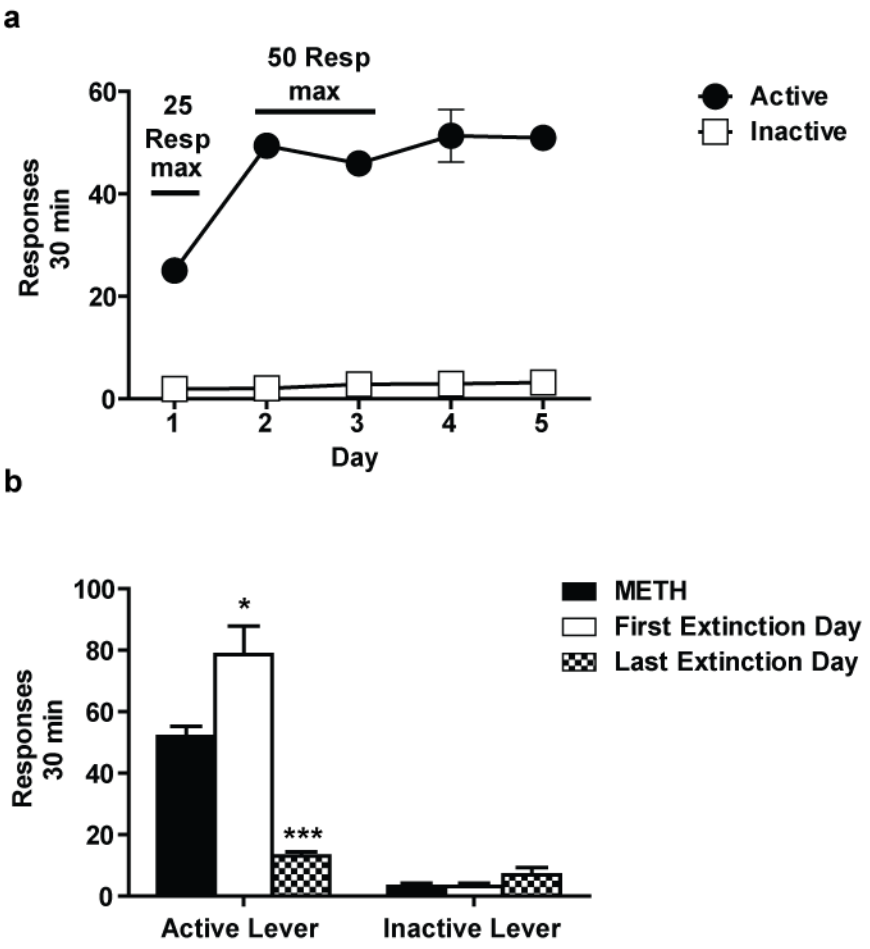


Figure 25. Mean (±SEM) number of responses on the active (black circles) and inactive (white squares) levers during 30 min METH self-administration sessions on an FR-5 schedule (**a**). METH intake was limited on the first three days to 5, 10 and 10 infusions, respectively. Mean (±SEM) number of responses during last 2 days of METH self-administration (black bar), first day of extinction (white bar) and last day of extinction (checked bar) on the active (left) and inactive (right) levers is shown (**b**). Mice showed an increase in active lever responding on the first day of extinction ompared to responding for METH and to responding on the last day of extinction. Active lever responding on the last day of extinction was also signficantly lower than active responding on the first day of extinction. * $p < 0.05$ vs. Active lever responding for METH (black bar), *** $p < 0.001$ vs. Active lever responding for METH (black bar) and First Extinction Day (white bar).

were significant (p = 0.126). However, a main effect of Lever indicated that responding on the active lever remained higher than on the inactive lever ($F_{1,15}$ = 45.50, p < 0.0005). Following a 1.0 mg/kg METH-prime there were significant main effects of Day ($F_{2,30}$ = 14.06, p < 0.0005) and Lever ($F_{1,15}$ = 63.86, p < 0.0005) and a significant Day x Lever interaction ($F_{2,30}$ = 5.76, p = 0.008; Fig. 26b). Follow up analyses indicated active lever responses decreased following a 1.0 mg/kg METH prime compared to baseline (p = 0.002) and the day following the reinstatement day (p = 0.003). In addition, mice responded less on the inactive lever on the reinstatement day compared to baseline (p = 0.001) and the day following reinstatement (p = 0.007). Neither active lever responding following 0.25 nor 1.0 mg/kg METH-primed reinstatement was significantly correlated with total or mean intake of food or METH (data not shown).

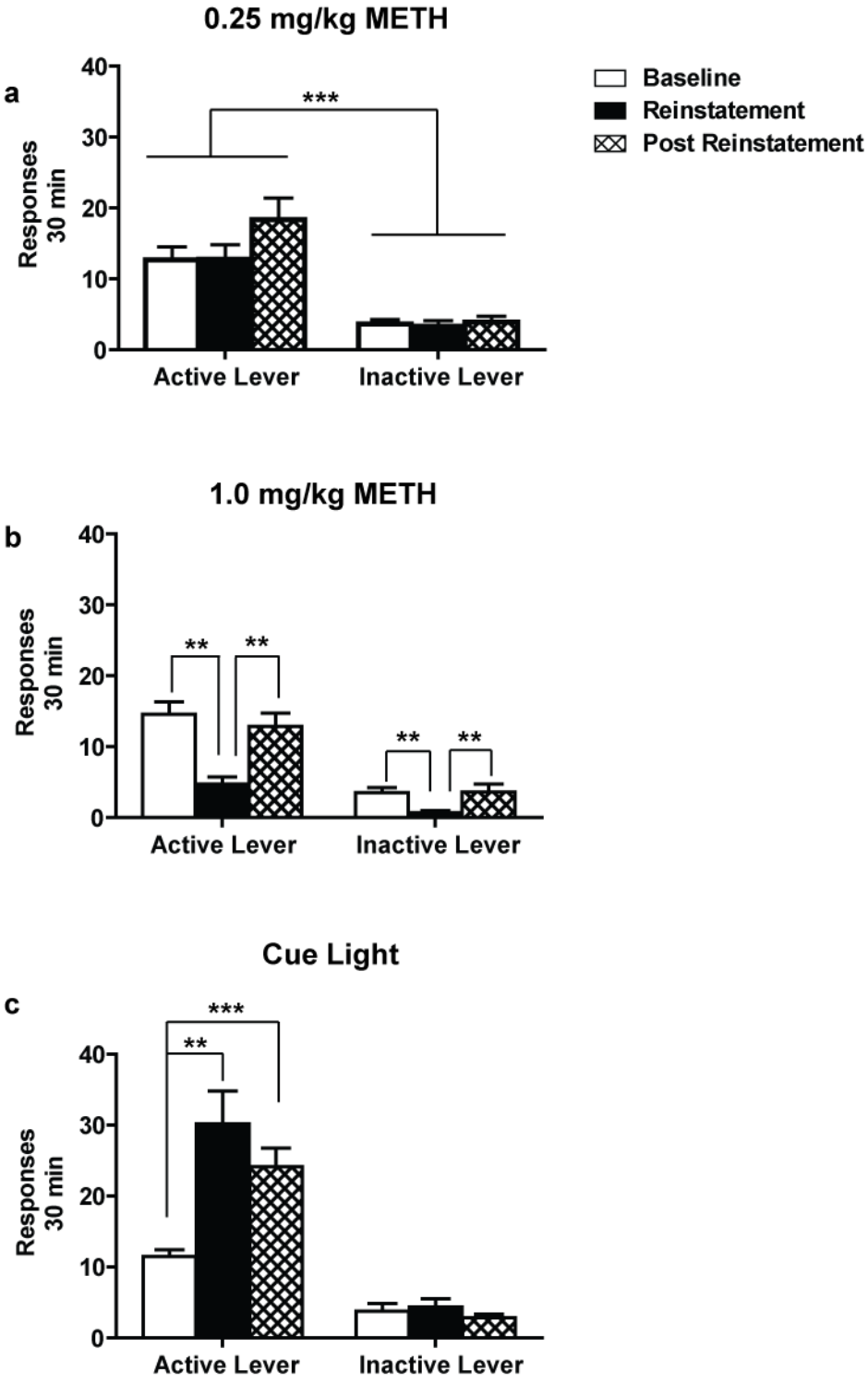
Cue-Induced Reinstatement

Cue light presentation significantly increased active lever pressing on the day of reinstatement and the following day (Fig. 26c). Analysis of cue-induced reinstatement indicated significant main effects of Day ($F_{1.5, 21.9}$ = 9.96, p = 0.002) and Lever ($F_{1,15}$ = 71.54, p < 0.005) and a significant Day x Lever interaction ($F_{2,30}$ = 13.85, p < 0.0005). Mice increased active lever pressing on the reinstatement day compared to baseline (p = 0.001), which perseverated to the next day (p < 0.0005). There was no change in inactive lever responding. Cue-induced active lever responding was not correlated with any of the other variables (data not shown).

There was no difference between baseline levels of responding before each reinstatement test (Lever x Day interaction: p = 0.305; main effect of Day: p = 0.456*)*; however, mice continued to discriminate between active and inactive levers ($F_{1,15}$ = 98.05, p < 0.0005).

Figure 26. Mean (±SEM) responding on the active (left) and inactive (right) levers is shown for the baseline day before reinstatement (white bar), the day of reinstatement (black bar) and the day after reinstatement (checked bar). 0.25 mg/kg METH prime did not affect active or inactive lever responding compared to baseline day or the day following reinstatement (**a**). 1.0 mg/kg METH prime inhibited responding compared to the baseline and day following reinstatement on the active and inactive levers (**b**). Presentation of the cue light significantly increased active lever responding compared to baseline, and this increase persisted to the day following the reinstatement test (**c**). ** $p < 0.01$; *** $p < 0.001$

Figure 26


0.25 mg/kg METH
a
Responses 30 min
40
30
20
10
0
***
Active Lever
Inactive Lever
Baseline
Reinstatement
Post Reinstatement
1.0 mg/kg METH
b
Responses 30 min
**
**
**
**
Active Lever
Inactive Lever
Cue Light
c
Responses 30 min
***
**
Active Lever
Inactive Lever

DISCUSSION

Consistent with previous studies using C57BL/6J mice, the current results suggest that cues previously presented during food and METH self-administration exert strong and persistent control over behavior (Yan et al. 2006; Yan et al. 2007a). In addition, not only did a METH-prime fail to reinstate lever pressing, the high dose actually inhibited operant responding. Several theories on reinstatement exist (de Wit 1996; Shaham et al. 2003) and previous studies in C57BL/6J and 129X1/SvJ mice have reported that IP cocaine prime also fails to reinstate drug-seeking behavior (Fuchs et al. 2003; Highfield et al. 2002).

One explanation for why 1.0 mg/kg METH did not reinstate drug-seeking is that animals were drug-satiated and therefore had no reason to drug-seek. However, by this theory, a non-satiating exposure to an unconditioned stimulus (in this study, the 0.25 mg/kg METH-prime) should have produced an increase in behaviors (i.e., drug seeking) associated with that US (Bindra 1974). It is also possible that 0.25 mg/kg METH was merely too low of a dose to induce reinstatement while 1.0 mg/kg METH was too high and induced excessive motor activation or stereotypic behaviors. A general activation effect of the high METH dose would also explain the decrease in lever pressing observed in the current study.

There were several methodological differences between the current study and the two previous studies, which demonstrated METH-primed reinstatement in mice. In contrast to the previous studies, we inserted additional extinction sessions between reinstatement tests, but did not find METH-primed reinstatement. In addition, the methods used during METH acquisition in the current study were very different compared to those used by Yan and colleagues. Specifically, the previous studies used

a nose poke operant, 3 h test sessions and inactivated the cue and hole lamps upon completion of the operant (Yan et al. 2006; Yan et al. 2007a). These procedural differences may explain why we did not replicate METH-primed reinstatement; however, cue-induced reinstatement was not affected. In addition, multiple consecutive days of low-dose METH exposure may have contributed to the success of the higher METH dose to reinstate active nose poke behavior.

In conclusion, we suggest that a passive IP METH-prime is not a reliable method to reinstate METH-seeking in the C57BL/6J mouse after extinction. In contrast, cue-induced reinstatement seems to be quite robust using a mouse model.

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