The Functional Neurocircuitry of Sign-tracking Behavior

by

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DEDICATION

"To give real service you must add something which cannot be bought or measured with money, and that is sincerity and integrity." -Douglas Adams

"The best way to find yourself is to lose yourself in service to others." -Mahatma Ghandi

I dedicate this dissertation to my family and friends who have supported me during my tenure in the neuroscience graduate program at the University of Michigan. To my parents, James and Linda Fitzpatrick, and my brother and sister-in-law, Jason and Marilyn Fitzpatrick, thank you for your continuous, unconditional love and support both before and during graduate school. Thank you for teaching me what it means to be ethical, have integrity, and give to others. These qualities have allowed me to prosper and engage in genuine, reciprocal relationships in and out of the workplace. To my niece, Jacquelyn, thank you for inspiring me to be the best uncle possible and a positive role model. To my cohort members—Chelsea Cummiford, Sivapratha Nagappan, Caitlin Rodriguez, James Roach, Natalie Navarez, Jonte Jones, Nicholas Silva, and Veronica Rios—thank you for being a part of my life and making graduate school a truly enjoyable and memorable experience. To Kat Miles, thank you for being my best friend: your friendship during graduate school has truly been an unforeseen joy in my life.

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"What you want in a mentor is someone who truly cares for you and who will look after your interests and not just their own. When you do come across the right person to mentor you, start by showing them that the time they spend with you is worthwhile." – Vivek Wadhwa

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LIST OF ABBREVIATIONS

3-MT	3-methoxytryptamine
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxyltryptamine, serotonin
ANOVA	analysis of variance
AP	anterior-posterior
BDNF	brain-derived neurotrophic factor
CNQX	cyanquixaline (6-cyano-7-nitroquinoxaline-2,3-dione)
CR	conditioned response
CS	conditioned stimulus
DA	dopamine
dHPC	dorsal hippocampus
DoD	Department of Defense
DOPAC	3,4-dihydrophenylacetic acid
DV	dorsal-ventral
FR	fixed ratio
GABA	γ-aminobutyric acid
Gln	glutamine
Glu	glutamate
Gly	glycine
GT	goal-tracker
^{1}H-MRS	hydrogen magnetic resonance spectroscopy
HPC	hippocampus
HPLC	high-pressure liquid chromatography
HSD	honest significant difference
HVA	homovanillic acid
Ins	*myo*-inositol
IR	intermediate-responder
LSD	least significant difference
ML	medial-lateral
mPFC	medial prefrontal cortex
NAA	N-acetylaspartate
NAc	nucleus accumbens
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline
NDSEG	National Defense Science and Engineering
NE	norepinephrine
NIDA	National Institute of Drug Abuse
NMDA	N-methyl-D-aspartate

NTS	nucleus tractus solitarius
PCA	Pavlovian conditioned approach
PFA	paraformaldehyde
PSB	Pontamine Sky Blue
PTSD	posttraumatic stress disorder
SPS	single prolonged stress
ST	sign-tracker
US	unconditioned stimulus
vHPC	ventral hippocampus
vSUB	ventral subiculum
VT	variable time
VTA	ventral tegmental area

ABSTRACT

Cues that are paired with unconditioned, rewarding stimuli can acquire rewarding properties themselves through a process known as the attribution of incentive salience. When previously neutral cues are imbued with incentive salience, they become attractive, "wanted" stimuli capable of motivating behavior. Pavlovian conditioned approach (PCA) procedures are commonly used to investigate the attribution of incentive salience in rodents. During PCA training, rats are presented with a lever (conditioned stimulus; CS) followed response-independently by food delivery (unconditioned stimulus; US), and three patterns of conditioned responses (CRs) develop: sign-tracking (CS-directed CR), goal-tracking (US-directed CR), or an intermediate response (both CRs). Goal-trackers (GTs) and sign-trackers (STs) both use the reward-related cue as a predictor of reward delivery, but only in STs are reward-related cues attributed with incentive salience, making STs more vulnerable to addiction-like behaviors, such as cue-induced reinstatement of drug-seeking. Currently, it is known that sign-tracking behavior is dopamine (DA)-dependent in the nucleus accumbens (NAc), a central hub in the 'motive circuit,' an array of brain regions that processes incentive stimuli and includes the medial prefrontal cortex, ventral tegmental area, amygdala, sensory cortices, and thalamic relays. However, the role of other signaling pathways and the contribution of afferent brain regions within the motive circuit to sign-tracking behavior is still poorly understood.

In Chapter II, I investigated whether GTs, intermediate-responders (IRs), and STs differ in baseline levels of 19 neurochemicals measured in the NAc and two regions known to

modulate its activity: the medial prefrontal cortex (mPFC) and hippocampus (HPC). Hydrogen magnetic resonance spectroscopy was used to quantify neurochemicals involved in energy metabolism, neurotransmission, membrane synthesis, and osmosis. I demonstrated that basal levels of *myo*-inositol (Ins)—a marker of astrocyte activity and an important precursor molecule in signal transduction pathways—are elevated in the ventral HPC and NAc of STs compared to GTs and IRs, and levels of Ins correlate with sign-tracking behavior. Moreover, levels of Ins in the mPFC and ventral HPC positively correlated with PCA behavior (i.e., increased sign-tracking behavior was correlated with increased Ins levels).

In Chapter III, I investigated the role of the HPC and its subdivisions in regulating sign-tracking behavior and DA transmission in the NAc. I demonstrated that permanent lesions of the ventral, but not dorsal or total, HPC decrease the acquisition, but not expression, of sign-tracking behavior. In addition, ventral HPC lesions decrease DA turnover in the NAc.

In Chapter IV, I investigated how an environmental stressor affects sign-tracking behavior and DA transmission within the NAc. I used single prolonged stress (SPS), a prolonged stressor that affects the motive circuit, as the environmental stressor. I demonstrated that SPS decreases the acquisition, but not expression, of sign-tracking behavior. In addition, using *in vivo* microdialysis, I showed that SPS decreases potassium-evoked DA release in the NAc, which may underlie SPS-induced decreases in sign-tracking behavior.

In Chapter V, I investigated how novel pharmacological interventions targeting the motive circuit affect sign-tracking behavior. I used subanesthetic ketamine as a pharmacological intervention, because it has been shown to activate top-down inhibitory control pathways within the motive circuit and reduce cue-induced craving in addicted patients. I demonstrated that a

single dose of subanesthetic ketamine causes a long-term reduction in the expression of sign-tracking behavior.

In summary, the results from this thesis (1) identifies Ins in the NAc and ventral HPC as a potential biomarker of sign-tracking behavior, (2) expands the motive circuit underlying sign-tracking behavior to include the ventral HPC, which can modulate DA turnover in the NAc, (3) demonstrates that a prolonged stressor can decrease sign-tracking behavior by decreasing DA release in the NAc, and (4) shows that subanesthetic ketamine, which activates top-down inhibitory control pathways in the motive circuit, decreases sign-tracking behavior.

CHAPTER I

Introduction

Note: Some of the text has appeared previously in the Journal of Visualized Experiments (Fitzpatrick and Morrow, 2016) and is used with the permission of the publisher, Wiley-Blackwell.

Addiction is a debilitating neuropsychiatric disorder that has a lifetime prevalence of 26.6% in the United States population (Kessler et al., 1994). Chronic tobacco and alcohol consumption are responsible for 16.6% and 3.5% of deaths in the United States, respectively, and are two of the leading causes of mortality (Mokdad et al., 2004). Moreover, the total economic burden of addiction[1] is $740 billion annually in costs related to crime, lost work productivity, and healthcare costs (National Institute on Drug Abuse, 2017). In addition, the recent opioid epidemic in the United States has been responsible for the majority of deaths caused by drug overdose (Rudd et al., 2016), rising rates of maternal morbidity and mortality (Maeda et al., 2014) as well as drug-affected births (Admon et al., 2018) and an increased estimated economic burden of $78.5 billion (Florence et al., 2016).

Currently, little is known why some individuals can try potentially addictive drugs without developing addiction, while others try the same drug and are quickly rendered incapable of controlling their urges to repeat the experience despite adverse consequences. Individual

[1] This statistic refers to tobacco, alcohol, illicit drugs, and prescription opioids.

variation in the incentive-motivational value of reward-related cues is believed to contribute to this individual vulnerability to addiction (Flagel et al., 2009). The attribution of incentive salience is typically measured in both model organisms and humans using a Pavlovian conditioned approach (PCA) procedure. In the most commonly used procedure, a conditioned stimulus (CS; e.g., a lever) response-independently predicts the delivery of an unconditioned stimulus (US; e.g., food). During training, three behavioral phenotypes may emerge: sign-trackers (STs), which express primarily CS-directed conditioned responses (CRs); goal-trackers (GTs), which develop US-directed CRs toward the location of reward delivery; and intermediate-responders (IRs), which display both CRs. Previously, it has been demonstrated that STs are more susceptible to cue-related, addiction-like behaviors than GTs or IRs, exhibiting increased cue-induced reinstatement of drug-seeking behavior (Saunders & Robinson, 2010; Yager et al., 2015) and even seeking drug cues despite adverse consequences (Saunders et al., 2013)[2]. Over the years, PCA procedures have been used to further our understanding of reinforcement learning and the pharmacological signaling pathways that underlie individual variation in the attribution of incentive-motivational value to reward-related cues. Chapter I discusses the history of and neurobiology underlying PCA procedures. In addition, it establishes the background of and summarizes the experiments in this thesis, which investigate the functional neurocircuitry of sign-tracking behavior.

A brief history of the Pavlovian conditioned approach procedure and sign-tracking behavior

[2] These observations have been observed following short-access schedules of cocaine self-administration. Following a prolonged, intermediate-access schedule of cocaine self-administration, STs and GTs do not differ on measures of addiction-like behavior, such as increased cocaine demand (a behavioral economics measure) and cue-induced reinstatement of cocaine-seeking behavior (Kawa et al., 2016). Therefore, STs may only show vulnerability to addiction-like behaviors in some situations, but not others.

"The fundamental phenomenon which I find presented in animal consciousness is one which can harden into inherited connections and reflexes, on the one hand, and thus connect naturally with a host of the phenomenon in animal life; on the other hand, it emphasizes the fact that our mental life has grown up as a mediation between stimulus and reaction" (Thorndike, 1898). This concept, known as the Law of Effect[3] became the basis for Burrhus Frederic (B.F.) Skinner's operant conditioning and reinforcement[4] learning theories in the 1930s and 1940s. Over the decades, reinforcement learning has been the subject of many theoretical debates, which have highlighted the strengths, limitations, and even the very nature of reinforcement itself. At the core of reinforcement learning is that (1) organisms will naturally approach a biologically relevant stimulus (US; i.e., a reinforcer/reward) and (2) other stimuli associated with the biologically relevant stimulus (a CS; i.e., an interoceptive or environmental cue) can serve as predictors of reinforcement, originally noted by Pavlov (1955) as orienting responses during cue presentation. The nature of "attraction" towards reward-related cues, was heatedly debated during the early to middle 20th century among prominent behaviorists, such as Thorndike (1911), Hull (1943), and Schnierla (1959).

One challenge to reinforcement learning theory was first described by B.F. Skinner (1948). In the experiment, a timer was attached to a solenoid and food hopper, which contained grain and was accessible at regular intervals. During these intervals, Skinner noticed that each pigeon performed 'superstitious' behaviors during the inter-trial intervals, such as turning counter-clockwise or head-tossing "as if placing its head between an invisible bar and lifting it repeatedly" (p. 168). Many of these experiments were performed by Marian and Keller Breland,

[3] The Law of Effect can be summarized as: a satisfactory event following a behavioral response to a stimulus results in stimulus-response associations and learning.
[4] Reinforcement is the strengthening of a behavior when it is preceded by an antecedent stimulus, or environmental cue that signals an organism should behave in a manner that maximizes positive consequences (reinforcement) and minimizes negative consequences (punishment).

Skinner's first graduate students who continued studying reinforcement learning as animal trainers for zoos, fairs, and television commercials. From their farm in Hot Springs (Arkansas, USA), they were instrumental in commercializing concepts of operant conditioning and are considered the first applied animal psychologists (Breland & Breland, 1951). In their seminal publication, "The Misbehavior of Organisms" (1961), they documented many of their challenges using reinforcement learning in animals and described them as "a clear and utter failure of conditioning theory" (p. 683). For instance, pigs were trained to deposit a coin into a container for a reward, but over the course of training every pig eventually stopped receiving rewards, because they instead dropped the coins on the ground and rooted. Unlike the experiment in Skinner's laboratory, which involved unconditioned reinforcement, the operant conditioning used on the farm resulted in unwanted conditioned responding toward reward-related cues.

This behavior had been first described by Zener (1937) when he expanded upon Pavlov's initial experiments by investigating CRs directed at the US as well as those directed at the CS. It was not until after the publication of the "Misbehavior of Organisms" (1961), however, that the behavior was officially named. Brown & Jenkins (1968) discovered during Pavlovian conditioning that pigeons would reliably move towards and peck illuminated keys that response-independently predicted the delivery of grain. In the experiment, pigeons underwent two sessions of Pavlovian conditioning (160 trials), and every pigeon pecked at the illuminated key at least once during the 160 trials. Initially, Brown & Jenkins described the key-peck response as a superstitious behavior, which developed because it occurred incidentally before reinforcement. Unlike the superstitious conditioning performed by Skinner, which involved unconditioned reinforcement, they defined superstitious conditioning as one during which the reinforcement is conditional on stimulus values, but not stimulus responses, and promotes unconditioned

associations among stimuli, responses, and reinforcements. In other words, pigeons peck illuminated keys, because they believe that the response might result in access to the grain. Brown & Jenkins decided to name this behavior 'autoshaping,' because it could replace the manual hand shaping that experimenters previously used to establish conditioned responding. Later, Hearst & Jenkins (1974)[5] advocated the more descriptive term, 'sign-tracking,' for the cue-directed behavior observed during Pavlovian conditioning; however, it wasn't until the 1990s and 2000s that this term was widely adopted. Also, around this time, 'goal-tracking' (i.e., approach and interaction with the location of reward delivery) was demonstrated using similar appetitive Pavlovian conditioning procedures with rats (Boakes, 1977).

During the 1970s, autoshaping was widely adopted and studied in pigeons (Franklin & Hearst, 1977; Wasserman et al., 1974) and applied to other organisms[6], leading to significant advances in reinforcement learning theory. Based on autoshaping procedures, contemporary theories were created (e.g., the theory that operant conditioning is actually Pavlovian in nature; Moore, 1973), disproven (e.g., the theory that autoshaping is superstitious; Gamzu and Schwam, 1974; Williams and Williams, 1969; Woodard et al., 1974), or reupdated (e.g., behavioral contrast theory; Gormley, 1976; Morris, 1976). It was even suggested that autoshaping itself was the basis of a new theory involving the "incentive-motivational values of situational stimuli" (Lajoie & Bindra, 1976). All these advances were elegantly described by Locurto and colleagues (1980), which Perkins (1982) reviewed and wrote:

> *Even a quick reading of the book indicated that the contributions of autoshaping are no longer primarily negative. Investigators are not just interested in showing that older conceptual frameworks […] are inadequate. The emphasis is on*

[5] It should be noted that the experiments in Brown & Jenkins (1968) involved aspects of operant conditioning more relevant to shaping procedures (i.e., the lever retracted and immediately resulted in food pellet delivery if the rat contacted it). On the other hand, the experiments in Hearst & Jenkins (1974) was a purely Pavlovian procedure (i.e., the lever did not retract and immediately result in food pellet delivery if the rat contacted it).

[6] See Appendix A for a discussion on the evolution of sign-tracking and Table S1.1 for a list of species that have exhibited sign-tracking behavior.

> *constructing approaches that will provide a better framework from which performance can be viewed systematically.* (p. 180)

Individual variation in the attribution of incentive salience

In the 1990s, Robinson & Berridge (1993) published their incentive-sensitization theory of addiction. Seeking to address shortcomings of previous theories of addiction, such as negative reinforcement (addiction is driven purely by withdrawal and/or tolerance) and positive reinforcement (addiction is driven purely by pleasure and liking a drug), which did not fully address the underlying neurobiology of craving, relapse, and addiction pathophysiology. According to the incentive-sensitization theory of addiction: (1) addictive drugs share a common neural system, (2) repeated administration of addictive drugs gradually hypersensitize this system, (3) sensitization-induced neuroadaptations are enduring, (4) sensitization-related neuroadaptations are amenable to CS control, (5) the mesolimbic dopamine (DA) system mediates incentive motivation, and finally (6) DA mediates incentive salience, not pleasure. In summary, repeated use of addictive drugs rapidly and enduringly sensitizes the mesolimbic DA system, which promotes the transition to addiction through DA-mediated attribution of incentive salience to drug-related cues ('wanting'), not DA-mediated seeking of pleasure ('liking').

Since Robinson & Berridge (1993) proposed the incentive-sensitization theory, autoshaping procedures (now referred to in the literature as PCA procedures) have been utilized to investigate incentive salience, particularly individual variation in the attribution of incentive salience. During a PCA procedure, a CS (e.g., a lever) is response-independently presented during a training session and predicts the delivery of a US (e.g., a food pellet). Over the course of multiple training sessions, three CRs develop (Figure 1.1): sign-tracking (CS-directed CR), goal-tracking (US-directed CR), and an intermediate-response (both CRs). Following the last PCA

training session, rats are divided into PCA phenotypes based on a PCA index score, which combines the number, latency, and probability of lever presses (sign-tracking) and magazine entries (goal-tracking) in a PCA training session (Meyer et al., 2012)[7]. GTs and STs both use the reward-related cue as a predictor of impending reward delivery; however, only in STs is the reward-related cue attributed with incentive-motivational. Meanwhile, IRs vacillate between attraction to the reward-related cue and the location of reward delivery. For STs, the CS is attributed with incentive salience, becoming attractive and desirable in and of itself as a 'motivational magnet.'

One of the many benefits of PCA procedures is that they experimentally isolate the incentive-motivational value from the predictive value of Pavlovian stimuli. Many procedures investigating reward in rodents do not permit the disentanglement of predictive versus incentive-motivational learning. In drug self-administration procedures, for example, both operant and Pavlovian contingencies are typically employed, such that rats learn to perform an action (e.g., nose pokes, lever presses, etc.) to receive an outcome (i.e., intravenous drug infusion). Yet, the rewarding outcome is also paired with Pavlovian stimuli (e.g., illumination of the nose-poke port, presentation of a cue light, etc.). It is often unclear in these procedures whether the cue is supporting goal-directed actions simply because of its predictive relationship with the reward, or whether the cue has acquired incentive-motivational properties of its own.

Other benefits of PCA procedures are the (1) *a priori* selection of differences in a population and (2) study of individual variation in *behavior*. Historically, individual variation has been studied through bred lines (Flagel et al., 2010; Mabrouk et al., 2018) or dividing a

[7] Generally, PCA index scores are averaged over the last two PCA training sessions, and PCA phenotypes are determined with the following cutoffs: GTs ($x \leq -0.5$), IRs ($-0.5 < x < 0.5$), and STs ($x \geq 0.5$). On the index, -1.0 represents absolute goal-tracking whereas +1.0 represents absolute sign-tracking

population of outbred animals using deviation from the mean (Lehner et al., 2008), terciles (Rada et al., 2010) or a median-split (Nelson et al., 2009). Each of these procedures, however, has inherent drawbacks, such as post hoc identification of behavioral differences or complications with turning a continuous variable into a categorical one (i.e., a median split procedure does not adequately separate two populations). Moreover, individual variation in behavior has been historically investigated as consequences of genes and environment, yet little attention has been given to individual variation in behavior because of learning principles and strategies, which is fundamental to the understanding of individual differences and behavior (Byrom & Murphy, 2018). This is a crucial point, because it directly contrasts the general process learning theory, which was established over the course of the 20th century, remains a fundamental theory in modern research, and posits that all learning phenomenon are generalizable irrespective of the organism, situation, stimuli, or response (Bower & Hilgard, 1981).

Pavlovian conditioned approach procedures as a translational tool to study addiction

Early studies using PCA procedures were critical in investigating individual variation in the attribution of incentive salience and supporting criteria of the incentive-sensitization theory of addiction. Flagel et al. (2011b) demonstrated that DA in the nucleus accumbens (NAc) core mediates sign-tracking, but not goal-tracking, behavior (i.e., NAc DA signals incentive-motivational but not predictive value). Later, it was demonstrated that sign-tracking behavior is DA-dependent[8] in the NAc core (Fraser & Janak, 2017; Saunders & Robinson, 2012; Saunders et al., 2013) and leads to different adaptations in the mesolimbic DA system than goal-tracking behavior (Flagel et al., 2007; Singer et al., 2016). Goal-tracking requires dopaminergic

[8] After extended training, sign-tracking becomes DA-independent (Clark et al., 2013).

neurotransmission outside the NAc core, but the exact node or pathway within the mesocorticolimbic DA system has not yet been determined (Chow et al., 2016; Fraser et al., 2016).

As previously mentioned, the attribution of incentive-motivational value to drug-related cues is believed to underlie craving and relapse in addiction (Robinson & Berridge, 1993). In support of this, reactivity to drug-related cues before and during treatment has been consistently associated with craving and relapse (Childress et al., 1993; Garland et al., 2012; Janes et al., 2010; Papachristou et al., 2014; Witteman et al., 2015). In animal models, cue-induced reinstatement of drug-seeking behavior is considered the gold standard of modeling the neurobiology of relapse in rodents (Knackstedt & Kalivas, 2009; Robinson et al., 2014). Using a combination of PCA and drug reinstatement procedures, it has been demonstrated that STs have increased cue-induced reinstatement following self-administration of ethanol (Cunningham & Patel, 2007; Krank et al., 2008; Villaruel & Chaudhri, 2016), cocaine (Saunders &Robinson, 2010; Yager & Robinson, 2013), nicotine (Yager & Robinson, 2015), heroin (Peters & De Vries, 2014), and remifentanil, a short-acting opioid (Yager et al., 2015).

Moreover, a history of drug exposure (developmentally or acutely) increases sign-tracking behavior, which is predicted by the incentive-sensitization theory (McClory & Spear, 2014; Overby et al., 2018; Palmatier et al., 2014; Palmatier et al., 2013; Spoelder et al., 2015; Versaggi et al., 2016)[9]. In addition, STs exhibit a variety of traits observed in patients suffering from addiction, such as impulsivity (Lovic et al., 2011), increased sensitization (Flagel et al., 2008), impaired Pavlovian extinction (Beckmann & Chow, 2015), cocaine-seeking behavior (Kawa et al., 2016), drug-induced 'relapse' (Saunders & Robinson, 2011), and preference of

[9] Paradoxically, psychostimulants, hypothesized to increase sign-tracking behavior, decrease sign-tracking behavior, most likely by disrupting cue-induced DA signaling in the NAc (Holden & Peoples, 2010; Schuweiler et al., 2018; Simon et al., 2009).

cocaine over natural rewards (Tunstall & Kearns, 2015)[10]. Importantly, sign-tracking has been demonstrated in humans (Joyner et al., 2018; Kimura et al., 1990; Versace et al., 2016; Wilcove & Miller, 1974), and currents studies in our laboratory and others are optimizing the procedure to investigate individual variation in the attribution of incentive salience in addicted patients.

The motive circuit: Neuroanatomy of sign-tracking behavior

The "motive circuit" is an array of cortical and subcortical brain regions that regulates motivated behaviors and reward processing of incentive stimuli (Figure 1.2; Baker et al., 2002). In addition, it includes the all of the cortical and thalamic brain regions underlying sensory processing and integration (i.e., coding visual, olfactory, auditory, tactile, and interoceptive signals with affective valence; Haber, 2011). It overlaps greatly with the mesocorticolimbic DA system, and includes signaling pathways (e.g., DA, glutamate, γ-aminobutyric acid [GABA]) and connections between the prefrontal cortex (PFC), ventral tegmental area (VTA), NAc, basolateral amygdala, and thalamic relays between the cortex and the limbic system or brainstem[11]. Immunohistochemistry and in situ hybridization studies of immediate-early genes (e.g., c-Fos) as markers of neural activity have identified that the motive circuit mediates the acquisition and expression of sign-tracking behavior in STs[12] (Figure 1.3; Flagel et al., 2011a; Haight et al., 2017; Yager et al., 2015). Findings from these mapping studies have also been supported by lesion studies (see Table S1.2), disconnection studies (see Table S1.3), and

[10] It has also been demonstrated that STs exhibit increased posttraumatic stress disorder (PTSD)-like behaviors in rodents (Morrow & Flagel, 2016; Morrow et al., 2011; Morrow et al., 2015), demonstrating that individual variation in the attribution of incentive-motivational value can manifest from both positively and negatively valenced cues and suggesting that sign-tracking may underlie vulnerability to PTSD and comorbid PTSD/addiction.

[11] The basolateral amygdala and thalamic relays are believed to permit access of sensory information from the CS to mesolimbic DA neurons originating from the VTA and terminating in the NAc (Ono et al., 1995).

[12] Due to the complexity of isolating goal-tracking behavior without turning the location of reward delivery into a CS, it is currently unknown what neural circuitry underlies goal-tracking in GTs. Based on previous studies of appetitive Pavlovian conditioning procedures, however, it is likely that goal-tracking behavior is regulated by a circuit including the mPFC, dorsal striatum, amygdala, and hypothalamus (Cole et al., 2015; Keefer & Petrovich, 2017).

electrophysiological studies (Ahrens et al., 2016; Stringfield et al., 2017). Within this circuit, it has been demonstrated that sign-tracking is modulated by other neurotransmitter systems (e.g., glutamatergic, cannabinoid, cholinergic, adrenergic, opioidergic, etc.) outside DA signaling (Bacharach et al., 2018; Chow & Beckmann, 2018; DiFeliceantonio and Berridge, 2012; Pasquariello et al., 2018).

The motive circuit and ventral hippocampus: Regulation of accumbal dopamine activity

It is now fully understood that the dorsal hippocampus (dHPC) and ventral hippocampus (vHPC) are functionally unique structures (Fanselow & Dong, 2010) with the former regulating space and context and the latter regulating stress and reward (O'Mara et al., 2009). Historically, the vHPC has been viewed as modulating contextual information in the environment during reward-seeking behavior (Bossert et al., 2016; Burhans & Gabriel, 2007; Komorowski et al., 2013; Lasseter et al., 2010; Luo et al., 2011). Recently, however, it has been proposed that the vHPC is part of a 'ventral emotional network' (i.e., NAc, vHPC, mPFC, and amygdala), which encodes conditioned responding with affective valence (i.e., approach to positively valenced stimuli and avoidance of negatively valenced stimuli; Gruber & McDonald, 2012) and integrates this information with downstream regions in the mesolimbic reward system, such as the NAc and VTA (Floresco et al., 2001; Floresco et al., 2003). Taken together, the vHPC is proposed to link affectively valenced cues and contexts with appropriate modes of behavior, which are translated into behavioral actions by the NAc and mPFC (Behrendt, 2013).

The vHPC influences reward processing via the ventral subiculum (vSUB; the main output structure of the vHPC), which sends efferents to the NAc, VTA and mPFC. The vHPC regulates mesocortiocolimbic DA signaling (Grace, 2010; Grace et al., 2007), and stimulation of

the vHPC increases DA release in the mPFC, VTA, and NAc (Peleg-Raibstein & Feldon, 2006; Taepavarapruk et al., 2008; Zornoza et al., 2005). Behavioral reinforcement and approach to incentive stimuli are the explicit consequence of strong excitatory drive to the NAc (Britt et al., 2012), and glutamatergic inputs from the vHPC to the NAc are one of many. Glutamatergic inputs from the mPFC and BLA convey different types of reward-related information to gate information flow and guide goal-directed behavior (Papp et al., 2012). For example, glutamatergic efferents from the mPFC to the NAc potentiate and selectively tune encoding of reward-related cues (McGlinchey et al., 2016; Otis et al., 2017). Little is known how these neurotransmitter systems signal in the mPFC and NAc to modulate sign-tracking, however, and nothing is known regarding the contribution of the vHPC to sign-tracking behavior or DA signaling in STs.

Summary of present experiments

It is evident from the literature that DA signaling in the NAc and connections between the NAc and other brain regions are critical for the acquisition and expression of sign-tracking behavior; however, several questions remain unanswered: (1) What other neurochemicals in the motive circuit besides DA in the NAc underlie sign-tracking behavior? (2) What role does the vHPC have in the motive circuit and does it regulate sign-tracking behavior and DA signaling in STs? (3) How do environmental stressors and pharmacological interventions known to affect the vHPC and NAc influence sign-tracking behavior? In this thesis, I investigate these questions through four aims. First, I examine basal neurochemical differences within multiple regions of the motive circuit (mPFC, NAc, and HPC) in STs using proton magnetic resonance imaging (^{1}H-MRS). Second, I investigate the vHPC as a component of the motive circuit that regulates sign-

tracking behavior as well as DA signaling in the NAc. Third, I explore how an environmental stressor (single prolonged stress; SPS), relevant to addiction pathophysiology with known effects on the motive circuit, affects sign-tracking behavior and DA release in the NAc. Finally, I investigate whether a subanesthetic dose of ketamine, known to activate top-down inhibitory control within the motive circuit and reduce craving/relapse in humans, reduces the attribution of incentive-motivational value to reward cues in STs.

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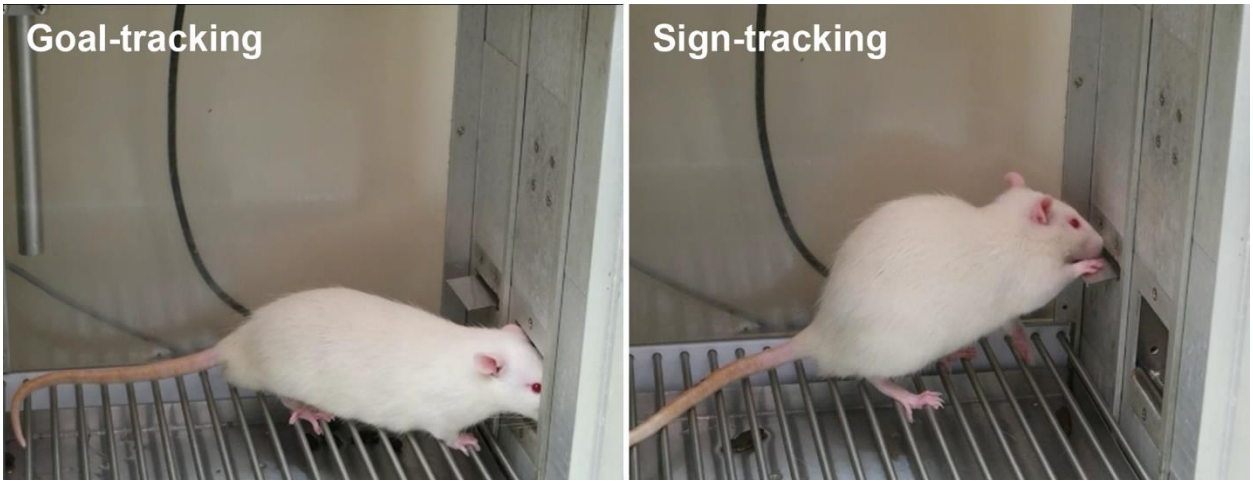

Figure 1.1. Photographic representation of goal- and sign-tracking behaviors. Goal-tracking behavior is characterized by conditioned responding to the location of reward delivery (i.e., the pellet magazine). On the other hand, sign-tracking behavior is characterized by conditioned responding to the reward-related cue (i.e., the lever). Both sign- and goal-trackers use the lever as a predictor of reward; however, only sign-trackers attribute the lever with incentive salience, imbuing it with motivational properties.

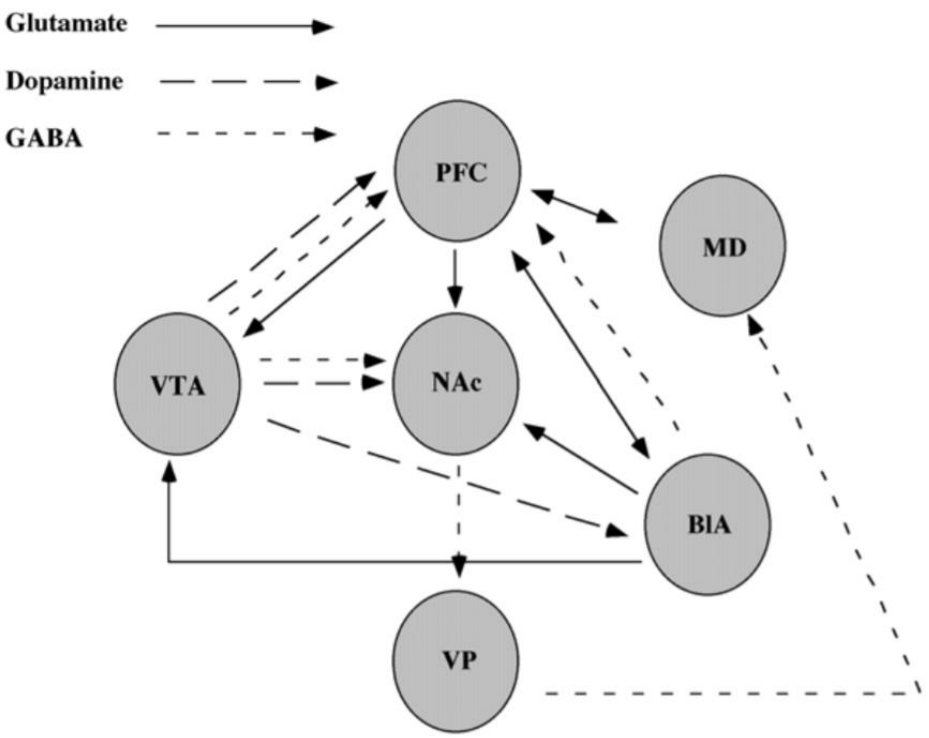

Figure 1.2. Schematic of the 'motive circuit' that regulates motivated behaviors and processing of incentive stimuli. BLA – basolateral amygdala, MD – mediodorsal nucleus of the thalamus, NAc – nucleus accumbens, PFC – prefrontal cortex, VP – ventral pallidum, VTA – ventral tegmental area. *Image adapted from Baker et al. (2002).*

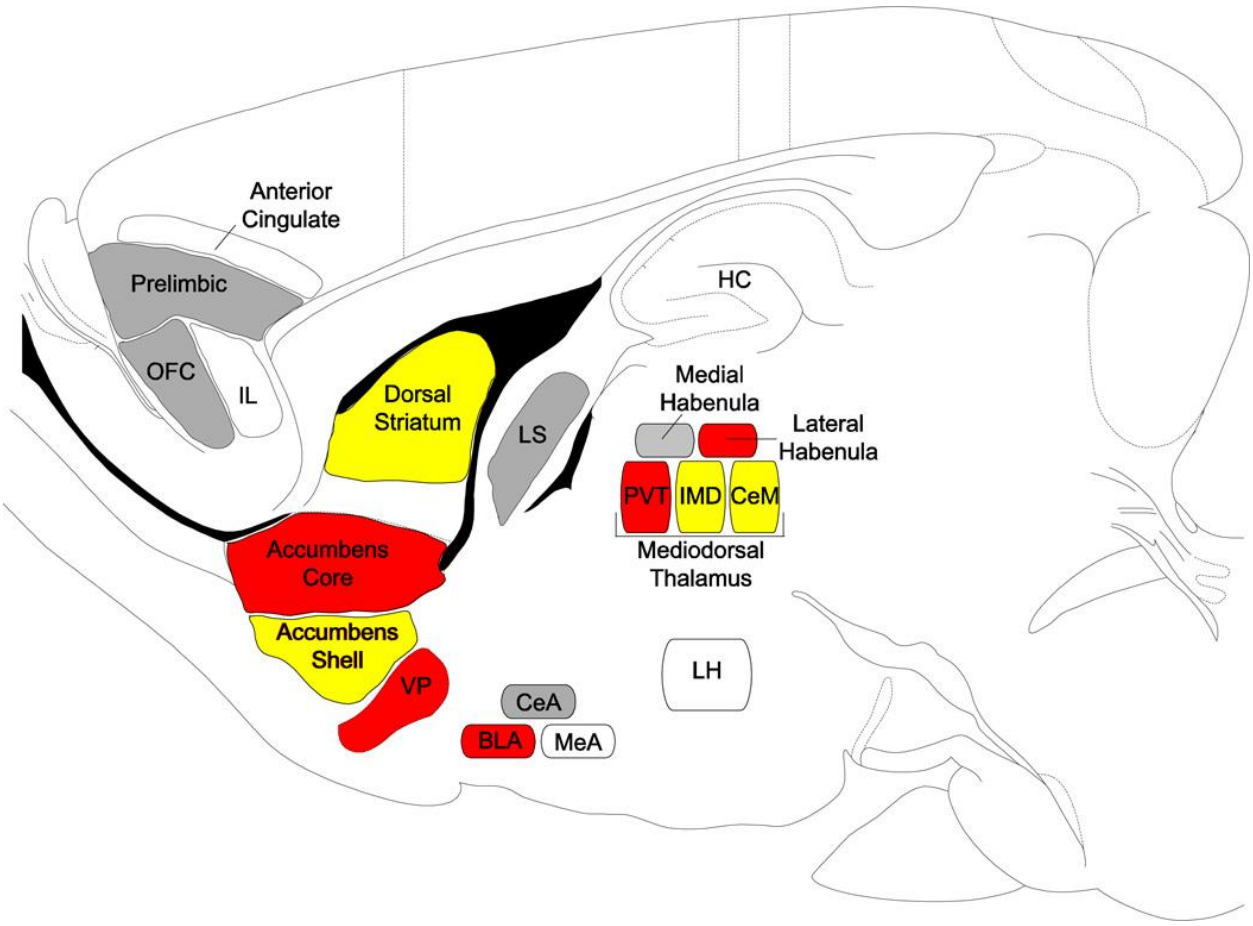

Figure 1.3. Schematic of the brain regions involved in the attribution of incentive salience to reward-related cues. Grey – brain regions with increased neural activity to either food- or drug-related cues, yellow – brain regions with increased neural activity to both food- and drug-related cues, red – brain regions with increase neural activity to both food- and drug-related cues that have been validated using additional techniques (e.g., chemogenetics, lesions, etc.). BLA – basolateral nucleus of the amygdala, CeA – central nucleus of the amygdala, CeM – centromedial nucleus of the thalamus, HC – hippocampus, IL – infralimbic cortex, IMD – interomediodorsal nucleus of the thalamus, LH – lateral hypothalamus, LS – lateral septum, MeA – medial nucleus of the amygdala, OFC – orbitofrontal cortex, PVT – paraventricular nucleus of the thalamus, VP – ventral pallidum. *Image adapted from Flagel & Robinson (2017) and Yager et al. (2015).*

Appendix A: : Chapter I Supplemental Information

Evolutionary perspective of sign-tracking:

Breland & Breland (1961) originally noted (in relation to the sign-tracking behavior of their trained animals) that "the behavior of any species cannot adequately be understood, predicted, or controlled without knowledge of its instinctive patterns, evolutionary history, and ecological niche" (p. 684). It is now understood that sign-tracking is an evolutionarily conserved survival mechanism that is intimately tied to foraging and predation (Killeen, 2003). To date it has been demonstrated in 26 species[13]—including birds, amphibians, reptiles, mollusks, and mammals (Table S1.1)—and manifests species-specifically. For example, Breland & Breland (1961) gave wooden coins to raccoons that they had to deliver into a coin bank, and during sign-tracking the racoons would "wash" the coins as they would do a piece of food. In addition, species vary in optimal sign-tracking behavior regarding the distance between the CS and US, which most likely reflects distances from an environmental feature that the organism would naturally find prey or other sources of food (Purdy et al., 1999)[14].

In support of sign-tracking being related to foraging/hunting strategies, the degree of similarity between the CS and US influences the strength and vigor of sign-tracking behavior (Tomie, 1989). Although sign-tracking may reduce feeding *efficiency* in nature, it has been suggested that it is ultimately advantageous in natural environments by increasing feeding

[13] Sign-tracking is the result of convergent evolution, representing selective pressure for a beneficial behavior in organisms that forage or hunt. Because the common ancestor of cuttlefish and mammals (two sign-tracking species) predates the Cambrian explosion, this behavior is evolutionary ancient and has been robustly preserved in organisms that forage or hunt.
[14] Sign-tracking depends on CS modality, and sign-tracking is not present when a nonlocalizable CS response-independently predicts a US during appetitive PCA procedures (Beckmann & Chow, 2015; Meyer et al., 2014; Ploog, 2014).

opportunities through association of a feature in the environment (the CS) with the presence of prey or opportunity to forage (the US; Purdy et al., 1999). In the laboratory, the malleability of sign-tracking in response to conditioning parameters most likely reflects complex behavioral adaptations to the reliability and temporal relationship of cues in predicting the availability of food in a natural environment, such that sign-tracking increases with increasing reward probability or spatial/temporal contiguity of the CS and US (Anselme et al., 2013; Burns & Domjan, 2001; Christie, 1996).

Most sign-tracking species are omnivorous except for horses, which are herbivores. Consequently, many of these species actively predate on other organisms, making sign-tracking behavior either an immediate or speculative hunting response based on cues in the environment surrounding food sources. Indeed, one of the best examples of sign- and goal-tracking behavior was a procedure during which a ball bearing rolled on a track across an apparatus and signaled food presentation (Timberlake, 1983). Sign-tracking rats pounced and contacted the rolling ball as they would insects or small mammals (e.g., mice) in the wild. Notably, sign-tracking is present in species with active foraging/hunting strategies (Atlantic cod), yet absent in species, such as the Atlantic halibut, which have adapted 'sit-and-wait' foraging/hunting strategies that require them to remain motionless and undetected by prey (Nilsson et al., 2008; Nilsson et al., 2010).

Finally, sign-tracking is enduringly and robustly expressed during development and into adulthood. Sign-tracking responses are rapidly expressed early in development in both newborn animals (Starr, 1978) and young children (3-5 years old; Zeiler, 1973). In addition, sign-tracking is acquired and expressed even after neurophysiological insults. For example, neonatally decorticated (Oakley et al., 1981) or microcephalic (Goldstein & Oakley, 1989) rats have equal

to or greater levels of acquisition and expression of sign-tracking behavior as healthy controls. Similarly, sign-tracking is still present in children with severe cognitive impairments (Deckner et al., 1980).

Class	Organism (Species)	References
Actinopterygii	Archer fish (Toxotes chatareus)	(Waxman & McCleave, 1978)
Actinopterygii	Atlantic cod (Gadus morhua)	(Nilsson et al., 2008)
Actinopterygii	Goldfish (Carassius Auratus)	(Bitterman, 1974; Scobie, 1977)
Actinopterygii	Mozambique tilapia (Oreochromis mossambicus)	(Squier, 1969)
Amphibia	American bullfrog (Rana catesbeiana)	(Van Bergeijk, 1967)
Cephalapoda	Cuttlefish (Sepiida officinalis)	(Cole and Adamo, 2005; Purdy et al., 1999)
Chondrichthyes	Port Jackson shark (Heterodontus portusjacksoni)	(Guttridge and Brown, 2014)
Mammalia	Chicken (Gallus gallus domesticus)	(Breland & Breland, 1961; Starr, 1978)
Mammalia	Common rat (Rattus norvegicus)	(Locurto, 1981)
Mammalia	Common squirrel monkey (Saimiri sciureus)	(Gamzu and Schwam, 1974) (Schwam & Gamzu, 1975)
Mammalia	Cynomolgus monkey (Macaca fascicularis)	(Bullock and Myers, 2009)
Mammalia	Domestic pig (Sus domesticus)	(Breland & Breland, 1961; Dantzer, 1978)
Mammalia	Horse (Equus ferus caballus)	(Miyashita et al., 1999)
Mammalia	House mouse (Mus musulus)	(Campus et al., 2016; Dickson et al., 2015; Tomie et al., 2012)
Mammalia	Human (Homo erectus)	(Wilcove & Miller, 1974) (Joyner et al., 2018; Kimura et al., 1990; Versace et al., 2016)
Mammalia	Racoon (Procyon lotor)	(Breland & Breland, 1961)
Mammalia	Rhesus macaque (Macaca mulatta)	(Likely, 1974)
Ornithurae	Blue jay (Cyanocitta cristata)	(Mauldin, 1981)
Ornithurae	Common crow (Corvus brachyrhynchos)	(Powell and Kelly, 1976)
Ornithurae	Domesticated pigeon (Columba livia)	(Brown and Jenkins, 1968; Powell and Kelly, 1976; Silva et al., 1992)
Ornithurae	Japanese quail (Coturnix japonica)	(Burns and Domjan, 1996; 2001)
Ornithurae	Ring-necked dove (Streptopelia capicola)	(Ohyama et al., 1999)
Ornithurae	Robin (Turdus migratorius)	(Mauldin, 1981)
Ornithurae	Starling (Sturnus vulgaris)	(Mauldin, 1981)
Reptilia	Benegal monitor (Varanus bengalensis)	(Loop, 1976)
Reptilia	Painted turtle (Chrysemys picta)	(Yeh and Powers, 2005)

Table S1.1. Phylogenetic classes and species exhibiting sign-tracking behavior.

Brain Region	Name	Method	PCA Phase	Sign-tracking	Goal-tracking	Reference
Amygdala	BLA	NMDA[15]	Acquisition	↓	↑	(Chang et al., 2012b)
	BLA	Quinolinic (acid)	Acquisition	No effect	—	(Parkinson et al., 2000a)
	BLA	NMDA	Acquisition	No effect	No effect	(Naeem and White, 2016)
	BLA/CeA	NMDA	Acquisition	↓	No effect	(Naeem and White, 2016)
	CeA	Ibotenic (acid)	Acquisition	↓	—	(Parkinson et al., 2000a)
	CeA	Ibotenic	Acquisition	No effect	—	(Chang et al., 2012a)
	CeA	NMDA	Acquisition	No effect	No effect	(Naeem and White, 2016)
Cortex	Cg1/2[16]	Quinolinic	Acquisition	No effect*	—	(Cardinal et al., 2003)
	Cg1/2[1]	Quinolinic	Acquisition	No effect*	—	(Bussey et al., 1997)
	Cg1/2[1]	Quinolinic	Acquisition	↑	—	(Parkinson et al., 2000b)
	IL	Quinolinic	Acquisition	No effect	—	(Chudasama and Robbins, 2003)
	IL/PL	Quinolinic	Acquisition	No effect	—	(Bussey et al., 1997)
	OFC	Quinolinic	Acquisition	↓	—	(Chudasama and Robbins, 2003)
	OFC	NMDA	Acquisition	No effect	—	(Chang, 2014)
	PrC	NMDA	Acquisition	No effect	—	(Bussey et al., 2000)
	PoC	NMDA	Acquisition	No effect	—	(Bussey et al., 2000)
	RSA/RSG[17]	Quinolinic	Acquisition	No effect	—	(Bussey et al., 1997)
Hippocampus	dHPC	NMDA	Acquisition	No effect	↑	(Fitzpatrick et al., 2016)
	dHPC	NMDA	Acquisition	No effect	No effect	(Naeem and White, 2016)
	dSUB	Quinolinic	Acquisition	No effect	—	(Parkinson et al., 2000a)
	HPC	NMDA	Acquisition	No effect	No effect	(Fitzpatrick et al., 2016)
	HPC	NMDA	Acquisition	↑	—	(Ito et al., 2005)
	vHPC	NMDA	Acquisition	↓	↑	(Fitzpatrick et al., 2016)
	vHPC	NMDA	Expression	No effect	No effect	(Fitzpatrick et al., 2016)
	vSUB	Quinolinic	Acquisition	No effect	—	(Parkinson et al., 2000a)
Pons	PPT	Ibotenic	Acquisition	↓	—	(Inglis et al., 2000)
Basal ganglia	DMS	NMDA	Acquisition	↓	↑	(Naeem & White, 2016)
	DLS	NMDA	Acquisition	↓	↑	(Naeem & White, 2016)
	NAc	NMDA	Acquisition	↓	↑	(Chang et al., 2012b)
	NAcC	Ibotenic	Acquisition	No effect	No effect	(Chang & Holland, 2013)
	NAcC	Quinolinic	Acquisition	↓	—	(Parkinson et al., 2000b)
	NAcSh	Ibotenic	Acquisition	No effect	No effect	(Chang & Holland, 2013)
	NAcSh	Ibotenic	Acquisition	No effect	—	(Parkinson et al., 2000b)
	VP	DREADDs (hM4di)	Acquisition	↓	No effect	(Chang et al., 2015)
Subthalamus	STN	Ibotenic	Acquisition	↑	—	(Uslaner et al., 2008)
	STN	Ibotenic	Expression	↑	—	(Uslaner et al., 2008)
Thalamus	PVT	Ibotenic	Acquisition	↑	↓	(Haight et al., 2015)
	PVT	Ibotenic	Expression	↑	↓	(Haight et al., 2015)
	VPMpc	Electrolytic	Acquisition	↑	—	(Reilly & Pritchard, 1997)
White matter	Fornix	Electrolytic	Acquisition	No effect	—	(Bussey et al., 2000)

[15] Excitotoxic chemicals (N-methyl-D-aspartate, NMDA; ibotenic acid; quinolinic acid) lesion cell bodies while sparing fibers of passage. Electrolytic lesions target both cell bodies and fibers of passage.
[16] Cg1/2 is the rodent homologue of the human anterior cingulate cortex.
[17] RSA/RSG is the rodent homologue of the human posterior cingulate cortex.
* The lesion did not affect conditioned responding to the CS+, however, it affected discrimination between the CS+ and CS-.

	FR[18]	Electrolytic	Acquisition	↑	No effect	(Danna et al., 2013)

Table S1.2. Lesion studies of sign- and goal-tracking behaviors in rats. BLA – basolateral nucleus of the amgydala, CeA – central nucleus of the amygdala, Cg1/2 – cingulate cortex (area 1/2), DREADD – designer receptor exclusively activated by designer drugs, dHPC – dorsal hippocampus, dSUB – dorsal subiculum, FR – fasciculus retroflexus, HPC – hippocampus, IL – infralimbic cortex, NAcC – nucleus accumbens core, NAcSh – nucleus accumbens shell, PCA – Pavlovian conditioned approach, PrC – perirhinal cortex, PoC – postrhinal cortex, PVT – paraventricular nucleus of the thalamus, STN – subthalamic nucleus, vHPC – ventral hippocampus, VPMpc – ventral posteromedial thalamic nucleus (parvocellular part), vSUB – ventral subiculum, PL – prelimbic cortex, PPT – pedunculopontine tegmental nucleus, VP – ventral pallidum. Rows shaded in gray are electrolytic lesions of white matter tracts, not electrolytic/neurochemical lesions of gray matter in brain regions.

[18] The fasciculus retroflexus is the main output tract from the habenular nuclei.

Pathway	Method	PCA Phase	Sign-tracking	Goal-tracking	References
NAcSh ↔ VP	AAV-FLEX-DREADD (hM4Di)	Acquisition	↑	No effect	(Chang et al., 2018)
IC ↔ BLA	Baclofen/muscimol	Expression	↓	↓	(Nasser et al., 2018)
NAc ↔ BLA	NMDA	Acquisition	↓	No effect	(Chang et al., 2012b)
Cg1/2 ↔ NAcC	Quinolinic acid (Cg1/2), ibotenic acid (NAc)	Acquisition	↓	—	(Parkinson et al., 2000b)
mPFC[19] ↔ mCPu	Quinolinic	Acquisition	No effect	—	(Christakou et al., 2005)

Table S1.3. Disconnection studies of sign- and goal-tracking behavior in rats. AAV – adeno-associated virus, BLA – basolteral nucleus of the amygdala, Cg1/2 – cingulate cortex (area 1/2), DREADD – designer receptor exclusively activated by designer drugs, IC – insular cortex, mPFC – medial prefrontal NAc – nucleus accumbens, NAcC – core of the nucleus accumbens, NAcSh – nucleus accumbens shell, PCA – Pavlovian conditioned approach, VP – ventral pallidum.

[19] The lesion encompassed the prelimbic cortex (PL) and Cg1.

CHAPTER II

Sign-trackers have elevated *myo*-inositol in the nucleus accumbens and ventral hippocampus following Pavlovian conditioned approach

Note: Some of the text and figures have appeared previously in print in the Journal of Neurochemistry (Fitzpatrick et al., 2016) and are used with the permission of the publisher, Wiley-Blackwell.

Abstract

Pavlovian conditioned approach (PCA) is a behavioral procedure that can be used to assess individual differences in the attribution of incentive-motivational value to reward-related cues. Using proton magnetic resonance spectroscopy (^{1}H-MRS) *ex vivo*, we simultaneously analyzed concentrations of multiple neurochemicals throughout the mesocorticolimbic system two weeks after PCA training to identify potential addiction vulnerability factors in drug naïve rats for future investigations. Neurochemicals of interest were those in the glutamate/GABA-glutamine cycle as well as N-acetylaspartate (a marker of neuronal integrity) and *myo*-inositol (Ins; a marker of glial activity/proliferation). Levels of Ins were increased in the nucleus accumbens (NAc) and ventral hippocampus (vHPC), but not dorsal hippocampus or medial prefrontal cortex, of sign-trackers compared to goal-trackers or intermediate-responders. In addition, Ins levels positively correlated with PCA behavior in the NAc and vHPC. These results

suggest that alterations in glial activity/proliferation within these regions underlie individual variation in the attribution of incentive-motivational value to reward-related cues.

Introduction

^{1}H-MRS has been used to detect drug-related alterations of brain neurochemicals in preclinical models of addiction (Hu et al., 2012; Perrine et al., 2010; Yang et al., 2015) and in human subjects (Licata & Renshaw, 2010). Importantly, ^{1}H-MRS has strong translational potential for comparing the results of preclinical experimental manipulations in animal models to clinical observations in addicted patients (Hermann et al., 2012). A long-standing question in ^{1}H-MRS studies, however, has been whether any observed neurochemical differences reflect a preexisting vulnerability rather than a transitory or compensatory neuroplasticity after drug exposure. This question remains a challenge due to the inherent difficulty of determining addiction vulnerability in drug-naïve subjects. Preclinical models can be used to address this challenge by investigating reward-related behaviors in the absence of any potential confounds related to drug exposure.

PCA is a behavior that develops when a CS (e.g., a retractable lever) is paired with the response-independent presentation of an appetitive, US (e.g., a food pellet). Rats trained with a PCA procedure manifest one of three behavioral phenotypes: sign-tracking (CS-directed CRs), goal-tracking (US-directed CRs), or intermediate-responding (both CRs). STs attribute motivational salience to the CS and are more vulnerable to cue-induced reinstatement of drug-seeking behavior (Saunders and Robinson, 2010; 2011) than GTs or IRs even in the presence of adverse consequences (Saunders et al., 2013). Thus, sign-tracking behavior in a PCA procedure predict cue-related addiction-like behaviors in rats.

Previous studies have demonstrated that the 'motive circuit,' an array of mesocorticolimbic brain regions that process incentive stimuli, is activated during sign-tracking behavior and cue-induced reinstatement of drug-seeking behavior in STs (Flagel et al., 2011a; Yager et al., 2015). In addition, regions within the motive circuit (e.g., NAc and mPFC) have been implicated in cue-induced drug craving (Li et al., 2012; Pickens et al., 2011), and activation of these brain regions by drug-related cues predicts relapse (Grusser et al., 2004; Li et al., 2015), making this circuit an important target for the identification, intervention, and treatment of addiction. More specifically, functional magnetic resonance imaging (fMRI) studies have demonstrated that drug administration in both addicted patients (Gu et al., 2010) and sensitized rats (Febo et al., 2005) alters fMRI responses within the motive circuit, including the mPFC, NAc, and HPC. The HPC can be divided into dorsal and ventral regions in rats (corresponding to posterior and anterior regions in humans, respectively), and both uniquely process motivationally salient stimuli (Behrendt, 2013).

Differences in ^{1}H-MRS neurochemical profiles within brain regions of the motive circuit may therefore inform the neurobiology of addiction vulnerability as well as provide a basis for the discovery of neurochemicals associated with PCA behavior and addiction vulnerability. Although it is known that dopaminergic transmission within the NAc core is critical for the expression of sign-tracking behavior (Flagel et al., 2011b; Saunders & Robinson, 2012; Yager et al., 2015), the relationship of other neurochemicals in the NAc, mPFC and HPC to sign-tracking behavior is poorly understood. In the present study, high-resolution ^{1}H-MRS *ex vivo* was used to assess unbiased neurochemical profiles two weeks[20] after PCA training within the mPFC, NAc,

[20] Measurements of baseline levels of neurochemicals can be measured more accurately in the future using *in vivo* instead of *ex vivo* ^{1}H-MRS. Because *in vivo* ^{1}H-MRS was not available during the time of testing, *ex vivo* ^{1}H-MRS was performed following a two-week quiescent period, because it is outside the period of postconditioning memory consolidation during which synaptic alterations occur and stabilize (Xu et al., 2009).

dHPC, and vHPC to discover neurochemical differences that distinguish drug-naïve subjects that are either vulnerable to cue-related, addiction-like behaviors (STs) or more resistant to them (GTs and IRs). Neurochemicals of interest were those in the glutamate (Glu)/GABA-glutamine (Gln) cycle, due to their recently discovered role in sign-tracking behavior (Batten et al., 2018; Chow & Beckmann, 2018), as well as N-acetylaspartate (NAA; a marker of neuronal integrity) and *myo*-inositol (Ins; a marker of glial[21] activity/proliferation)[22].

Materials and Methods

Animals

Twenty-seven adult male Sprague Dawley rats (250-300 g) were purchased from Charles River Laboratories, Harlan Laboratories, and Taconic Biosciences in order to ensure a diversity of behavioral phenotypes (Fitzpatrick et al., 2013)[23]. Rats were maintained on a 12-h light/dark cycle, and standard rodent chow and water were available *ad libitum*. All procedures were approved by the University Committee on the Use and Care of Animals (University of Michigan; Ann Arbor, MI).

Pavlovian Conditioned Approach: Apparatus

Modular conditioning chambers (24.1 cm width × 20.5 cm depth x 29.2 cm height; MED Associates, Inc.; St. Albans, VT) were used for Pavlovian conditioning. Each chamber was

[21] Although the role of glial cells has not been explicitly studied in PCA procedures, it has been previously demonstrated that nonneuronal cells (e.g., mast cells) are different between phenotypes and have a critical role in the acquisition of sign-tracking behavior (Fitzpatrick and Morrow, 2017). Given the immense role of glial cells in the regulation of synaptic transmission and plasticity, it is likely that these cells contribute to PCA behavior.
[22] See Table S2.1 for a summary of the ^{1}H-MRS studies performed in addicted patients that have investigated Glu, GABA, Gln, Ins, and NAA.
[23] Rats differ in PCA behavior as a result of strain (Andrews et al., 1995; Kearns et al., 2006; Rodriguez et al., 2008) and vendor/barrier (i.e., the individual breeding company and its facilities; Fitzpatrick 2013).

inside a sound-attenuating cubicle equipped with a ventilation fan to provide ambient white noise. Chambers were equipped with a pellet magazine, an illuminated retractable lever (counterbalanced on the left or right of the pellet magazine), and a red house light on the wall opposite to the pellet magazine. When inserted into the chamber, the retractable lever was illuminated by an LED light within the lever housing. A pellet dispenser delivered banana-flavored food pellets into the pellet magazine, and an infrared sensor inside the pellet magazine detected head entries.

Pavlovian Conditioned Approach: Procedure

For two days prior to pretraining, rats were familiarized with banana-flavored food pellets (45 mg; Bioserv; Flemington, NJ) in their home cages. Twenty-four hours later, rats were placed into the conditioning chambers and underwent one pretraining session during which the red house light remained on, but the lever was retracted. Fifty food pellets were delivered on a variable time (VT) 30-s schedule (i.e., one food pellet was delivered on average every 30 s, but actual delivery varied between 0-60 s). All rats consumed all the food pellets by the end of the pretraining session. Twenty-four hours later, rats underwent daily PCA training sessions over five days. Each trial during a test session consisted of extension of the illuminated lever (the CS) into the chamber for 8 s on a VT 90-s schedule (i.e., one food pellet was delivered on average every 90 s, but actual delivery varied between 60-120 s). Retraction of the lever was immediately followed by the response-independent delivery of one food pellet (the US) into the pellet magazine. Each test session consisted of 25 trials of CS-US pairings, resulting in a total session length of approximately 40 min. All rats consumed all the food pellets that were delivered.

Tissue Collection

Two weeks after the fifth PCA training session, rats were rapidly decapitated, and their brains were frozen in isopentane over dry ice and stored at -80°C. The two-week waiting period was imposed to minimize acute PCA training-induced neurochemical changes in the brain. Frozen brains were then placed into an acrylic adult rat brain matrix, and 2-mm coronal sections were dissected, using *The Rat Brain in Stereotaxic Coordinates 6th Edition (Paxinos and Watson, 2007).* Next, samples from coronal sections were removed using a 2-mm tissue biopsy punch, and tissue punches from the mPFC (AP: +2.7), NAc (AP: +1.2), dHPC (AP: -2.8), and vHPC (AP: -4.8) were extracted (Figure 2.3). Tissue punches were stored at -80°C until ^{1}H-MRS measurement.

High-resolution magic angle spinning proton magnetic resonance spectroscopy (^{1}H-MRS)

The details of the methodology have been previously published (Ghoddoussi et al., 2010; Perrine et al., 2014; Sajja et al., 2012). After weighing samples (~2.5 mg), the frozen tissue punches were placed into a 12-µl Bruker zirconium rotor with 8 µl ice-cold buffer (pH 7.4; 100 mM K_2HPO_4/KH_2PO_4, 200 mM HCOONa, 1 g/L NaN_3 diluted 50% with D_2O containing 3 mM trimethylsilylpropinoate as a chemical shift [ppm = 0.0] reference). A vertical 8.9-cm bore Bruker 11.7-T magnet, controlled with an Avance DRX-500 console (Bruker Biospin Corp.; Billerica, MA), was used to analyze the sample by placing the rotor (with tissue sample and buffer) into a multi-nuclear Bruker magic angle spinning probe. Sample temperature was maintained at 4°C, and rotors were spun at 4.2 ± 0.002 kHz and 54.7° relative to the static magnetic field, B_0. A 1-D Carr–Purcell–Meiboom–Gill pulse sequence with a pre-saturation pulse, for water suppression, and a semi-automated shimming procedure, to compensate for field

inhomogeneities, was used to acquire data using Bruker-XWINNMR (Version 3.6). Raw spectral data (see Figure 2.5) were analyzed with a custom LCModel (Version 6.1-4), utilizing a custom basis set of individual neurochemical spectra and non-specific lipid signals designed to fit the brain tissue spectrum and quantify concentrations of neurochemicals with resonance peaks between 1.0 and 4.2 ppm (Provencher, 1993; 2001). Neurochemical signals were corrected for tissue weight and expressed as nmol neurochemical/mg tissue wet weight. Cramér–Rao bounds provided an estimate of the precision of the LCModel fit to the spectral data and were typically 10% or less for most neurochemicals and acceptable under 25%. Using an unbiased experimental approach, the following MR-visible neurochemicals were assessed in each tissue punch (see Figure 2.4): lactate, GABA, N-acetylaspartate (NAA), N-acetylaspartylglutamate, glutamate (Glu), glutamine (Gln), succinate, glutathione, creatine, glycerophosphorylcholine, choline, phosphocholine, glycerophosphocholine, phosphorylethanolamine, taurine, *myo*-inositol (Ins), glycine (Gly), cholines (a combined signal of choline, phosphocholine, and glycerophosphocholine) and Ins-Gly (a combined signal of the closely overlapping resonances of Ins and Gly)[24]. Some samples were unable to be analyzed (e.g., tissue loss or Cramér–Rao bounds outside the acceptable range following processing), for the mPFC (n = 6 out of 27), dHPC (n = 4 out of 27), and vHPC (n = 6 out of 27).

Statistical Analysis

SPSS (Version 21; IBM, Inc.; Armonk, NY) was used for all statistical analysis. PCA behavior was scored using an index that averages the number, latency, and probability of lever presses (sign-tracking CRs) and magazine entries (goal-tracking CRs) during CS presentations

[24] ^{1}H-MRS is not able to detect catecholamines (e.g., DA).

within a session (Meyer et al., 2012). Briefly, we averaged together the response bias (i.e., number of lever presses and magazine entries for a session; [lever presses – magazine entries] / [lever presses + magazine entries]), latency score (i.e., average latency to perform a lever press or magazine entry during a session; [magazine entry latency – lever press latency]/8 s), and probability difference (i.e., proportion of lever presses or magazine entries; [lever press probability – magazine entry probability]). The index scores behavior from +1.0 (absolute sign-tracking) to -1.0 (absolute goal-tracking), with 0 representing no bias. The PCA index score from Session 5 was used to classify animals as STs (score $\geq$ 0.5), GTs (score $\leq$ -0.5) or IRs (-0.5 < x < 0.5).

Repeated measures were analyzed using a linear mixed model with a covariance structure selected using Akaike's information criterion (Akaike, 1974). Group differences in neurochemical levels in each brain region were analyzed using one-way analysis of variance (ANOVA) uncorrected[25] for multiple comparisons between brain regions. With a significant ANOVA, post hoc comparisons were performed using Tukey's honest significant difference (HSD). Correlations between PCA behavior and neurochemical levels were analyzed using Pearson's r.

Results

Rats underwent five daily sessions of PCA training and were classified as STs (n = 8), IRs (n = 9), or GTs (n = 10). Figure 2.1 shows that phenotypes differed in their lever press number (effect of Phenotype: $F_{(2,30.03)} = 15.47$, $p = 2.42 \times 10^{-5}$), latency (effect of Phenotype: $F_{(2,30.36)} = 14.89$, $p = 3.12 \times 10^{-5}$), and probability (effect of Phenotype: $F_{(2,28)} = 16.54$, $p = 1.81 \times$

[25] Measurements between brain regions were not corrected for multiple comparisons, because the neurochemical levels in these brain regions (e.g., NAA and Ins) result from local dynamics and are largely independent.

10^{-5}) as well as magazine entry number (effect of Phenotype: $F_{(2,27.21)} = 7.53$, $p = 0.002$), latency (effect of Phenotype: $F_{(2,28.49)} = 12.03$, $p = 1.63 \times 10^{-4}$), and probability (effect of Phenotype: $F_{(2,29.36)} = 16.18$, $p = 1.83 \times 10^{-5}$). In addition, rats differed in their PCA index scores, and the PCA index score from Session 5 was used to phenotype rats (Figure 2.2; effect of Phenotype: $F_{(2,25.23)} = 15.75$, $p = 3.64 \times 10^{-5}$).

To determine baseline levels of ^{1}H-MRS-detectable neurochemicals across phenotypes, tissue punches from the mPFC, NAc, dHPC, and vHPC were obtained two weeks after the final PCA training session (Figure 2.3). (Table 2.1 shows a complete list of neurochemicals and functions, Tables 2.2-2.5 show statistical analyses for every neurochemical, and Table 2.6 summarizes significant findings between phenotypes and across brain regions.) Neurochemicals of interest were: Glu, GABA, Gln, NAA, and Ins. Although the current experiment utilized a magnet that was able to differentiate between Ins and Gly signals, which are spectrally similar, , the Ins-Gly signal was also investigated, because it is often reported in studies utilizing lower tesla magnets for ^{1}H-MRS studies..

In the mPFC (Figure 2.6), there were significant differences in Glu (effect of Phenotype: $F_{(2,20)} = 4.19$, $p = 0.032$), but not Gln (effect of Phenotype: $F_{(2,20)} = 2.31$, $p = 0.13$) or GABA (effect of Phenotype: $F_{(2,20)} = 2.37$, $p = 0.12$). Post hoc comparisons revealed that levels of Glu were increased in STs compared to IRs ($p = 0.01$) but not GTs ($p = 0.11$). PCA behavior and levels of Glu ($r = 0.17$, $p = 0.46$), Gln ($r = 0.29$, $p = 0.20$), or GABA ($r = -0.07$, $p = 0.78$), however, were not correlated. In the NAc (Figure 2.7), there were no significant differences in Glu (effect of Phenotype: $F_{(2,26)} = 2.56$, $p = 0.098$), Gln (effect of Phenotype: $F_{(2,26)} = 2.87$, $p = 0.076$), or GABA (effect of Phenotype: $F_{(2,26)} = 0.87$, $p = 0.43$). PCA behavior and levels of Glu ($r = 0.003$, $p = 0.99$), Gln ($r = 0.20$, $p = 0.32$), or GABA ($r = 0.20$, $p = 0.33$) were not correlated.

In the dHPC (Figure 2.8), there were no significant differences in Glu (effect of Phenotype: $F_{(2,22)} = 0.45$, $p = 0.65$), Gln (effect of Phenotype: $F_{(2,22)} = 0.64$, $p = 0.54$), or GABA (effect of Phenotype: $F_{(2,22)} = 0.23$, $p = 0.79$). PCA behavior and levels of Glu ($r = 0.17$, $p = 0.43$), Gln ($r = 0.16$, $p = 0.46$), or GABA ($r = 0.19$, $p = 0.39$) were not correlated. In the vHPC (Figure 2.9), there were no significant differences in Glu ($F_{(2,19)} = 0.39$, $p = 0.72$), Gln ($F_{(2,19)} = 1.50$, $p = 0.25$), or GABA ($F_{(2,19)} = 0.035$, $p = 0.97$). PCA behavior and levels of Glu ($r = 0.14$, $p = 0.55$), Gln ($r = 0.32$, $p = 0.16$), or GABA ($r = 0.05$, $p = 0.82$) were not correlated.

Figure 2.10 shows that levels of NAA did not differ between GTs, IRs, and STs in the mPFC (effect of Phenotype: $F_{(2,20)} = 2.58$, $p = 0.10$), NAc (effect of Phenotype: $F_{(2,26)} = 2.48$, $p = 0.11$), dHPC (effect of Phenotype: $F_{(2,22)} = 0.52$, $p = 0.60$), or vHPC (effect of Phenotype: $F_{(2,19)} = 0.48$, $p = 0.63$). In addition, PCA behavior was not correlated with levels of NAA in the mPFC ($r = 0.09$, $p = 0.70$), NAc ($r = 0.50$, $p = 0.14$), dHPC ($r = 0.34$, $p = 0.45$), or vHPC ($r = 0.14$, $p = 0.55$). Figure 2.11, however, shows that levels of Ins differed in the mPFC (effect of Phenotype: $F_{(2,20)} = 6.18$, $p = 0.009$), NAc (effect of Phenotype: $F_{(2,26)} = 4.18$, $p = 0.028$), and vHPC (effect of Phenotype: $F_{(2,19)} = 4.57$, $p = 0.025$), but not dHPC (effect of Phenotype: $F_{(2,22)} = 0.44$, $p = 0.65$). Post hoc comparisons revealed that levels of Ins were decreased in IRs compared to STs ($p = 0.003$) or GTs ($p = 0.032$) in the mPFC, increased in STs compared to IRs ($p = 0.01$) or GTs ($p = 0.039$) in the NAc, and increased in STs compared to IRs ($p = 0.014$) or GTs ($p = 0.014$) in the vHPC. In addition, levels of Ins-Gly in the NAc were different between phenotypes (effect of Phenotype: $F_{(2,26)} = 4.43$, $p = 0.023$), and post hoc comparisons revealed that levels of Ins-Gly were increased in STs compared to IRs ($p = 0.013$) or GTs ($p = .018$). Finally, PCA behavior was correlated with Ins-Gly ($r = 0.42$, $p = 0.031$) in the NAc and Ins in the vHPC ($r = 0.47$, $p = 0.033$), but not with Ins in the mPFC ($r = 0.065$, $p = 0.78$) or dHPC ($r = 0.056$, $p = 0.80$).

Discussion

The present study was an unbiased assessment of ^{1}H-MRS-detectable neurochemicals in GTs, IRs, and STs following PCA training to identify addiction vulnerability factors in drug-naïve animals. The analysis was restricted to select brain regions of the motive circuit (NAc, mPFC, dHPC, and vHPC), and neurochemicals of interest were part of the Glu/GABA-Gln system or markers of neuronal integrity (NAA) or glial activity/proliferation (Ins). Levels of Glu were increased in the mPFC of STs compared to IRs, but there was no correlation between levels of Glu in the mPFC and PCA index scores. In addition, we found increased levels of Ins-Gly (a combined signal of the closely overlapping resonances of Ins and Gly) in the NAc and Ins in the vHPC of STs compared to GTs or IRs. In addition, Ins-Gly and Ins correlated positively with PCA behavior in the NAc and vHPC, respectively.

Ins is higher in the brains of addicted patients than healthy controls (Ernst et al., 2000; Sung et al., 2007) and in preclinical models of drug use (Perrine et al., 2010). Even in asymptomatic, abstinent addicts (average duration of 66.4 months), Ins is increased in the brain (Chang et al., 1997). Interestingly, Ins normalizes over withdrawal, therapeutic intervention, and abstinence in both animals and humans (Gao et al., 2007; Mon et al., 2012; Verdejo-Garcia et al., 2013; Yang et al., 2015). In addition, Ins in the brain is positively correlated with monthly drug consumption and negatively correlated with neurocognitive ability one week into abstinence (Mon et al., 2012). It is important to note that drug consumption in addicted patients (Jovanovski et al., 2005; Vonmoos et al., 2014) and self-administering rodents (Briand et al., 2008) causes cognitive impairment[26], and cognitive impairments induced by trauma or neurodegeneration negatively correlate with Ins levels in the brains of humans (Rami et al., 2007; Watanabe et al.,

[26] The referenced studies were performed with cocaine-dependent patients and cocaine self-administrating rodents. Cognitive impairment, however, is widely observed across different drugs of abuse (Vik et al., 2004).

2012) and rodents (Sajja et al., 2014). However, the animals in our study were never exposed to any drugs or treatments, and it has previously been demonstrated that STs learn at the same rate as GTs despite expressing different CRs (Yager & Robinson, 2013). Because Ins is increased in STs and correlates positively with PCA behavior, it suggests that higher baseline levels of Ins in a subset of individuals may potentiate the effects of drug use and thereby predispose toward addiction.

Ins, the most common physiological isomer of polyhydroxylated cyclohexane (Agranoff, 2009), is considered a marker of glial cell activity/proliferation. In support of this, intracellular transport of Ins, which uses the sodium/Ins cotransporter (SLC5A3) occurs to a much greater extent in glia compared to neurons, maintaining a 30-times greater intracellular concentration (Glanville et al., 1989). In addition, Ins is detectable in the ^{1}H-MRS spectra of glia but not neurons in primary cultures (Brand et al., 1993). More specifically, it has been suggested that ^{1}H-MRS-detected Ins reflects astrocyte activity/proliferation, because ^{1}H-MRS-detected Ins increases in parallel with S100B and glial fibrillary acidic protein (GFAP), two astrocytic markers (Hammen et al., 2008; Rothermundt et al., 2007). In astrocytes, Ins is a critical substrate for the synthesis of membrane-bound phosphoinositides, which are precursors of the second messengers, phosphatidylinositol 4,5-bisphosphate (PIP_2) and diacylglycerol. Chemogenetic activation *in vivo* of G_q protein-coupled receptors, expressed exclusively in glia, has profound effects on physiology and behavior (Agulhon et al., 2013), supporting the contention that Ins-based receptor systems (e.g., mGluR1 and mGluR5) may be related to distinct behavioral phenotypes. Given the modulation of VTA dopaminergic neurons in the NAc by vHPC glutamatergic projection neurons (Floresco et al., 2001), disrupted efficacy of G_q protein-coupled

signaling in this pathway may conceivably alter the sensitivity of mesolimbic DA systems to behavioral reinforcement.

Acute and chronic cocaine increases astrocyte proliferation and morphology (Fattore et al., 2002), and glial dysfunction is believed to contribute to addiction by altering glia-neuron interactions (Vijayaraghavan, 2009) and Glu homeostasis (Scofield & Kalivas, 2014). Despite the role of glutamatergic transmission within the NAc for both sign-tracking behavior (Di Ciano et al., 2001) and cue-induced reinstatement of drug-seeking (Backstrom & Hyytia, 2007; Gipson et al., 2013), basal levels of ^{1}H-MRS-detectable Glu, Gln, or GABA in the NAc or the vHPC did not differ among phenotypes. Nonetheless, astrocytes play a crucial role in recycling synaptic Glu (Lebon et al., 2002), regulating extrasynaptic Glu (Fellin et al., 2004; Jourdain et al., 2007), and modulating drug-seeking behavior (Reissner et al., 2014). Therefore, the possibility that glutamatergic transmission during drug-seeking behavior is subject to differential regulation by astrocytes across phenotypes warrants further investigation.

Over the past decade, the role of glial cells in drug reward and addiction-like behaviors has been increasingly investigated. For example, methamphetamine treatment increases astrocyte signaling, and propentofylline, a modulator of glial intracellular signaling, reduces both astrocyte activation and drug reward (Narita et al., 2006; Narita et al., 2008). In addition, intra-accumbal administration of astrocyte-conditioned medium enhances the rewarding effects of methamphetamine (Narita et al., 2006; Narita et al., 2008). Drugs that cause behavioral sensitization, such as methamphetamine, also cause enduring astrocytic activation in the limbic system, an effect that is not observed in drugs that do not cause behavioral sensitization, such as methylphenidate (Suzuki et al., 2007). Based on these observations, gliomodulators have been investigated as a novel, efficacious treatment of addiction (Beardsley & Hauser, 2014; Haydon et

al., 2009). Our results suggest that individual variation in Ins represents a potential vulnerability factor associated with an animal phenotype of addiction, and pharmacologically manipulating glial activity/proliferation may be a viable therapeutic strategy for mitigating or even preventing the development of addiction in certain individuals.

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Function	Neurochemical	Other Function	References
Energy metabolism	Creatine		(Zhu & Barker, 2011)
Energy metabolism	Lactate		(Tholey & Ledig, 1990)
Energy metabolism	Succinate		(Tholey & Ledig, 1990)
Intracellular signaling	Glutathione	Antioxidation	(Dringen, 2000; Janaky et al., 1999)
Membrane synthesis	Choline		(Sanders & Zeisel, 2007)
Membrane synthesis	Glycerophosphocholine		(Zhu & Barker, 2011)
Membrane synthesis	Phosphorylethanolamine		(Araki & Wurtman, 1998)
Membrane synthesis	Phosphocholine		(Zhu & Barker, 2011)
Neurotransmission	Alanine		(Tiedje et al., 2010)
Neurotransmission	Aspartate		(Herring et al., 2015)
Neurotransmission	GABA		(McCormick, 1989)
Neurotransmission	Glutamate		(Zhu & Barker, 2011)
Neurotransmission	Glutamine		(Zhu & Barker, 2011)
Neurotransmission	Glycine		(Dingledine et al., 1990; Legendre, 2001)
Neurotransmission	N-acetylaspartylglutamate		(Neale, 2011; Neale et al., 2000)
Osmosis	Betaine	Methyl metabolism	(Lever & Slow, 2010)
Osmosis	Inositol	Intracellular signaling	(Zhu & Barker, 2011)
Osmosis	N-acetylaspartate		(Baslow, 2003)
Osmosis	Taurine	Neurotransmission	(Oja & Saransaari, 2007; Wu & Prentice, 2010)

Table 2.1. ^{1}H-MRS-detectable neurochemicals and their functional significance.

Category	Neurochemical	GT	IR	ST
Energy Metabolism	Creatine	1.83 ± 0.08	2.00 ± 0.08	1.91 ± 0.10
	Lactate	3.56 ± 0.18	3.79 ± 0.14	3.76 ± 0.23
	Succinate	0.14 ± 0.01	0.14 ± 0.01	0.13 ± 0.01
Intracellular Signaling	Glutathione	0.40 ± 0.03	0.42 ± 0.02	0.41 ± 0.02
Membrane Synthesis	Choline	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.02
	Glycerophosphocholine	0.24 ± 0.02	0.27 ± 0.03	0.25 ± 0.04
	Phosphocholine	0.27 ± 0.04	0.25 ± 0.02	0.27 ± 0.03
	Phosphorylethanolamine	0.84 ± 0.10	0.84 ± 0.05	0.82 ± 0.04
Neurotransmission	Alanine	0.18 ± 0.02	0.20 ± 0.01	0.19 ± 0.02
	Aspartate	0.36 ± 0.04	0.44 ± 0.06	0.45 ± 0.07
	GABA	0.98 ± 0.08	1.03 ± 0.08	1.06 ± 0.07
	Glutamate	2.58 ± 0.18	2.79 ± 0.16	2.69 ± 0.13
	Glutamine	1.05 ± 0.06	1.16 ± 0.07	1.18 ± 0.11
	Glycine	0.34 ± 0.10	0.31 ± 0.05	0.29 ± 0.03
	N-acetylaspartylglutamate	0.35 ± 0.02	0.37 ± 0.01	0.39 ± 0.02
Osmosis	Betaine	0.29 ± 0.01	0.33 ± 0.05	0.26 ± 0.03
	Inositol	2.37 ± 0.14	2.54 ± 0.14	2.39 ± 0.15
	N-acetylaspartate	1.88 ± 0.11	2.03 ± 0.10	1.92 ± 0.09
	Taurine	2.21 ± 0.14	2.48 ± 0.14	2.19 ± 0.10
Combined	Cholines	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.02
	Glx	3.63 ± 0.24	3.94 ± 0.21	3.87 ± 0.22
	Inositol-glycine	2.62 ± 0.19	2.81 ± 0.17	2.64 ± 0.16
	NAAX	2.23 ± 0.13	2.40 ± 0.10	2.32 ± 0.10

Table 2.2. ^{1}H-MRS-detectable neurochemical concentrations (nmol/mg) in the dorsal hippocampus. GT – goal-tracker, IR – intermediate-responder, ST – sign-tracker. Combined signals - Cholines (choline + glycerophosphocholine + phosphocholine), Glx (Glutamate + Glutamine), NAAX (N-acetylaspartate + N-acetylaspartylglutamate). Data are mean and S.E.M.

Category	Neurochemical	GT	IR	ST
Energy Metabolism	Creatine	1.76 ± 0.06	1.94 ± 0.07	1.95 ± 0.08
	Lactate	3.29 ± 0.26	3.77 ± 0.19	3.91 ± 0.39
	Succinate	0.11 ± 0.01	0.11 ± 0.01	0.12 ± 0.02
Intracellular Signaling	Glutathione	0.31 ± 0.01	0.31 ± 0.03	0.34 ± 0.03
Membrane Synthesis	Choline	0.15 ± 0.01	0.15 ± 0.02	0.16 ± 0.03
	Glycerophosphocholine	0.27 ± 0.03	0.22 ± 0.02	0.23 ± 0.04
	Phosphocholine	0.21 ± 0.03	0.28 ± 0.04	0.24 ± 0.04
	Phosphorylethanolamine	0.60 ± 0.05	0.71 ± 0.04	0.69 ± 0.09
Neurotransmission	Alanine	0.16 ± 0.02	0.21 ± 0.02	0.19 ± 0.03
	Aspartate	0.38 ± 0.04	0.39 ± 0.03	0.51 ± 0.09
	GABA	1.04 ± 0.10	1.07 ± 0.07	1.04 ± 0.12
	Glutamate	2.29 ± 0.19	2.46 ± 0.12	2.48 ± 0.24
	Glutamine	0.96 ± 0.06	1.03 ± 0.07	1.16 ± 0.12
	Glycine	0.28 ± 0.05	0.32 ± 0.04	0.37 ± 0.05
	N-acetylaspartylglutamate	0.36 ± 0.04	0.39 ± 0.03	0.40 ± 0.05
Osmosis	Betaine	0.20 ± 0.02	0.28 ± 0.03	0.23 ± 0.04
	Inositol	2.12 ± 0.12	2.12 ± 0.05	2.53 ± 0.13$^{*,\#}$
	N-acetylaspartate	1.65 ± 0.12	1.79 ± 0.08	1.75 ± 0.12
	Taurine	1.81 ± 0.22	1.86 ± 0.13	1.93 ± 0.14
Combined	Cholines	0.15 ± 0.01	0.15 ± 0.02	0.16 ± 0.03
	Glx	3.25 ± 0.24	3.49 ± 0.15	3.64 ± 0.33
	Inositol-glycine	2.45 ± 0.16	2.44 ± 0.07	2.90 ± 0.18
	NAAX	2.01 ± 0.14	2.17 ± 0.09	2.15 ± 0.17

Table 2.3. ^{1}H-MRS-detectable neurochemical concentrations (nmol/mg) in ventral hippocampus. GT – goal-tracker, IR – intermediate-responder, ST – sign-tracker. Combined signals – cholines (choline + glycerophosphocholine + phosphocholine), Glx (Glutamate + Glutamine), NAAX (N-acetylaspartate + N-acetylaspartylglutamate). Data are mean and S.E.M. * = ST differs from GT, $^{\#}$ = ST differs from IR. $^{*,\#}$ = $p < 0.05$.

Category	Neurochemical	GT	IR	ST
Energy Metabolism	Creatine	4.04 ± 0.15	3.68 ± 0.27	4.48 ± 0.28
	Lactate	5.90 ± 0.22	5.32 ± 0.49	6.49 ± 0.51
	Succinate	0.23 ± 0.01	0.22 ± 0.03	0.25 ± 0.02
Intracellular Signaling	Glutathione	0.60 ± 0.08	0.51 ± 0.04	0.71 ± 0.08
Membrane Synthesis	Choline	0.11 ± 0.01	0.09 ± 0.01	0.09 ± 0.01
	Glycerophosphocholine	0.62 ± 0.03	0.54 ± 0.07	0.68 ± 0.07
	Phosphocholine	0.31 ± 0.02	0.25 ± 0.03	0.40 ± 0.07
	Phosphorylethanolamine	1.77 ± 0.10	1.49 ± 0.09	1.86 ± 0.17
Neurotransmission	Alanine	0.33 ± 0.02	0.29 ± 0.03	0.29 ± 0.02
	Aspartate	0.70 ± 0.05	0.64 ± 0.06	0.86 ± 0.10
	GABA	1.64 ± 0.05	1.45 ± 0.12	1.69 ± 0.05
	Glutamate	7.76 ± 0.36	6.88 ± 0.54	8.90 ± 0.54$^{\#}$
	Glutamine	1.28 ± 0.13	1.17 ± 0.17	1.71 ± 0.25
	Glycine	0.40 ± 0.06	0.37 ± 0.05	0.39 ± 0.03
	N-acetylaspartylglutamate	0.55 ± 0.05	0.49 ± 0.06	0.77 ± 0.12
Osmosis	Betaine	1.86 ± 0.17	1.53 ± 0.26	2.05 ± 0.08
	Inositol	3.54 ± 0.11	2.98 ± 0.25	3.87 ± 0.14$^{\#\#}$
	N-acetylaspartate	4.30 ± 0.16	3.93 ± 0.34	4.76 ± 0.23
	Taurine	3.39 ± 0.16	3.08 ± 0.25	3.72 ± 0.28
Combined	Cholines	1.02 ± 0.03	0.86 ± 0.09	1.04 ± 0.09
	Glx	9.04 ± 0.45	8.05 ± 0.67	10.61 ± 0.77$^{\#}$
	Inositol-glycine	3.89 ± 0.15	3.24 ± 0.28	4.06 ± 0.21
	NAAX	4.85 ± 0.20	4.42 ± 0.38	5.53 ± 0.33

Table 2.4. Neurochemical concentration (nmol/mg) in medial prefrontal cortex. Combined signals – cholines (choline + glycerophosphocholine + phosphocholine), Glx (Glutamate + Glutamine), NAAX (N-acetylaspartate + N-acetylaspartylglutamate). GT – goal-tracker, IR – intermediate-responder, ST – sign-tracker. Data are mean and S.E.M. $^{\#}$ = ST differs from IR. $^{\#}$ - $p < 0.05$, $^{\#\#}$ - $p < 0.01$.

Category	Neurochemical	GT	IR	ST
Energy Metabolism	Creatine	4.95 ± 0.14	4.73 ± 0.17	5.13 ± 0.09
	Lactate	6.75 ± 0.24	6.45 ± 0.30	7.16 ± 0.27
	Succinate	0.28 ± 0.01	0.27 ± 0.02	0.29 ± 0.02
Intracellular Signaling	Glutathione	0.87 ± 0.06	0.79 ± 0.04	0.99 ± 0.07
Membrane Synthesis	Choline	0.21 ± 0.02	0.19 ± 0.02	0.18 ± 0.02
	Glycerophosphocholine	0.87 ± 0.06	0.97 ± 0.08	1.11 ± 0.07
	Phosphocholine	0.69 ± 0.07	0.53 ± 0.04	0.58 ± 0.05
	Phosphorylethanolamine	2.08 ± 0.16	1.90 ± 0.14	2.17 ± 0.15
Neurotransmission	Alanine	0.41 ± 0.02	0.34 ± 0.03	0.42 ± 0.02
	Aspartate	0.67 ± 0.06$^{\&}$	0.98 ± 0.10	0.74 ± 0.02
	GABA	2.89 ± 0.25	3.49 ± 0.45	3.32 ± 0.31
	Glutamate	7.87 ± 0.23	7.14 ± 0.38	8.09 ± 0.30
	Glutamine	2.40 ± 0.18	2.08 ± 0.09	2.71 ± 0.25
	Glycine	0.39 ± 0.03	0.36 ± 0.03	0.35 ± 0.02
	N-acetylaspartylglutamate	0.65 ± 0.05	0.73 ± 0.07	0.75 ± 0.09
Osmosis	Betaine	1.89 ± 0.09	1.91 ± 0.11	1.80 ± 0.21
	Inositol	4.70 ± 0.22	4.46 ± 0.23	5.47 ± 0.30$^{\#}$
	N-acetylaspartate	4.96 ± 0.18	4.68 ± 0.20	5.22 ± 0.10
	Taurine	4.92 ± 0.24	4.29 ± 0.33	4.63 ± 0.45
Combined	Cholines	1.77 ± 0.10	1.69 ± 0.10	1.88 ± 0.07
	Glx	10.3 ± 0.38	9.22 ± 0.40	10.8 ± 0.43$^{\#}$
	Ins-Gly	4.82 ± 0.21	4.74 ± 0.21	5.65 ± 0.28$^{*,\#}$
	NAAX	5.61 ± 0.20	5.40 ± 0.22	5.97 ± 0.13

Table 2.5. Neurochemical concentration (nmol/mg) in nucleus accumbens. Combined signals – cholines (choline + glycerophosphocholine + phosphocholine), Glx (Glutamate + Glutamine), NAAX (N-acetylaspartate + N-acetylaspartylglutamate). GT – goal-tracker, IR – intermediate-responder, ST – sign-tracker. Data are mean and S.E.M. * – ST differs from GT, $^{\#}$ – ST differs from IR, $^{\&}$ – GT differs from IR. $^{*,\#,\&}$ – $p < 0.05$.

Category	Neurochemical	GT	IR	ST
mPFC[*]	Glu			↑[*]
mPFC	Ins		↓[#,&]	
NAc	Ins-Gly			↑[*,#]
vHPC	Ins			↑[*,#]

Table 2.6. Summary of significant neurochemical differences between sign-trackers (STs), intermediate-responders (IRs), and goal-trackers (GTs). [*] – ST differs from GT, [#] – ST differs from IR, [&] – GT differs from IR.

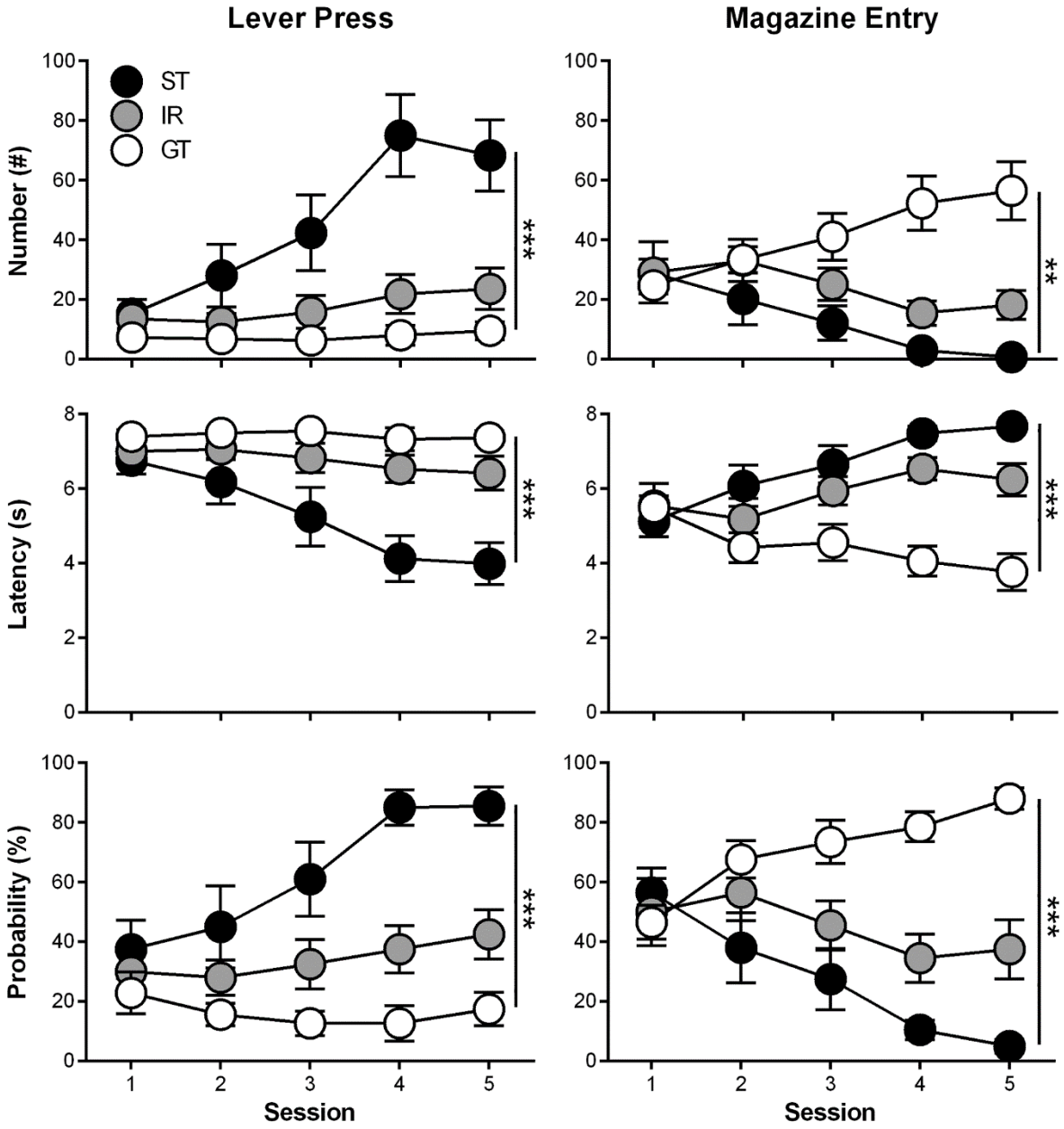

Figure 2.1. Pavlovian conditioned approach training. Rats underwent PCA training over five daily sessions and were classified as sign-trackers (STs), intermediate responders (IRs), or goal-trackers (GTs) based on their lever press and magazine entry number, latency, and probability during Session 5. Data are mean and S.E.M. ** – $p < 0.01$, *** – $p < 0.001$.

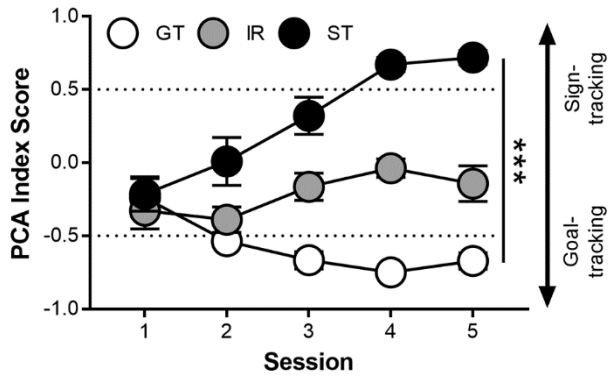

Figure 2.2. Pavlovian conditioned approach (PCA) index. Scores were calculated for each PCA training session by combining the number, latency, and probability of lever presses and magazine entries: a value of +1.0 represents absolute sign-tracking and a value of -1.0 represents absolute goal-tracking. The index score from the final PCA training session was used to phenotype rats, and the dashed lines represent the cut-offs: GTs ($x \leq -0.5$), IRs ($-0.5 < x < 0.5$), STs ($x \geq 0.5$). Data are mean and S.E.M. *** – $p < 0.001$.

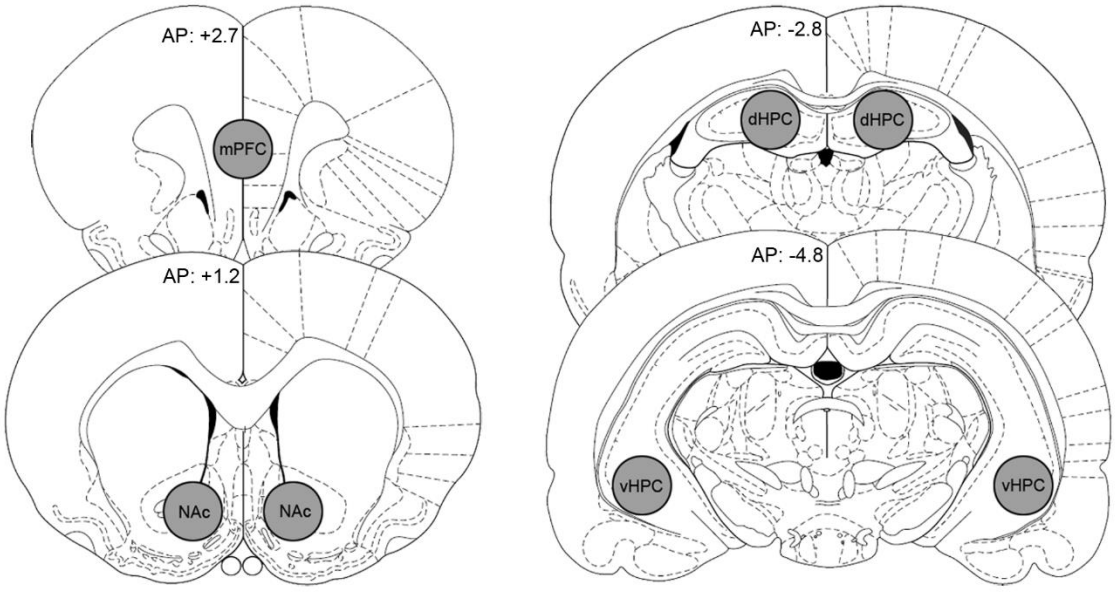

Figure 2.3. Schematic of brain tissue punches for ^{1}H-MRS. Tissue punches were taken for neurochemical analysis from the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), dorsal hippocampus (dHPC), and ventral hippocampus (vHPC) of the rat brain. Sections are labeled with anterior-posterior (AP) coordinates from bregma (in mm). *Images are modified from Paxinos & Watson (2007).*

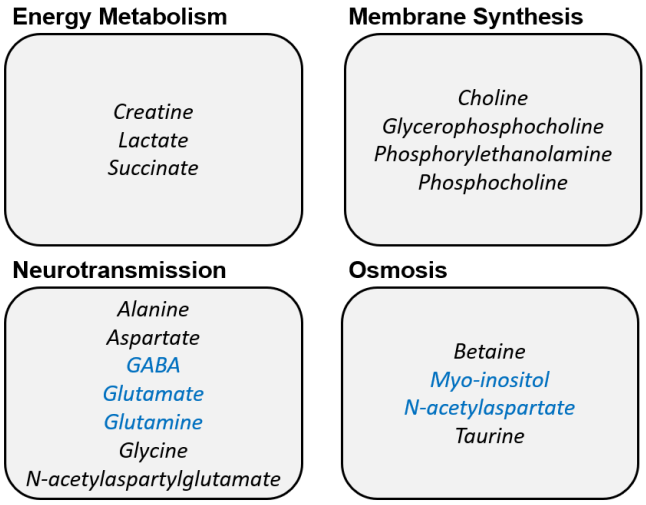

Figure 2.4. ^{1}H-MRS-detectable neurochemicals. Neurochemicals fall into four distinct categories: energy metabolism, membrane synthesis, and osmosis. The exception is glutathione, which is an antioxidant and does not fall into any of these categories. Many of the neurochemicals also have other functions, such as taurine, which is an osmolyte and neurotransmitter. *A priori* neurochemicals of interest are highlighted in blue.

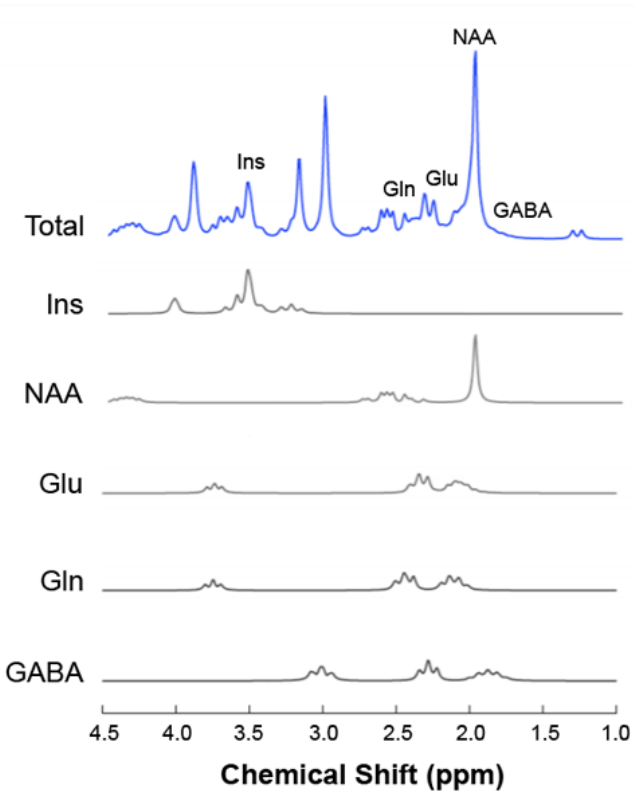

Figure 2.5. ^{1}H-MRS sample spectrograms. Examples of total and isolated neurochemical peaks of *myo*-Inositol (Ins), N-acetylaspartate (NAA), glutamate (Glu), glutamine (Gln), and γ-aminobutyric acid (GABA) as measured by proton magnetic resonance spectroscopy.

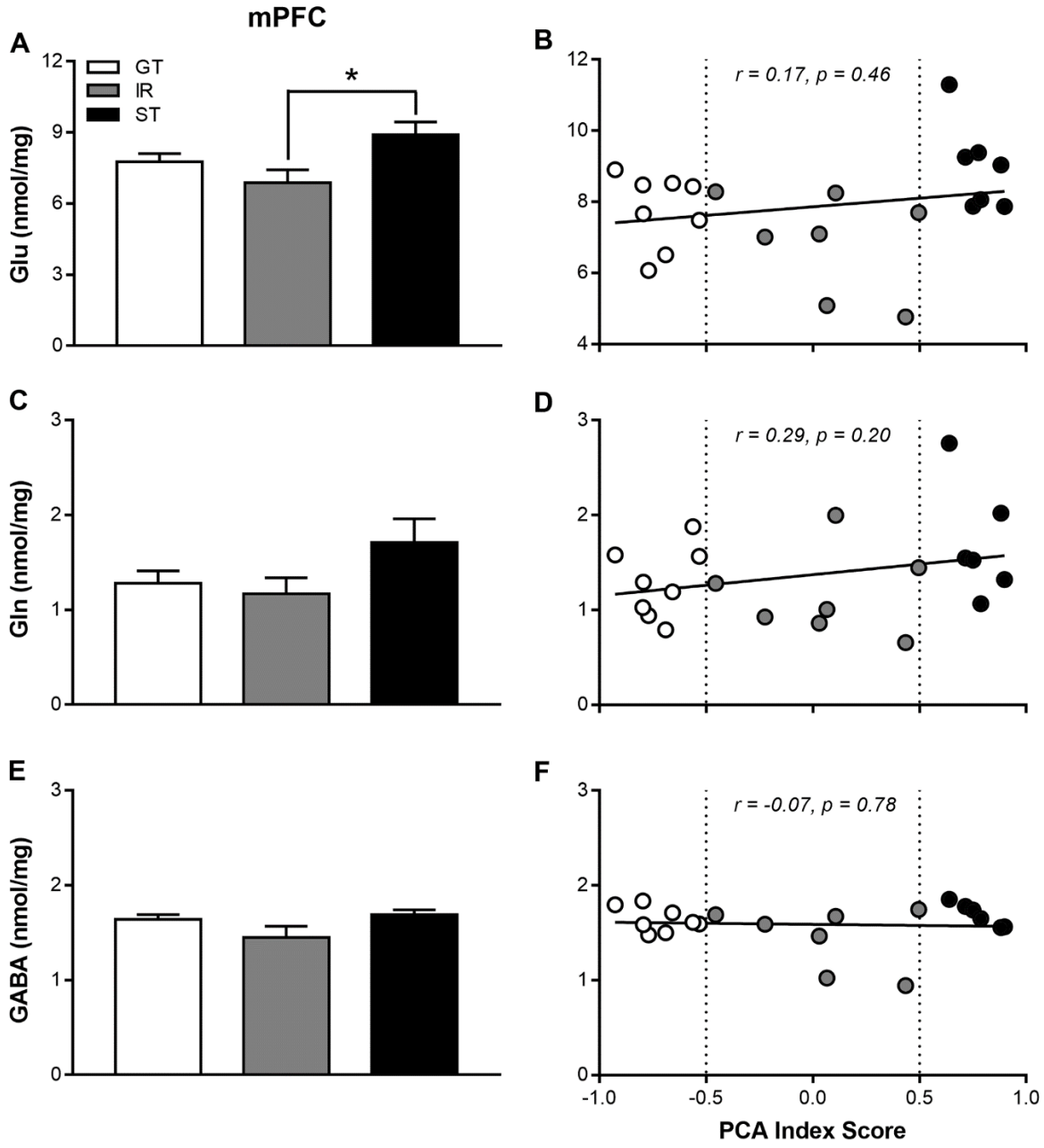

Figure 2.6. Glutamate (Glu), glutamine (Gln), and γ-aminobutyric acid (GABA) in the medial prefrontal cortex (mPFC). Baseline levels of (A) Glu, (C) Gln, and (E) GABA are not different between goal-trackers (GTs), intermediate-responders (IRs), and sign-trackers (STs) in the mPFC. In addition, there is no correlation between Pavlovian conditioned approach (PCA) index scores and baseline levels of (B) Glu, (D) Gln, and (F) GABA in the mPFC. Data are mean and S.E.M. * – $p < 0.05$.

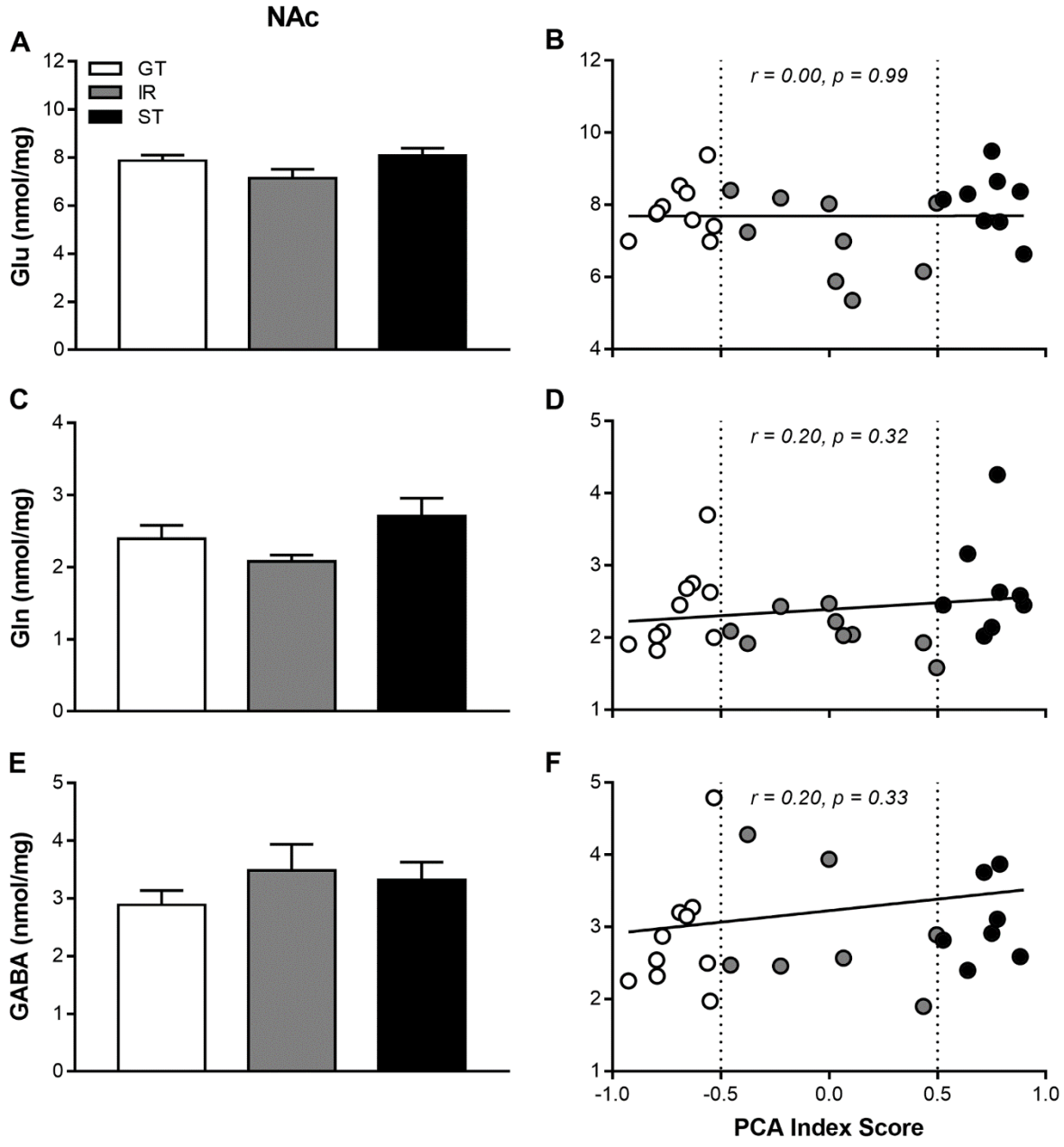

Figure 2.7. Glutamate (Glu), glutamine (Gln), and γ-aminobutyric acid (GABA) in the nucleus accumbens (NAc). Baseline levels of (A) Glu, (C) Gln, and (E) GABA are not different between goal-trackers (GTs), intermediate-responders (IRs), and sign-trackers (STs) in the NAc. In addition, there is no correlation between Pavlovian conditioned approach (PCA) index scores and baseline levels of (B) Glu, (D) Gln, and (F) in the NAc. Data are mean and S.E.M.

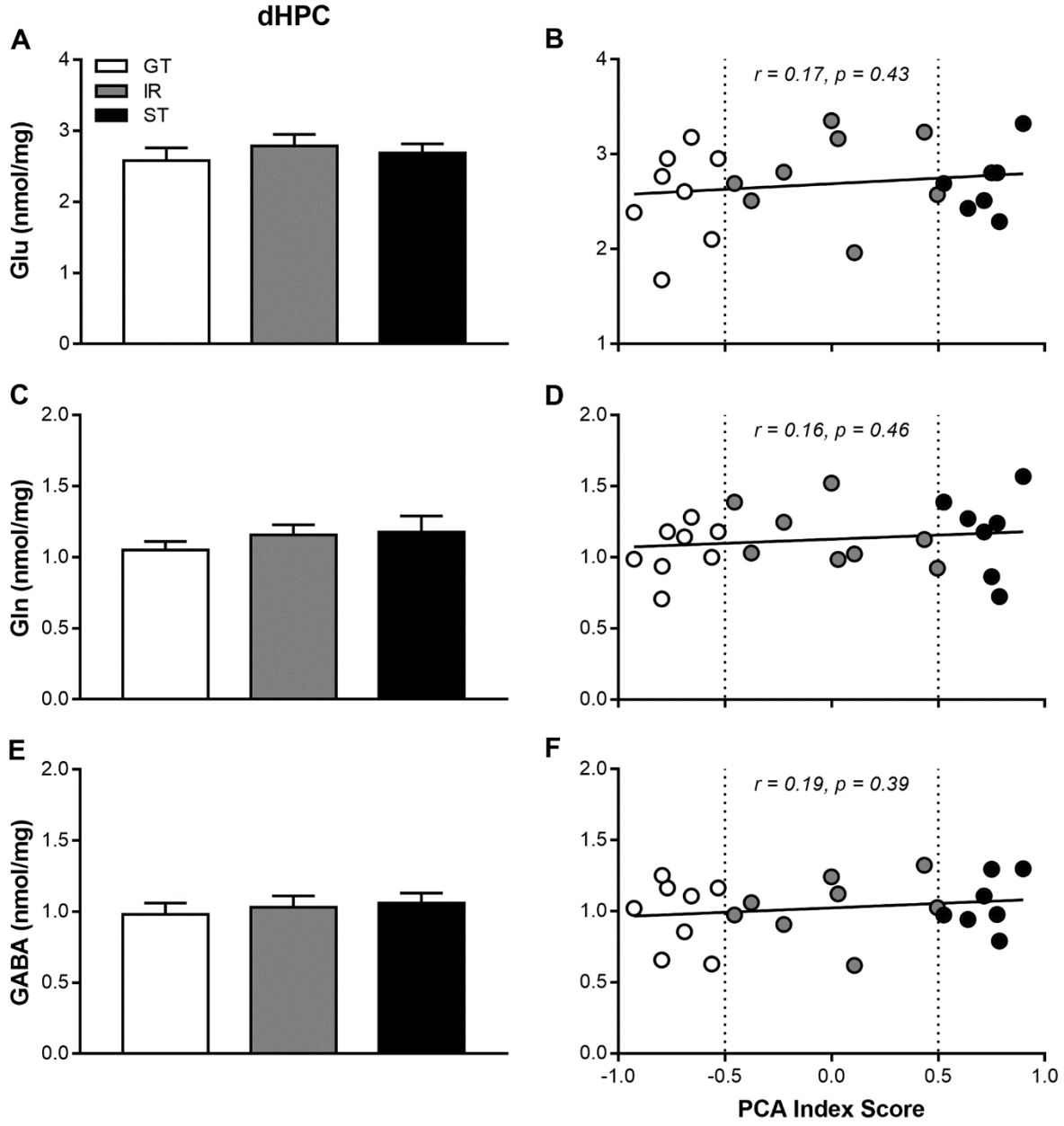

Figure 2.8. Glutamate (Glu), glutamine (Gln), and γ-aminobutyric acid (GABA) in the dorsal hippocampus (dHPC). Baseline levels of (A) Glu, (C) Gln, and (E) GABA are not different between goal-trackers (GTs), intermediate-responders (IRs), and sign-trackers (STs) in the dHPC. In addition, there is no correlation between Pavlovian conditioned approach (PCA) index scores and baseline levels of (B) Glu, (D) Gln, and (F) in the dHPC. Data are mean and S.E.M.

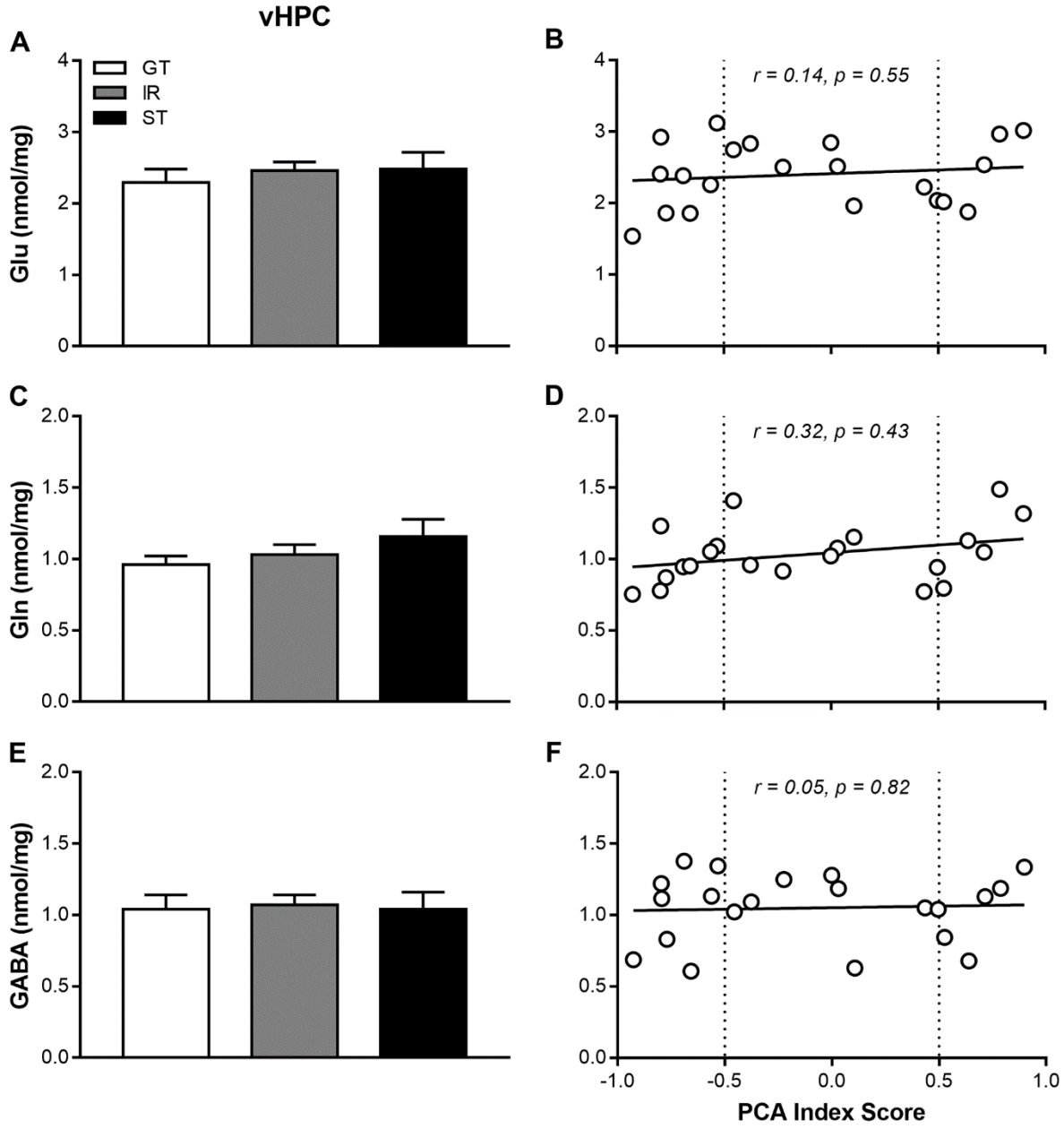

Figure 2.9. Glutamate (Glu), glutamine (Gln), and γ-aminobutyric acid (GABA) in the ventral hippocampus (vHPC). Baseline levels of (A) Glu, (C) Gln, and (E) GABA are not different between goal-trackers (GTs), intermediate-responders (IRs), and sign-trackers (STs) in the vHPC. In addition, there is no correlation between Pavlovian conditioned approach (PCA) index scores and baseline levels of (B) Glu, (D) Gln, and (F) GABA in the vHPC. Data are mean and S.E.M.

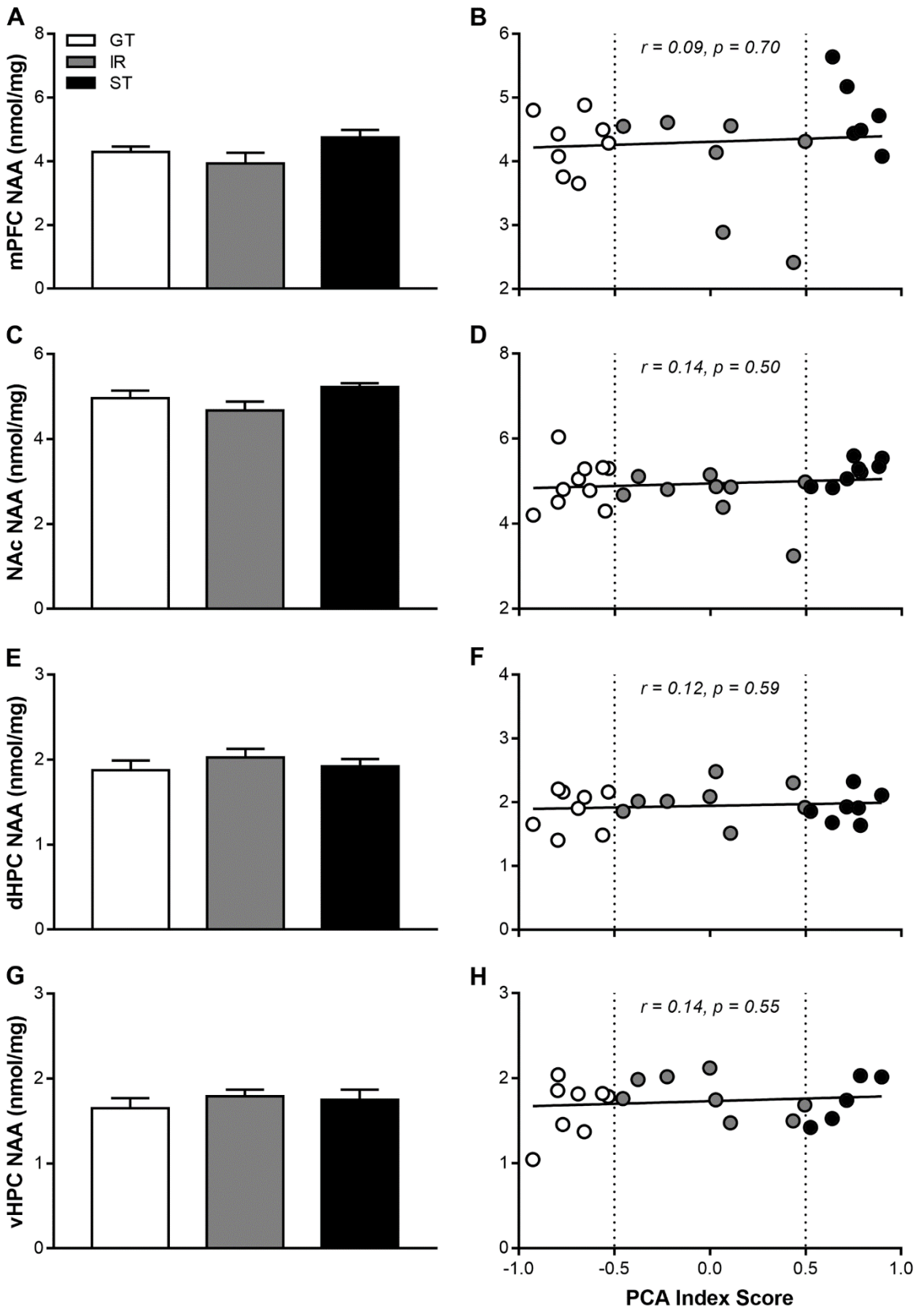
A
GT
IR
ST
mPFC NAA (nmol/mg)
B
r = 0.09, p = 0.70
C
NAc NAA (nmol/mg)
D
r = 0.14, p = 0.50
E
dHPC NAA (nmol/mg)
F
r = 0.12, p = 0.59
G
vHPC NAA (nmol/mg)
H
r = 0.14, p = 0.55
PCA Index Score

Figure 2.10. N-acetylaspartate (NAA) in the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), dorsal hippocampus (dHPC), and ventral hippocampus (vHPC). Baseline levels of NAA are not different in the (A) mPFC, (C) NAc, (E) dHPC, or (G) vHPC between goal-trackers (GTs), intermediate-responders (IRs), and sign-trackers (STs). In addition, there is no correlation between Pavlovian conditioned approach (PCA) index scores and baseline levels of NAA in the (B) mPFC, (D) NAc, (F) dHPC, or (H) vHPC. Data are mean and S.E.M.

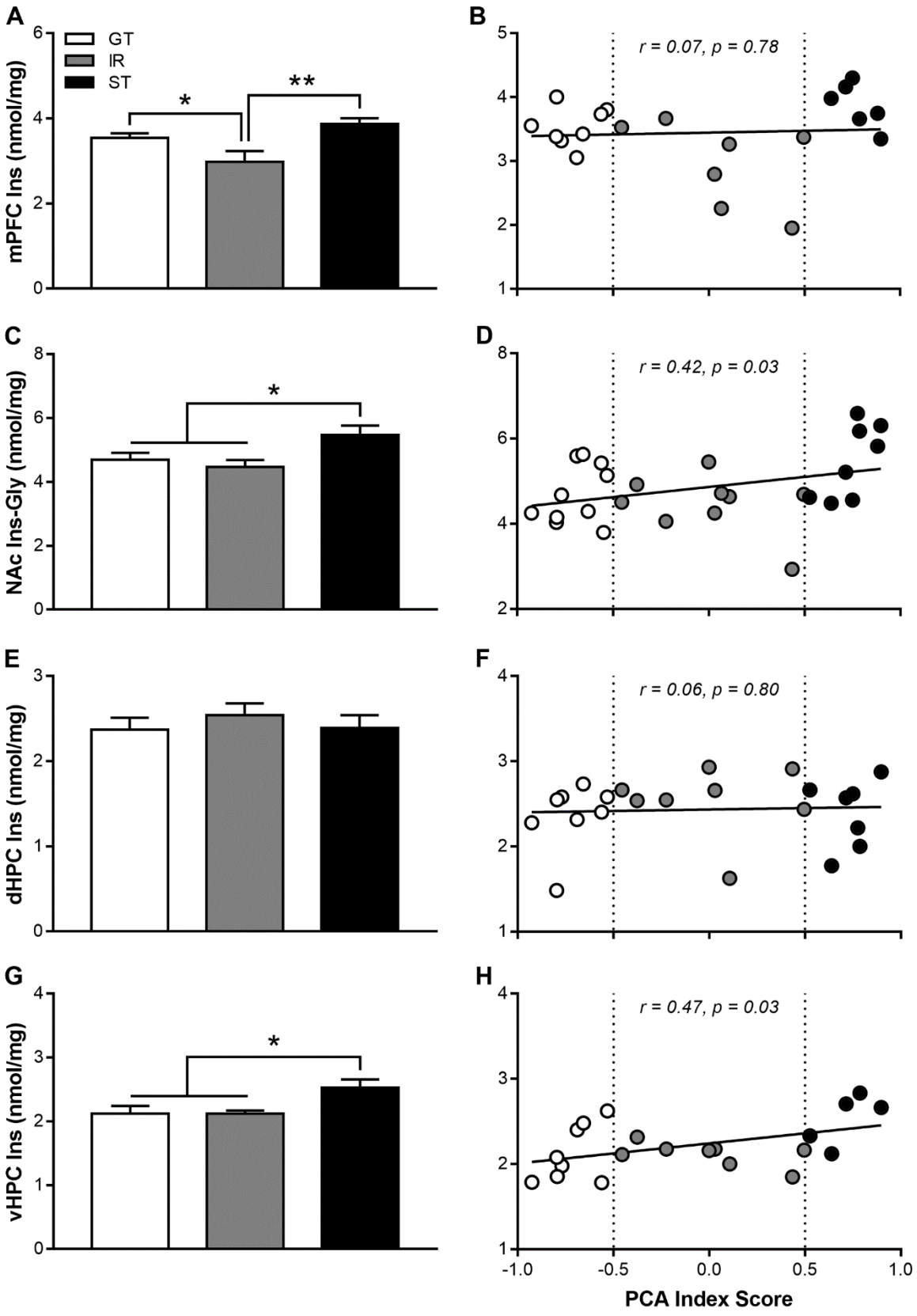
A
GT
IR
ST
mPFC Ins (nmol/mg)
*
**
B
r = 0.07, p = 0.78
C
NAc Ins-Gly (nmol/mg)
*
D
r = 0.42, p = 0.03
E
dHPC Ins (nmol/mg)
F
r = 0.06, p = 0.80
G
vHPC Ins (nmol/mg)
*
H
r = 0.47, p = 0.03
-1.0
-0.5
0.0
0.5
1.0
PCA Index Score

Figure 2.11. *Myo*-inositol (Ins) in the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), dorsal hippocampus (dHPC), and ventral hippocampus (vHPC). (A) Baseline levels of *myo*-inositol (Ins) are decreased in intermediate-responders (IRs) compared to goal-trackers (GTs) and sign-trackers (STs) in the mPFC, but (B) they do not correlate with Pavlovian conditioned approach (PCA) index scores. (C) Levels of Ins are increased in STs compared to IRs and goal-trackers (GTs) in the NAc, and (D) they positively correlate with PCA index scores. (E) Levels of Ins are not different between phenotypes in the dHPC, and (F) they do not correlate with PCA index scores. (G) Finally, levels of Ins are increased in STs compared to IRs and GTs in the vHPC, and (H) they positively correlate with PCA index scores. Data are mean and S.E.M. * – $p < 0.05$, ** – $p < 0.01$.

Appendix B: Chapter II Supplemental Information

Chemical	Brain Region	Drug	Stage	Effect	Reference
GABA	ACC	Alcohol	Abuse	↓	(Silveri et al., 2014)
Glu	ACC	Alcohol	Dependence & PTSD	↓	(Pennington et al., 2014)
GABA	ACC	Alcohol	Dependence & BPD	↓	(Prisciandaro et al., 2017)
GABA	Occipital cortex	Alcohol	Dependence	↓	(Behar et al., 1999)
Gln	ACC	Alcohol	Dependence	↑	(Thoma et al., 2011)
Glu	ACC	Alcohol	Dependence	↓	(Cheng et al., 2018)
Glu	ACC	Alcohol	Dependence	↓	(Thoma et al., 2011)
Glu	ACC	Alcohol	Abstinence	↓	(Mon et al., 2012)
Glu	ACC	Cocaine	Dependence	↑	(Schmaal et al., 2012)
Glu/Cr[27]	ACC	Cocaine	Dependence	↓	(Yang et al., 2009)
Gln	Insula	Nicotine	Withdrawal	↑	(Gutzeit et al., 2013)
Glu/Gln	ACC	Opiate	Dependence	↓	(Yucel et al., 2007)
Glu	Brainstem	Methamphetamine	Dependence	↑	(Yang et al., 2018)
Glu	dlPFC	Alcohol	Dependence	↑	(Frye et al., 2016)
Glu	mPFC	Methamphetamine	Dependence	↓	(Crocker et al., 2014)
Glu	NAc	Alcohol	Abstinence	↑	(Bauer et al., 2013)
Glu	NAc	Prescription opioids	Dependence	↑	(Liu et al., 2017)
Glu	PFC	Cocaine	Abstinence	↓	(Crocker et al., 2017)
Ins	ACC	Betel quid	Dependence	↑	(Liu et al., 2015)
Ins	Frontal cortex	Methamphetamine	Dependence	↓	(Ernst et al., 2000)
Ins/CR	mPFC	Methamphetamine	Dependence	↑	(Wu et al., 2018)
Glx/Cr[28]	ACC	Betel quid	Dependence	↑	(Liu et al., 2015)
Glx	Frontal cortex	Methamphetamine	Withdrawal	↓	(O'Neill et al., 2014)
Glx	HPC	Alcohol	Withdrawal	↑	(Frischknecht et al., 2017)
Glx	PCC	Methamphetamine	Withdrawal	↓	(O'Neill et al., 2014)
NAA	ACC	Opiate	Dependence	↓	(Yucel et al., 2007)
NAA/Cr	ACC	Betel quid	Dependence	↓	(Liu et al., 2015)
NAA	ACC	Alcohol	Abuse	↓	(Silveri et al., 2014)
NAA	ACC	Alcohol	Abstinence	↓	(Mon et al., 2012)
NAA/Cr	ACC	Methamphetamine	Abstinence	↓	(Salo et al., 2011)
NAA/Cr	ACC	Methamphetamine	Abuse	↓	(Salo et al., 2007)
NAA/Cr	ACC	Methamphetamine	Abstinence	↓	(Nordahl et al., 2002)
NAA/Cr	Brainstem	Alcohol	Abuse	↓	(Bloomer et al., 2004)
NAA	Frontal cortex	Methamphetamine	Dependence	↓	(Ernst et al., 2000)

[27] Some investigators prefer to report results as a ratio of the neurochemical of interest and creatine (Cr).
[28] Some magnets used for ^{1}H-MRS investigations do not have a high enough tesla to differentiate the nearby glutamate (Glu) and glutamine (Gln) signals, so investigators report the combined signal, Glx.

NAA	Frontal cortex	Alcohol	Withdrawal	↓	(Zahr et al., 2016)
NAA	Frontal cortex	Heroin	Dependence	↓	(Haselhorst et al., 2002)
NAA	mPFC	Methamphetamine	Dependence	↓	(Crocker et al., 2014)
NAA/CR	mPFC	Methamphetamine	Dependence	↓	(Wu et al., 2018)
NAA	PFC	Cocaine	Abstinence	↓	(Crocker et al., 2017)
NAA	Thalamus	Alcohol	Withdrawal	↓	(Zahr et al., 2016)

Table S2.1. Summary of ^{1}H-MRS studies investigating γ-aminobutyric acid (GABA), glutamine (Gln), glutamate (Glu), *myo*-inositol (Ins), and N-acetylaspartate (NAA) in addicted patients. ACC – anterior cingulate cortex, mPFC – medial prefrontal cortex, Cr – creatine, PFC – prefrontal cortex.

CHAPTER III

The ventral hippocampus regulates sign-tracking behavior and dopamine turnover in the nucleus accumbens

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Abstract

Individual variation in the attribution of motivational salience to reward-related cues is believed to underlie addiction vulnerability. Pavlovian conditioned approach (PCA) measures individual variation in motivational salience by identifying rats that are attracted to and motivated by reward cues (sign-trackers; STs) or motivationally fixed on the reward itself (goal-trackers; GTs). Previously, it has been demonstrated that STs are more vulnerable to addiction-like behavior[29]. Moreover, STs release more dopamine (DA) in the nucleus accumbens (NAc) than GTs in response to reward-related cues, and sign- but not goal-tracking behavior is DA-dependent. In the present study, we investigated whether the ventral hippocampus (vHPC), a potent driver of DA activity in the NAc, modulates the acquisition and expression of PCA behavior. In Experiment 1, lesions of the ventral, but not dorsal or total hippocampus, before the

[29] These observations have been observed following short-access schedules of cocaine self-administration. Following a prolonged intermediate-access schedule of cocaine self-administration, STs and GTs do not differ on measures of addiction-like behavior, such as increased cocaine demand (a behavioral economics measure) and cue-induced reinstatement of cocaine-seeking behavior (Kawa et al., 2016). Therefore, STs may only show vulnerability to addiction-like behaviors in some situations and not others.

acquisition of PCA behavior decreased the acquisition of sign-tracking behavior. In Experiment 2, lesions of the vHPC after the acquisition of PCA behavior did not affect the expression of sign- or goal-tracking behaviors (or conditioned reinforcement). In addition, temporary inactivation of the ventral subiculum, the main output pathway of the vHPC, did not affect the expression of sign- or goal-tracking behaviors. High-performance liquid chromatography of NAc tissue punches revealed that ventral hippocampal lesions decreased levels of the DA metabolite, homovanillic acid, and the homovanillic acid/DA ratio (a marker of DA release and metabolism) in only STs, but levels of 3,4-dihydroxyphenylacetic acid (another DA metabolite) were unchanged. These results suggest that the vHPC is important for the acquisition, but not expression, of sign-tracking behavior, possibly because of altered DA in the NAc.

Introduction

It is important to understand why some individuals can try potentially addictive drugs without developing addiction, while others try the same drug and are quickly rendered incapable of controlling their urges to repeat the experience. Individual variation in the incentive-motivational value of reward-related cues is believed to contribute to this individual vulnerability to addiction (Flagel et al., 2009). Incentive salience is typically measured using a PCA procedure, during which a CS (e.g., a lever) response-independently predicts the delivery of a US (e.g., a food pellet). During training, three behavioral phenotypes emerge: STs (CS-directed CRs), GTs (US-directed CRs), and IRs (both CRs). STs are more susceptible to cue-related, addiction-like behaviors than GTs or IRs, exhibiting more cue-induced reinstatement of drug-seeking (Saunders & Robinson, 2010; Yager et al., 2015) and even seeking drug cues despite adverse consequences (Saunders et al., 2013).

Understanding the brain regions that are activated during sign-tracking behavior is critical in determining the biological basis of individual variation in incentive salience and drug vulnerability. Previously, it has been demonstrated that food and drug cues activate the motive circuit in STs (Flagel et al., 2011a; Yager et al., 2015). In addition, lesion studies have confirmed the importance of brain regions (e.g., NAc, basolateral amygdala, and paraventricular nucleus of the thalamus) within the motive circuit in the acquisition of sign-tracking behavior (Chang et al., 2012; Haight et al., 2015). Other regions with important connections to this circuit have been implicated in sign-tracking behavior as well, such as the HPC (Ito et al., 2005), although little is known regarding how subregions of this heterogeneous structure individually regulate sign-tracking behavior.

The HPC can be broadly divided into dorsal and ventral regions, which have unique connectivity and functions (Fanselow and Dong, 2010). For example, efferents from the vHPC, but not dHPC, are potent drivers of dopaminergic activity in the NAc, and lesions of the vHPC, but not dHPC, decrease DA levels in the NAc (Lipska et al., 1992; Lipska et al., 1991). Moreover, stimulation of the ventral subiculum (vSUB), the main output structure of the HPC, increases DA release in the NAc (Blaha et al., 1997; Taepavarapruk et al., 2000). Because STs release more DA in the NAc than GTs in response to reward-related cues, and sign-tracking, but not goal-tracking, behavior is DA-dependent (Flagel et al., 2011b; Saunders & Robinson, 2012), it is possible that increased ventral hippocampal activity regulates NAc DA in STs, thereby enhancing the incentive-motivational value of reward-related cues. A reduction of activity in the vHPC would therefore be expected to decrease dopaminergic activity in response to reward-related cues and decrease sign-tracking behavior.

The present study aimed to determine how the HPC and its subregions contribute to the acquisition and expression of PCA behavior. We hypothesized that lesions of the vHPC, but not dHPC, would decrease sign-tracking behavior during the acquisition and expression of PCA behavior. In Experiment 1, rats received sham surgery or lesions of the vHPC, dHPC, or total HPC, before undergoing five daily sessions of PCA training. Based on our results from Experiment 1, in Experiment 2, rats underwent seven daily sessions of PCA training, followed by sham surgery or vHPC lesions, then tested for the expression of PCA behavior and conditioned reinforcement. Rats undergoing sham surgeries were implanted with cannulas targeting the vSUB, allowing us to temporarily inactivate and further probe the importance of the vHPC during the expression of PCA behavior. Following behavioral testing, tissue punches of the NAc were analyzed for monoamine and metabolite concentrations of DA as well as norepinephrine (NE) and serotonin (5-HT) using HPLC.

Materials and Methods

Animals

Adult male Sprague Dawley rats (275-300g) were purchased from Harlan Laboratories and Charles River Laboratories to increase phenotypic diversity (Fitzpatrick et al., 2013). Rats were maintained on a 12:12-hr light/dark cycle, and food and water were available *ad libitum* for the duration of experimentation. Rats were acclimatized to the housing colony for two days prior to handling. All procedures were approved by the University Committee on the Use and Care of Animals (University of Michigan; Ann Arbor, MI).

Drugs

N-methyl-D-aspartate (NMDA; #M3262; Sigma-Aldrich, Inc.; St. Louis, MO), lidocaine hydrochloride (#L5647; Sigma-Aldrich, Inc.), and Pontamine Sky Blue (PSB; also known as Chicago Sky Blue 6B; #C8679); Sigma-Aldrich, Inc.) were used. NMDA was dissolved in saline to make a 0.09 M solution (pH = 7.34-7.36). Lidocaine hydrochloride was dissolved in saline to make a 20% solution (200 mg/mL; pH = 5.0), as previously described (Kantak et al., 2002). Saline was used as the vehicle control.

Pavlovian conditioned approach: Apparatus

Modular conditioning chambers (24.1 cm width × 20.5 cm depth x 29.2 cm height; MED Associates, Inc.; St. Albans, VT) were used for Pavlovian conditioning. Each chamber was inside a sound-attenuating cabinet equipped with a ventilation fan to provide ambient white noise. Each chamber was equipped with a pellet magazine, an illuminated, retractable lever (counterbalanced on the left or right of the pellet magazine), and a red house light on the wall opposite of the pellet magazine. When inserted into the chamber, the retractable lever was illuminated by an LED light within the lever housing. A pellet dispenser delivered banana-flavored food pellets into the pellet magazine. An infrared sensor inside the pellet magazine measured head entries into the pellet magazine.

Pavlovian conditioned approach: Procedure

For two days prior to pretraining, rats were familiarized with banana-flavored food pellets (45mg; Bioserv; Frenchtown, NJ) in their home cages. On the third day, rats were placed into the chambers and underwent one pretraining session during which the red house light remained on, but the lever was retracted. Fifty food pellets were delivered on a variable time (VT) 30-s schedule (i.e., one food pellet was delivered on average every 30 s, but actual delivery

varied between 0-60 s). All rats consumed all the food pellets by the end of the pretraining session. Each trial during a PCA training session consisted of extension of the illuminated lever (the CS) into the chamber for 8 s on a VT 90-s schedule (i.e., one food pellet was delivered on average every 90 s, but actual delivery varied between 30-150 s). Retraction of the lever was immediately followed by the response-independent delivery of one food pellet (the US) into the pellet magazine. Each test session consisted of 25 trials of CS-US pairings, resulting in a total session length of approximately 40 min. Each rat consumed all the food pellets that were delivered.

Conditioned reinforcement: Procedure

For testing of conditioned reinforcement in Experiment 2, which lasted 40 min, each chamber was equipped with two nose-poke ports adjacent to a lever located in the center of the front wall of the conditioning chamber. Nose pokes into the active nose-poke port resulted in presentation of the lever-CS for 2 s on a fixed-ratio 1 (FR1) schedule, whereas nose pokes in inactive nose-poke port did not result in presentation of the lever-CS.

Surgery

For all surgical procedures, rats were administered carprofen (2 mg/kg; s.c.) for analgesia, anesthetized using a ketamine (90 mg/kg; i.p.) and xylazine (10 mg/kg; i.p.) solution, and placed in a stereotaxic frame (David Kopf Instruments; Tujunga, CA). For excitotoxic lesions, injectors (33-gauge; Plastics One, Inc.; Roanoke, VA), connected via PE-20 tubing to microsyringes (1 µL; Hamilton Company; Reno, NV) in a pump controller (Harvard Instruments; Holliston, MA) were lowered into the HPC. NMDA (0.09 M) was infused bilaterally. For dHPC infusions, 0.4 µL was infused at a rate of 0.4 µL/min per infusion site, and infusion cannulas

were left in place for an additional 4 min before removal. For vHPC infusions, 0.2 µL was infused at a rate of 0.2 µL/min per infusion site, and infusion cannulas were left in place for an additional 2 min before removal. In Experiment 1, lesions were targeted at the dHPC (two lesion sites), vHPC (four lesion sites), or total HPC (a combination of all six sites; see Table S3.1). previously described lesions for a total of six sites). AP coordinates were measured from bregma, DV coordinates were measured from dura, and all coordinates were referenced to Paxinos & Watson (2007). In Experiment 1, surgical controls received incisions and burr holes on the skull over the HPC, but no infusions were performed. In Experiment 2, surgical controls were implanted bilaterally with guide cannulas (26-gauge, cut 7 mm below the pedestal; Plastics One, Inc.) targeted at the vSUB (AP: -5.6 mm, ML: ±4.6 mm, DV: -5.8 mm). Injection cannulas that would later be inserted into these guide cannulas were 8 mm long, overhanging 1 mm past the guide cannula tip. Two jeweler's screws were secured both anterior and posterior to the burr holes, and dental acrylic (Fastray™; Harry J. Bosworth Company; Skokie, IL) was applied around the guide cannulas and screws to form a head stage. Dummy cannulas (8 mm, 1 mm overhang; Plastics One, Inc.) were inserted into the guide cannulas and removed only during testing. Following all surgeries, rats recovered for seven days prior to behavioral testing with food and water available *ad libitum*.

Experiment 1: Procedure

Rats underwent pre-conditioning hippocampal lesions (sham, dHPC, vHPC, or total HPC) and, following the surgical recovery period, five daily PCA training sessions to determine the effect of hippocampal lesions on the acquisition of PCA behavior. Following the last behavioral session, rats were transcardially perfused with a 4% paraformaldehyde solution (in 0.1 M phosphate-buffered saline; pH = 7.34-7.36). Brains were removed, immediately post-fixed

in 4% paraformaldehyde solution for one hour, and transferred to a 20% sucrose solution until saturated. Next, tissues were sectioned on a cryostat (35 μM; Leica CM1850; Leica Microsystems, Inc.; Buffalo Grove, IL), stained with Cresyl Violet (#C5042; Sigma-Aldrich, Inc.), and used for histological verification of lesions. Unilateral lesions were excluded from analysis (vHPC, n = 2; dHPC, n = 4, total HPC, n = 2).

Experiment 2: Procedure

Rats underwent seven daily PCA training sessions before hippocampal lesions (sham and vHPC). In this experiment, surgical controls were implanted with bilateral cannulas targeted at the vSUB. Following the seven-day surgical recovery period, rats underwent an additional seven daily PCA training sessions to determine the effect of post-conditioning lesions on the expression of PCA behavior[30]. Twenty-four hours later, rats underwent two more daily PCA training sessions during which surgical controls received bilateral infusions (0.5 μL/side) of a 20% lidocaine solution (100 μg/side; in saline) or vehicle at an infusion rate of 0.5 μL/min for 1 min in a counter-balanced manner (i.e., rats received lidocaine or saline during each session in a counter-balanced manner)[31]. For statistical analysis and graphical presentation, both sessions were collapsed into vehicle and lidocaine groups. Infusion cannulas were left in place for an additional minute to allow diffusion, and rats immediately underwent testing. Lesion rats also underwent two PCA training sessions to ensure that all subjects received equal amounts of PCA training. Twenty-four hours later, all rats underwent a test for conditioned reinforcement. Five

[30] Rats generally undergo five to seven daily PCA sessions during experiments on the acquisition or expression of PCA behavior. Sign-tracking and goal-tracking behavior generally asymptotes on the fifth session, and it is personal preference what number of sessions to choose.

[31] Functional assays are important to perform following cannulation of brain regions to determine whether a drug and dose truly inactivate the brain region. The only well-established behavioral outcome, however, of ventral subicular inactivation is decreased context-induced reinstatement of drug-seeking behavior (Bossert et al., 2016; Bossert & Stern, 2014; Marchant et al., 2016), which is not appropriate as a simple functional assay. In the future, if a simple functional assay is discovered (e.g., inactivation of the ventral subiculum reliably affects feeding behavior, locomotor activity, etc.), it should be incorporated as a positive control.

days after the conditioned reinforcement test, sham rats received bilateral infusions (0.5 µL/side) of a 2% pontamine sky blue solution at a flow rate of 0.5 µL/min to dye and visualize infusion sites in the vSUB. Infusion cannulas were left in place for an additional minute to allow diffusion. Next, all rats were rapidly decapitated, and brains were removed and placed into an ice-cold adult rat brain matrix. A 1-mm thick tissue section including the NAc (AP: +1.2 mm) was biopsied using 2-mm diameter tissue biopsy punches. Whole brains posterior to this section were kept for histological verification of lesions and cannula placements. All tissue was immediately flash-frozen in isopentane (Sigma Aldrich, Inc.) over dry ice. Tissue punches were later processed to quantify levels of the following monoamines and their metabolites: dopamine (DA), 3,4-dihydrophenylacetic acid (DOPAC), homovanillic acid (HVA), 3-methoxytryamine (3-MT), norepinephrine (NE), serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA). Tissue sections were stained with Cresyl Violet (Sigma-Aldrich, Inc.) and used for histological verification. For lesions, unilateral lesions were excluded from further analysis (ST, n = 2). For cannula placement, subjects with an absence of cannula tracks and dye were kept for statistical comparison between lesion and sham control; however, they were excluded from further analysis for testing of temporary inactivation of the vSUB (GT, n = 1; ST, n = 2).

High-performance liquid chromatography (HPLC)

Frozen tissue samples were weighed and sonically disrupted in 100 µL 0.2N $HClO_4$. Samples were then centrifuged at 4°C at 12,100 g for 10 min. A 50 µL aliquot of supernatant was removed and monoamine analysis was performed utilizing a Dionex Ultimate 3000 UHPLS system (Thermo Fisher Scientific, Inc.; Waltham, MA). At a flow rate of 0.6 mL/min, 10 µL of each sample was injected with an auto-sampler maintained at 4°C and with a 100 µL sample

loop onto a C18-RP (2-µL diameter) column maintained at 25°C. Test Mobile Phase (Thermo Fisher Scientific, Inc.) containing acetonitrile, phosphate buffer, and an ion-pairing reagent was used. Coulometric detection was achieved with an ultra-analytical dual electrode cell (Thermo Fisher Scientific, Inc.) set at -175 mV (reference electrode) and 300 mV (working electrode). Gain settings for both electrodes were set to 100 µA. A guard cell set to 350 mV and guard column (2.1/3.0 mm ID; Thermo Fisher Scientific, Inc.) were also used. Monoamine and metabolite levels were quantified by comparison to external standards (Sigma-Aldrich, Inc.) performed in sequence with tissue samples and corrected for tissue weight. The order of sample analysis within the run was randomized. Chromatograms were obtained and analyzed using Chromeleon 7 Chromatography Data System software (Dionex Corp.; Sunnyvale, CA) with peak heights being the output measure. The detection threshold was set at 5 nA, and samples with peak heights lower than 5 nA were excluded from analysis. Monoamine and metabolite levels (DA, DOPAC, HVA, 3-MT, NE, 5-HT and 5-HIAA) were expressed as absolute tissue values (ng neurochemical / mg wet tissue weight; ng/mg).

Statistical analysis

PCA behavior was scored using an index that incorporates the number, latency, and probability of lever presses (sign-tracking CR) and magazine entries (goal-tracking CR) during CS presentations within a session (Meyer et al., 2012). Briefly, we averaged together the response bias (i.e., [number of lever presses – number of magazine entries] / [lever presses + magazine entries]), latency bias (i.e., [magazine entry latency – lever press latency]/8), and probability difference (i.e., lever press probability – magazine entry probability). The PCA index scores behavior from +1.0 (absolute sign-tracking) to -1.0 (absolute goal-tracking), with 0 representing no bias. When applicable, the average PCA index scores of Sessions 6 and 7 were

used to classify rats as STs (score $\geq$ 0.5), GTs (score $\leq$ -0.5), and IRs (-0.5 < score < 0.5). In Experiment 2, IRs were excluded from further experimentation and statistical analysis. For conditioned reinforcement, active – inactive nose-poke port responses were quantified and compared between groups.

SPSS (Version 22; IBM, Inc.) was used for all statistical analyses. For all linear mixed models, the covariance structure was selected based upon Akaike's information criterion (i.e., the lowest number criterion using a given covariance structure represents the highest quality statistical model; Akaike, 1974). In Experiment 1, PCA behavior across training sessions was analyzed using a linear mixed model with Lesion (sham, vHPC, dHPC, total HPC) as the main factor. In Experiment 2, PCA behavior across training sessions was analyzed using a linear mixed model with Phenotype (GT and ST) and Lesion (sham and vHPC) as factors. For the test of temporary inactivation, PCA behavior was analyzed using a three-way analysis of variance (ANOVA) with Phenotype (GT and ST), Drug (Saline and Lidocaine), and Order (First and Second) as factors. For the conditioned reinforcement test, nose-poke port responses (active – inactive) were analyzed using a two-way ANOVA with Phenotype (GT and ST) and Lesion (sham and vHPC) as factors. For HPLC analysis, neurochemicals were analyzed using a two-way ANOVA with Lesion (sham and vHPC) and Phenotype (GT and ST) as factors. With a significant ANOVA, multiple comparisons were performed using Fisher's Least Significant Difference (LSD) post hoc test.

Results

Experiment 1

Excitotoxic lesions of the hippocampus (sham, n = 13; vHPC, n = 11; dHPC, n = 8; total HPC, n = 10) were performed prior to five daily PCA training sessions to determine whether the

lesions affected the acquisition of PCA behavior. Figure 3.1 shows a schematic representation of the extent of NMDA-induced lesions in the vHPC, dHPC, and total HPC. Figure 3.2 shows that there was an effect of Lesion on lever press number ($F_{(3,38)} = 2.85$, $p = 0.05$), latency ($F_{(3,38)} = 3.34$, $p = 0.029$), and probability ($F_{(3,38)} = 3.31$, $p = 0.03$) as well as magazine entry number ($F_{(3,38)} = 3.01$, $p = 0.042$), and latency ($F_{(3,38)} = 3.55$, $p = 0.023$), which were averaged over Sessions 4 and 5. There was no effect of Lesion on magazine entry probability ($F_{(3,38)} = 2.76$, $p = 0.055$). Post hoc comparisons revealed that, compared to the sham condition, vHPC lesions decreased lever press number ($p = 0.007$), latency ($p = 0.009$), and probability ($p = 0.006$) as well as increased magazine entry number ($p = 0.019$) and latency ($p = 0.007$). Lesions of the dHPC, compared to the sham condition, only increased magazine entry number ($p = 0.02$) and latency ($p = 0.017$). Lesions of the total HPC, compared to the sham condition, had no effect on any variable.

Across sessions, there was an interaction of Lesion and Session on lever press number ($F_{(16,148.84)} = 2.95$, $p = 3.06 \times 10^{-4}$), latency ($F_{(16,143.48)} = 2.99$, $p = 2.59 \times 10^{-4}$), and probability ($F_{(16,142.65)} = 2.70$, $p = 0.001$) as well as magazine entry number ($F_{(16,139.94)} = 3.89$, $p = 5.0 \times 10^{-6}$), latency ($F_{(16,138.61)} = 4.26$, $p = 1.0 \times 10^{-6}$), and probability ($F_{(16,138.09)} = 4.11$, $p = 2.0 \times 10^{-6}$). Post-hoc comparisons revealed that, compared to the sham condition, vHPC lesions decreased lever press number ($ps < 0.01$), latency ($ps < 0.01$), and probability ($ps < 0.01$) during Sessions 3-5 as well as increased magazine entry number ($ps < 0.05$), latency $ps < 0.05$), and probability ($ps < 0.05$) during Sessions 4-5. dHPC lesions, compared to the sham condition, increased magazine entry number ($ps < 0.05$), latency ($ps < 0.05$), and probability ($ps < 0.05$) during Sessions 3-5. Lesions of the total HPC, compared to the sham condition, had no effect on any variable. Figure 3.3 shows that there was an interaction of Lesion and Session on PCA index scores ($F_{(16,142.85)} =$

3.02, $p = 2.33 \times 10^{-4}$). Post-hoc comparisons revealed that only vHPC lesions, compared to the sham condition, decreased PCA index score (ps < 0.01) during Sessions 3-5. Lastly, there was no effect of Lesion on non-CS magazine entries over Sessions 1-5 (data not shown; $F_{(3,61.75)} = 0.80$, $p = 0.50$).

Experiment 2

Before surgery, rats underwent PCA training and were classified as STs, GTs, and IRs; however, only STs (n = 24) and GTs (n = 15) were used for further experimental testing. During seven daily PCA training sessions, STs and GTs differed in their lever press number ($F_{(1,37.63)} = 29.01$, $p = 4.0 \times 10^{-6}$), latency ($F_{(1,37.17)} = 37.71$, $p = 3.98 \times 10^{-7}$), and probability ($F_{(1,36.03)} = 48.61$, $p = 3.56 \times 10^{-8}$) as well as their magazine entry number ($F_{(1,47.15)} = 43.17$, $p = 3.62 \times 10^{-8}$), latency ($F_{(1,46.28)} = 54.81$, $p = 2.19 \times 10^{-9}$), and probability ($F_{(1,44.76)} = 53.70$, $p = 3.44 \times 10^{-9}$). STs and GTs differed on their PCA index scores over the seven daily PCA training sessions (Figure 5*A*; $F_{(1,40.10)} = 74.51$, $p = 1.10 \times 10^{-10}$), and the average PCA index scores of Sessions 6 and 7 were used to determine PCA phenotypes. Following training, rats underwent vHPC lesions (Figure 3.4*A*; ST, n = 12; GT, n = 6) or sham surgeries (ST, n = 12; GT, n = 9), which included cannulas targeted at the vSUB (Figure 3.4*B*). Following a surgical recovery period of seven days, rats underwent an additional seven daily PCA training sessions to determine the effect of vHPC lesions on the expression of PCA behavior. In GTs, there was no effect of Lesion on lever press number ($F_{(1,12.89)} = 0.51$, $p = 0.49$), latency ($F_{(1,13.95)} = 2.58$, $p = 0.40$), and probability ($F_{(1,13.86)} = 0.63$, $p = 0.44$), or magazine entry number ($F_{(1,14.24)} = 1.55$, $p = 0.23$), latency ($F_{(1,17.49)} = 0.58$, $p = 0.46$), and probability ($F_{(1,15.86)} = 0.50$, $p = 0.49$). Similarly, in STs, there was no effect of Lesion on lever press number ($F_{(1,23.19)} = 0.51$, $p = 0.48$), latency ($F_{(1,22.37)} = 0.02$, $p = 0.88$), and

probability ($F_{(1,20.1)} = 0.71$, $p = 0.79$) or magazine entry number ($F_{(1,19.51)} = 0.001$, $p = 0.97$), latency ($F_{(1,24.28)} = 0$, $p = 1.0$), and probability ($F_{(1,20.9)} = 0.024$, $p = 0.88$). Furthermore, Figure 3.5*B* shows that there was no effect of Lesion on PCA index scores in GTs ($F_{(1,14.29)} = 0.88$, $p = 0.36$) or STs ($F_{(1,17.62)} = 4.0 \times 10^{-5}$, $p = 1.0$).

Twenty-four hours after the final session of PCA training, sham controls were infused bilaterally in the vSUB with a 20% lidocaine solution or vehicle and tested in a counter-balanced manner across two PCA training sessions, which served as the test of temporary inactivation. Figure 3.4*B* shows a schematic representation of injection sites in the vSUB. Figure 3.5*C* shows that temporary inactivation of the vSUB did not affect the expression of PCA behavior in GTs (effect of Drug: $F_{(1,9)} = 0.02$, $p = 0.89$) or STs (effect of Drug: $F_{(1,13)} = 0.91$, $p = 0.36$). In addition, there was no effect of Order (i.e., receiving lidocaine infusion on the first test session and vehicle infusion on the second test session or vice versa; $F_{(1,22)} = 0.007$, $p = 0.93$). Rats with vHPC lesions underwent two PCA training sessions as well to ensure that all rats received similar behavioral testing procedures. Twenty-four hours later, all rats underwent a test for conditioned reinforcement to determine whether vHPC lesions affected the conditioned reinforcing properties of the lever. As expected, Figure 3.5*D* shows that STs exhibit greater conditioned reinforcement than GTs (i.e., higher active – inactive nose-poke port responses; effect of Phenotype: $F_{(1, 32)} = 6.28$, $p = 0.018$). However, vHPC lesions did not affect conditioned reinforcement in GTs (effect of Lesion: $F_{(1,13)} = 0.91$, $p = 0.36$) or STs (effect of Lesion: $F_{(1, 14)} = 0.51$, $p = 0.49$).

Using tissue punches of the NAc taken five days after the completion of testing (Figure 3.4*C*), HPLC analysis revealed an effect of Lesion on HVA levels (Figure 3.6*A*; $F_{(1,30)} = 7.63$, $p = 0.0097$) and HVA/DA (a marker of DA release and metabolism; Figure 3.6*B*; $F_{(1,30)} = 6.90$, $p =$

0.013). Post hoc comparisons showed that lesions decreased HVA levels in STs ($p = 0.0014$), but not GTs ($p = 0.51$), and decreased HVA/DA in STs ($p = 0.011$), but not GTs ($p = 0.26$). In addition, there was an effect of Lesion on NE levels across phenotypes (Figure 3.6*C*; $F_{(1,30)} = 4.21$, $p = 0.049$). There was no effect of Lesion (data not shown) on levels of DA ($F_{(1,30)} = 0.008$, $p = 0.93$), DOPAC ($F_{(1,30)} = 0.004$, $p = 0.95$), 3-MT ($F_{(1,30)} = 0.97$, $p = 0.33$), 5-HT ($F_{(1,30)} = 1.37$, $p = 0.25$), or 5-HIAA ($F_{(1,30)} = 0.22$, $p = 0.64$).

Discussion

The present study investigated the role of the HPC in the acquisition and expression of PCA behavior. In Experiment 1, we found that vHPC lesions decreased sign-tracking behavior and increased goal-tracking behavior during the acquisition of PCA training. Moreover, dHPC lesions also increased goal-tracking behavior, while total HPC lesions had no effect. Consequently, only the vHPC was further investigated in Experiment 2. During this experiment, we demonstrated that neither vHPC lesions nor temporary inactivation of the vSUB, the main output structure of the vHPC, affected the expression of PCA behavior. Postconditioning lesions of the vHPC also did not affect conditioned reinforcement. Finally, lesions of the vHPC decreased NE across both phenotypes, and decreased HVA and HVA/DA (a marker of DA release and metabolism) in STs, but not GTs.

In agreement with our hypothesis, vHPC lesions decreased the acquisition of sign-tracking (and increased the acquisition of goal-tracking) behavior in rats. vHPC lesions may have decreased sign-tracking behavior by attenuating activity of dopaminergic cell bodies in the VTA (Floresco et al., 2001) and/or VTA terminals in the NAc (Blaha et al., 1997). Alternatively, the loss of glutamatergic input from the vHPC to medium spiny neurons in the NAc may have also

decreased sign-tracking behavior by disrupting gating and outflow of NAc signals (French & Totterdell, 2002).

Interestingly, total HPC lesions did not have a similar effect as vHPC lesions, even though the region is included in the total HPC lesion. Previously, it has been reported that neonatal vHPC-induced amphetamine hyperlocomotion is abolished if the lesion encompasses both the vHPC and dHPC, similar to our total HPC lesion (Swerdlow et al., 2001). The authors suggested that the larger lesions may diminish a complex inhibitory control from the vHPC to dHPC that is critical for vHPC-induced behavioral alterations. A similar phenomenon may have occurred with our total HPC lesions, although future experiments, such as disconnection procedures, would need to be performed to investigate this question.

Unexpectedly, dHPC lesions also increased the acquisition of goal-tracking behavior. The dHPC has a role in the predictive value of a CS during Pavlovian conditioning (Munera et al., 2001) and cue-reward associations (Jacquet et al., 2013); therefore, it may seem counterintuitive that dHPC lesions increased goal-tracking behavior. It is possible, however, that neural activity in the dHPC competes with other brain regions during the acquisition of goal-tracking behavior, and dHPC lesions release these regions, facilitating goal-tracking behavior. For example, in an appetitive, conditioned cue preference procedure, it has been suggested that competition between the HPC and lateral amygdala balances learning strategies with the lateral amygdala facilitating approach behavior to the site of food delivery in the absence of hippocampal activity (Chai & White, 2004; Gaskin & White, 2006).

In addition, our data seem to be at odds with previous reports that total HPC lesions increase sign-tracking behavior (Ito et al., 2005). It is possible that this incongruence arises from experimental differences in CS-US proximity (Christie, 1996), CS modality (Beckmann &

Chow, 2015), or even rat strain differences in lesion-induced behavioral alterations (Lipska & Weinberger, 1995; Wood et al., 2001). However, the most likely explanation is that the PCA procedure employed by Ito et al. involves discrimination between a CS+ and CS- that are identical except for their physical location on the left or right side of the front wall. Rats in their study were required to be at the opposite side of the testing chamber before the start of each trial, which ensured that the CS+ was always on the same side of their body. Therefore, to discriminate between the two stimuli, the rats could either identify the actual location of the CS within the chamber, in a hippocampal-dependent manner, or simply identify whether the CS was on the left or right of their visual field. The latter strategy is both more efficient and hippocampal-independent, so elimination of competition from slower hippocampal-dependent processes would facilitate performance of such a task (Saunders & Robinson, 2012). The PCA procedure in the present study did not involve discrimination and would therefore not be expected to improve in this manner with hippocampal damage.

Following PCA training, vHPC lesions did not affect the expression of sign- or goal-tracking behaviors. In addition, vHPC lesions did not affect the conditioned reinforcing properties of the lever, although, consistent with previous findings, STs displayed higher conditioned reinforcement than GTs (Robinson & Flagel, 2009). Similarly, temporary inactivation of the vSUB did not affect the expression of either sign- or goal-tracking behaviors. Previously, it has been demonstrated that sign-tracking behavior becomes DA-independent after sufficient training (Clark et al., 2013; Darvas et al., 2014). Therefore, if the vHPC facilitates DA release in the NAc during PCA behavior, it may only be required during acquisition, and not

expression following extended training[32], during which dopaminergic activity is no longer required for the maintenance of conditioned responding.

Our neurochemical results agree with previous findings that HPC lesions decrease DA metabolites and NE in the NAc (Springer & Isaacson, 1982). Interestingly, we demonstrated that vHPC lesions attenuate HVA and HVA/DA in STs, but not GTs. Individual differences in the functional connectivity between the HPC, NAc, and VTA have been demonstrated in humans (Kahn and Shohamy, 2013), and differential connectivity within these regions may have resulted in our observed differences in HVA and HVA/DA following vHPC lesions. In the current experiment, we were unable to determine through which pathways the vHPC influences dopaminergic activity in the NAc; however, it is known that the vHPC modulates both dopaminergic cell bodies in the VTA, which project to the NAc (Floresco et al., 2001), and VTA terminals in the NAc (Blaha et al., 1997). Regarding NE, the NAc receives primary noradrenergic input from the A1 cell group of the nucleus tractus solitarius (NTS) in the ventral medulla (Delfs et al., 1998). Although the vHPC does not directly innervate the NTS, it can regulate NTS activity through an indirect circuit involving the infralimbic cortex, which projects directly to the NTS (Fisk & Wyss, 2000; Ruit & Neafsey, 1990). Transection of the ventral noradrenergic bundle, which contains noradrenergic projections from the NTS, decreases DA turnover in the NAc (O'Donohue et al., 1979). Therefore, the decreases in NE levels and DA turnover may be interconnected, especially given the fact that individual differences have been identified in the ability of noradrenergic compounds to modulate DA in the NAc (Tuinstra & Cools, 2000). Also, it is possible that tissue punches included adjacent NE-rich regions, such as the ventral pallidum or bed nucleus of the stria terminalis, which are contiguous with the NAc

[32] It should be noted that extended training in this experiment was considered over 15 daily sessions, which is much more than the training sessions in the current experiment.

(Berridge et al., 1997), receive noradrenergic innervation from the locus coeruleus (Jones & Yang, 1985), and may have contributed to observed differences in NE levels.

In summary, preconditioning lesions of the vHPC, but not dHPC or total HPC, decreased sign-tracking behavior while increasing goal-tracking behavior during the acquisition of PCA behavior. Moreover, postconditioning lesions of the vHPC did not affect the expression of PCA behavior or conditioned reinforcement. In addition, vHPC lesion-induced alterations in DA turnover and NE levels (Cogan et al., 2018) in the NAc may underlie the observed changes in PCA behavior. These results demonstrate that the vHPC is critical for the acquisition, but not expression, of sign-tracking behavior and adds to a growing body of literature indicating that the vHPC modulates motivated behavior and vulnerability to addiction (Jasinska et al., 2014; Robbins et al., 2008).

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Brain Region	Lesion (#)	AP (mm)	ML (mm)	DV (mm)
dHPC	1	-2.8	±1.6	-3.0
	2	-4.2	±2.6	-3.0
vHPC	1	-4.8	±4.8	-6.0
	2	-5.3	±4.6	-4.2
	3	-5.3	±4.6	-6.0
	4	-5.8	±4.6	-4.2
Total HPC	1	-2.8	±1.6	-3.0
	2	-4.2	±2.6	-3.0
	3	-4.8	±4.8	-6.0
	4	-5.3	±4.6	-4.2
	5	-5.3	±4.6	-6.0
	6	-5.8	±4.6	-4.2

Table 3.1. Stereotaxic coordinates of NMDA injections into the dorsal hippocampus (dHPC), ventral hippocampus (vHPC), or total HPC. AP – anterior-posterior, ML – medial-lateral, DV – dorsal-ventral. *Based on Paxinos & Watson, 2007.*

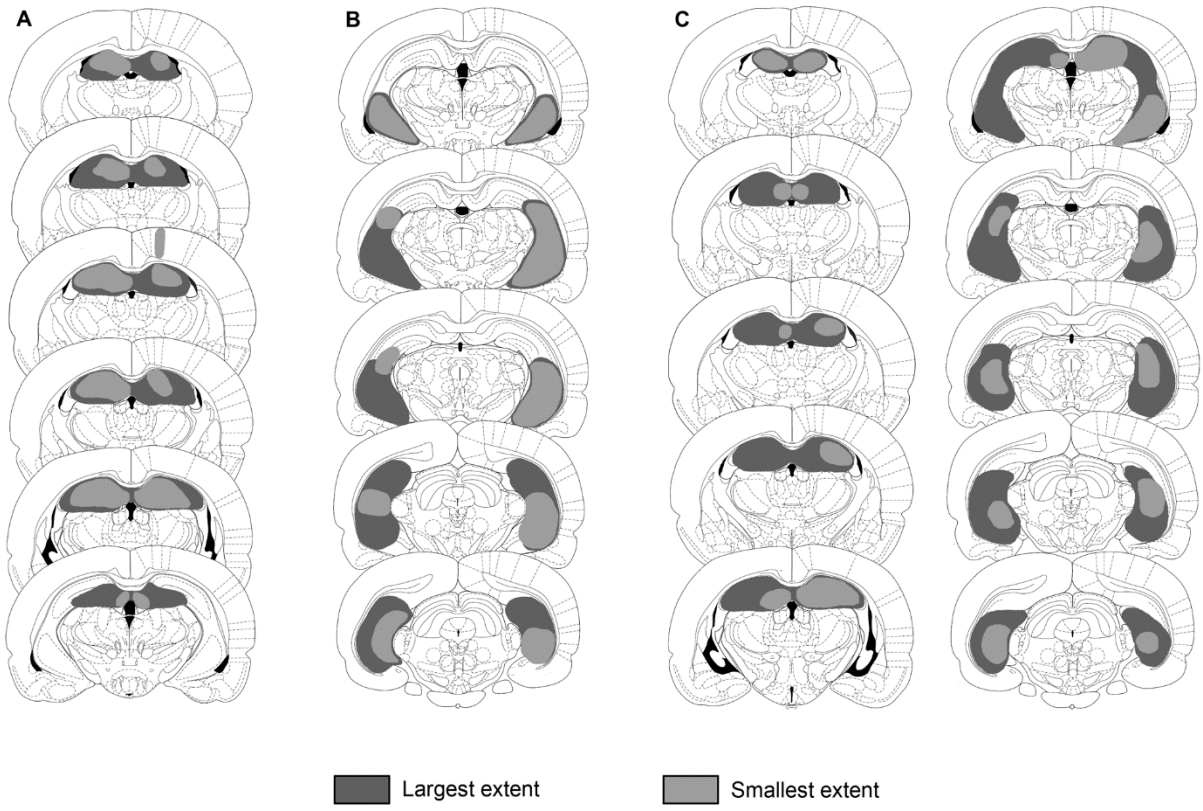

Figure 3.1. Schematic representation of NMDA lesions of the (A) dorsal, (B) ventral, and (C) total hippocampus. Cresyl violet-stained sections were visualized underneath a light microscope, and the largest (dark gray) and smallest (light gray) lesions were identified. Coronal sections are presented from 1.88 to 6.30 mm posterior to bregma. *Images adapted from Paxinos & Watson (2007).*

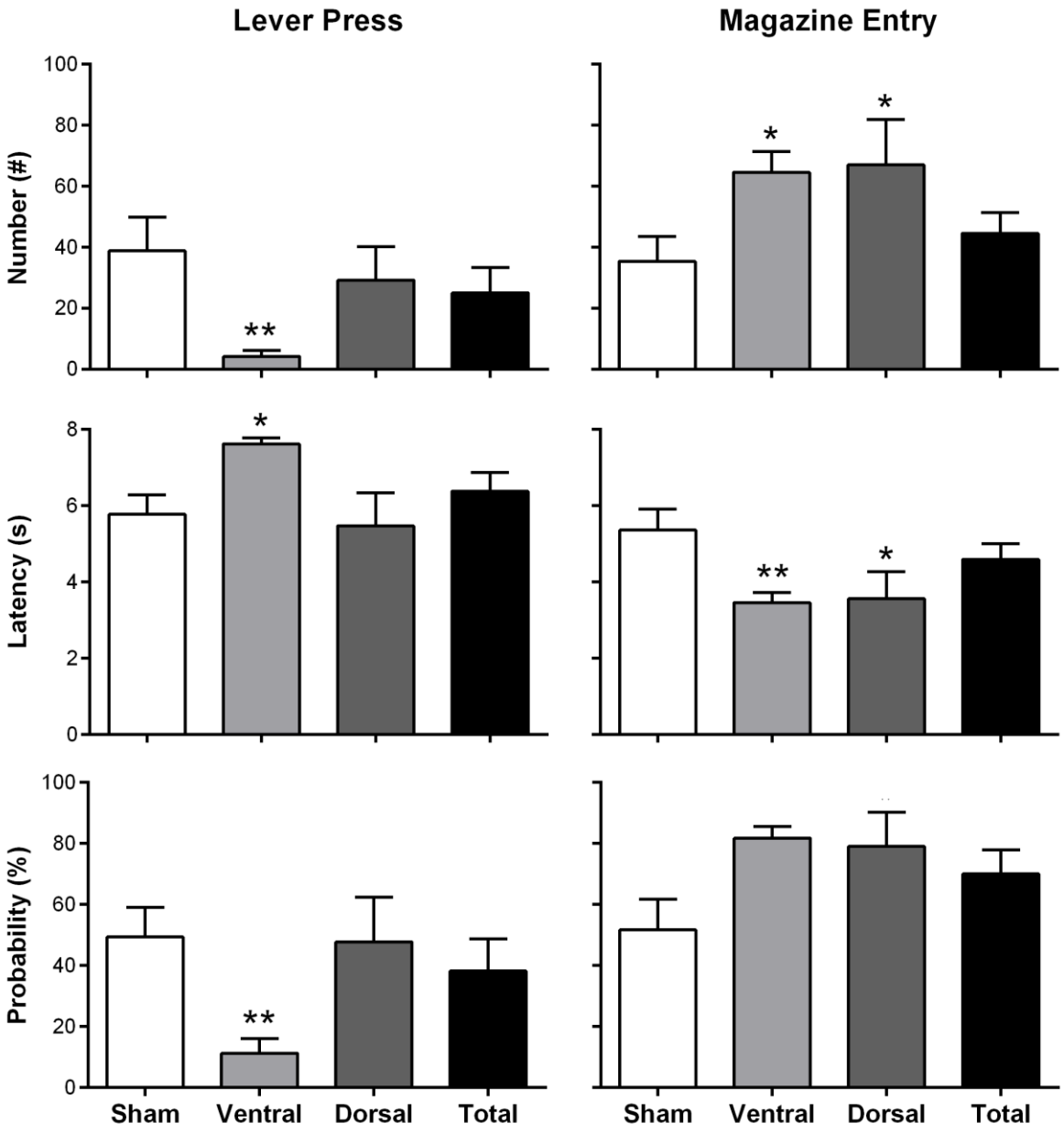

Figure 3.2. Ventral hippocampal lesions decrease the acquisition of Pavlovian conditioned approach (PCA) behavior. Before PCA approach training, surgeries were performed to lesion the ventral, dorsal, or total hippocampus. After surgical recovery, rats underwent five daily sessions of PCA training, and the number, latency, and probability of lever presses and magazine entries were averaged over Sessions 4 and 5. Data are mean and S.E.M. * — $p < 0.05$, ** — $p < 0.01$.

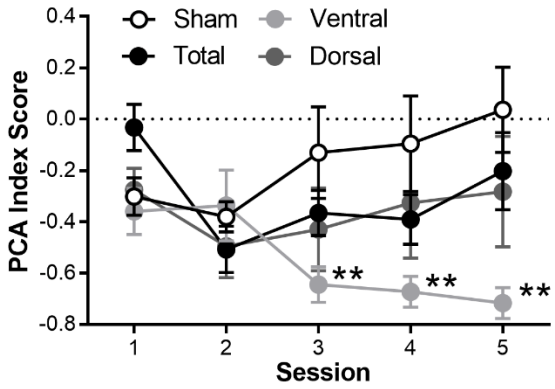

Figure 3.3. Ventral hippocampal lesions decrease Pavlovian conditioned approach (PCA) index scores. Rats underwent ventral, dorsal, or total hippocampal lesions before the acquisition of PCA training and were scored on the PCA index. Data are mean and S.E.M. ** — $p < 0.01$, compared to the sham group.

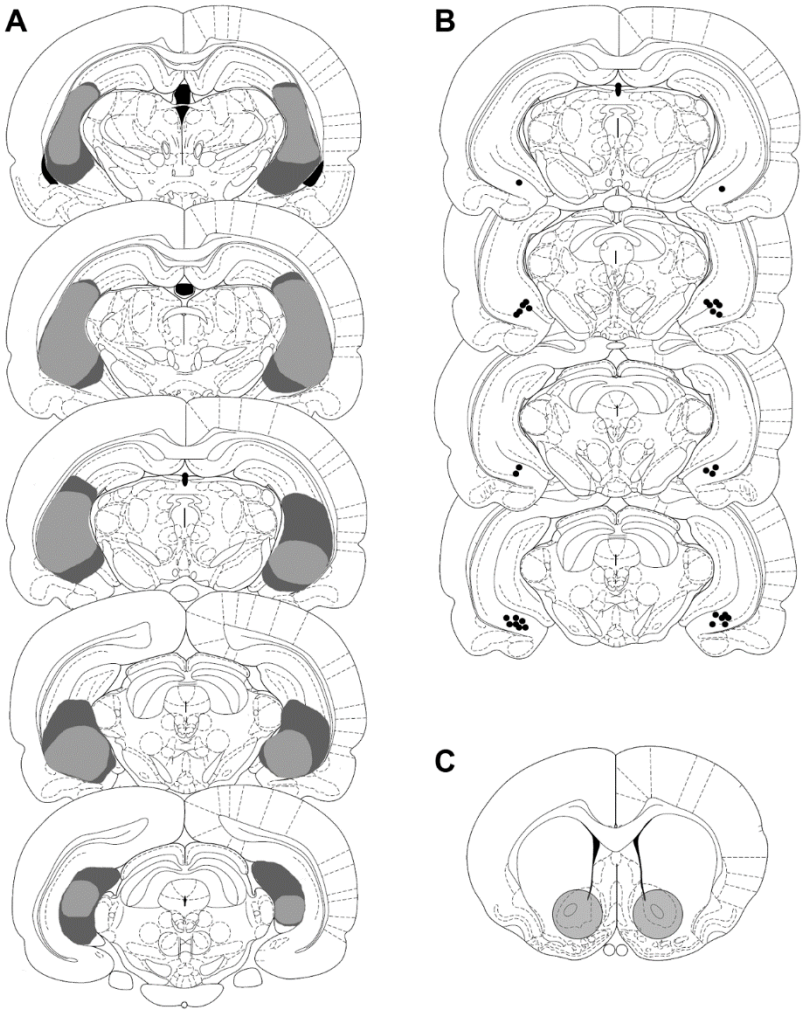

Figure 3.4. Schematic representation of ventral hippocampal lessions, cannulations of the ventral subiculum, and brain tissue punches of the nucleus accumbens. (A) NMDA-induced lesions of the ventral hippocampus (coronal sections presented from 4.52 to 6.30 mm posterior to bregma). (B) Cannulas bilaterally targeted at the ventral subiculum (coronal sections presented from 5.30 to 6.30 mm posterior to bregma). (C) Tissue punches taken from the nucleus accumbens (coronal section presented at 1.20 anterior to bregma). For ventral hippocampal lesions, the largest (dark gray) and smallest (light gray) lesions were identified. For cannula placements, black circles represent the location of the infusion cannula tip. For tissue punches, the location of the 2-mm tissue punch is shaded (light gray). *Images adapted from Paxinos & Watson (2007).*

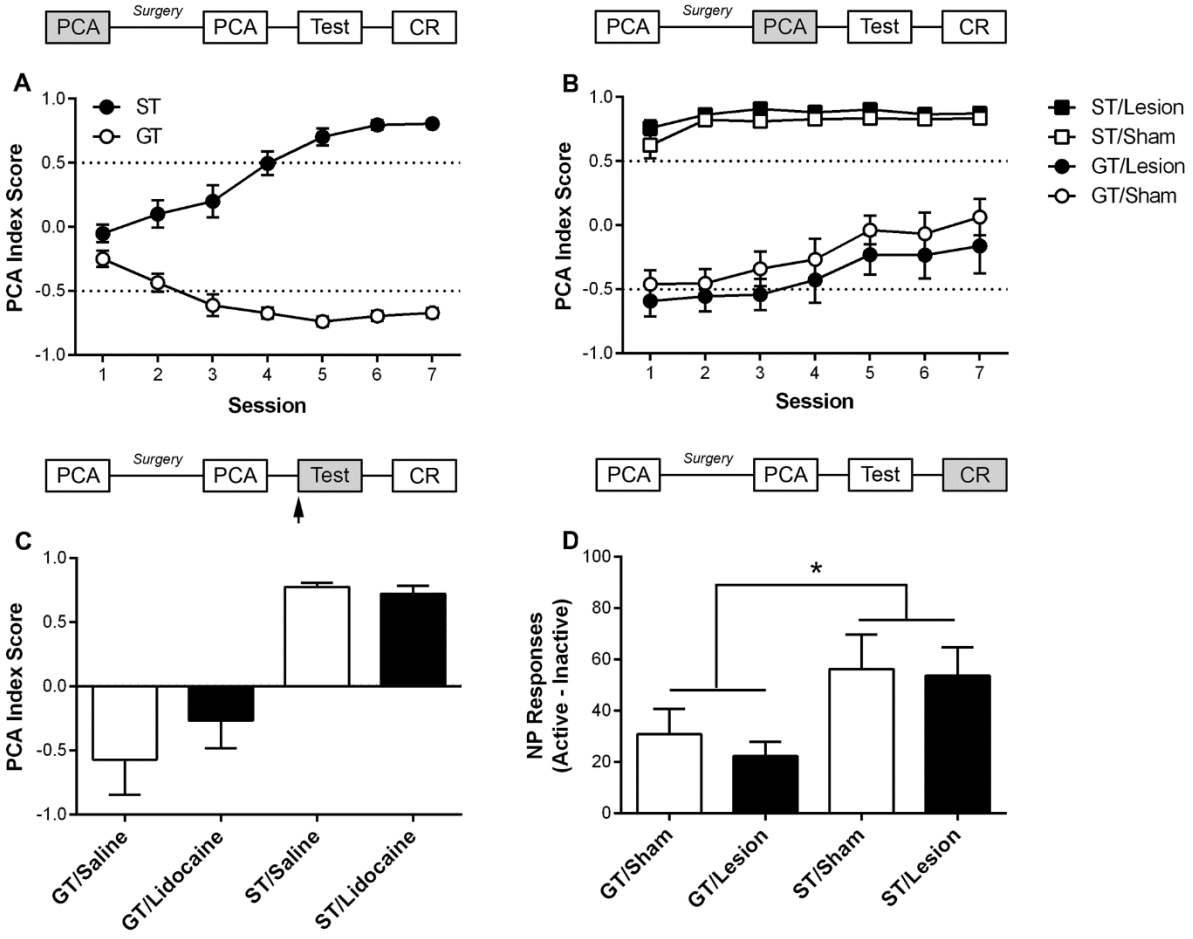

Figure 3.5. Ventral hippocampal lesions and temporary inactivation of the ventral subiculum do not affect the expression of Pavlovian conditioned approach (PCA) behavior or conditioned reinforcement. (A) Rats underwent seven daily sessions of PCA training, and the average PCA index scores of Sessions 6 and 7 were used to phenotype STs and GTs. (B) After performing surgeries to lesion the ventral hippocampus (Lesion) or implant guide cannulas targeted at the ventral subiculum (Sham), rats underwent another seven daily sessions of PCA training. (C) Next, sham rats were infused with either lidocaine (20% solution) or saline in a counter-balanced manner immediately before two additional PCA training sessions, which served as a temporary inactivation test. (D) Finally, all rats underwent a conditioned reinforcement test during which active, but not inactive, nose-poke (NP) responses resulted in 2-s presentations of the lever. Data are mean and S.E.M. * — $p < 0.05$.

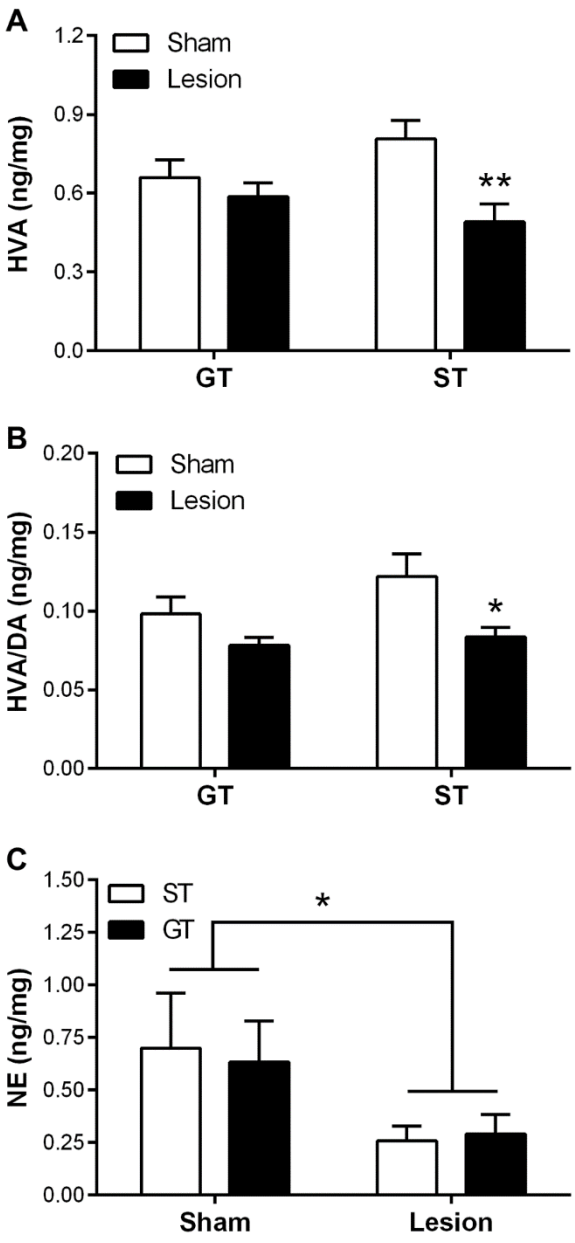

Figure 3.6. Ventral hippocampal lesions decrease accumbal levels of homovanillic acid (HVA) the ratio of HVA to dopamine (DA), and norepinephrine (NE). Five days following the last behavioral session (conditioned reinforcement test), tissue punches of the nucleus accumbens were taken to measure levels of monoamines and their metabolites using high-performance liquid chromatography. Significant differences between lesion (ventral hippocampus) and sham rats were observed with (A) HVA, (B) the ratio of HVA to DA, and (C) NE. Data are mean and S.E.M. * — $p < 0.05$, ** — $p < 0.01$.

CHAPTER IV

Prolonged stress decreases sign-tracking behavior and dopamine release in the nucleus accumbens

Note: Some of the text and figures have appeared previously in print in Behavioural Brain Research (Fitzpatrick et al., 2018) and are used with the permission of the publisher, Elsevier.

Abstract

Exposure to prolonged, uncontrollable stress reduces reward-seeking behavior, resulting in anhedonia in neuropsychiatric disorders, such as posttraumatic stress disorder. However, it is unclear to what degree stressed subjects lose interest in rewards themselves or in reward-related cues that instigate reward-seeking behavior. In the present study, we investigated the effects of single prolonged stress (SPS) using a Pavlovian conditioned approach (PCA) procedure. In Experiment 1, rats were exposed to SPS and tested for the acquisition of sign-tracking and goal-tracking behaviors during a PCA procedure. In Experiment 2, rats were exposed to SPS and tested for the expression of sign- and goal-tracking behaviors as well as conditioned reinforcement. In Experiment 3, rats were exposed to SPS, and *in vivo* microdialysis was used to measure baseline and evoked dopamine (DA) levels in the nucleus accumbens (NAc), which has been shown to underlie sign-tracking behavior. SPS decreased sign-tracking and increased goal-tracking behaviors during the acquisition of PCA behavior without affecting reward consumption, expression of PCA behaviors, or conditioned reinforcement. In addition, SPS

decreased evoked, but not baseline, levels of DA in the NAc. These results suggest that SPS decreases the motivational, but not consummatory, aspects of reward-seeking behavior, which may result from long-term, SPS-induced reductions in DA release in the NAc.

Introduction

Exposure to prolonged, uncontrollable stress can lead to the development of neuropsychiatric disorders, such as posttraumatic stress disorder (PTSD). One feature of PTSD is anhedonia, or the pathological lack of interest or pleasure in once desirable activities. Stress-induced anhedonia is typically modeled in animals by exposing them to prolonged or repeated uncontrollable stressors and then measuring the resultant decreases in reward-related behavior. For example, rats exposed to prolonged stress show decreases in exploratory behavior, sexual behavior, consumption of sweetened liquids, conditioned place preference for palatable foods and drug rewards, and operant responses for rewarding brain stimulation (Gronli et al., 2005; Moreau et al., 1992; Papp et al., 1991; Zacharko et al., 1983). The standard interpretation has been that in these situations reward-related activities are diminished, because, as thought for depressed patients, they no longer find these activities pleasurable.

However, the concept of anhedonia as a diminished capacity for pleasure has been recently challenged (Treadway & Zald, 2011). The most common way to assess anhedonia is simply to ask patients whether they are experiencing decreased enjoyment of activities that they would normally find pleasurable, and affected individuals reliably report a lack of pleasure (Watson & Naragon-Gainey, 2010). Yet, people's estimates of their own subjective enjoyment of future, past, or hypothetical activities are often inaccurate and can be heavily influenced by their own past decisions on whether to engage in those activities (Ariely & Norton, 2008; Brehm,

1956; Wenze et al., 2012; Wilson & Gilbert, 2005; Wilson et al., 2003). Several clinical studies have found that patients endorsing anhedonia often show normal hedonic responses to rewarding stimuli when affect is measured in real time (Klein, 1987; Kring & Moran, 2008; Strauss & Gold, 2012; Taylor et al., 2012; Treadway & Zald, 2011). It has been suggested, therefore, that the symptomatic deficit in patients may primarily be their motivation to pursue rewards, rather than their hedonic capacity to enjoy rewards (Myin-Germeys et al., 2000; Treadway & Zald, 2013).

A crucial process in generating motivated behavior is the attribution of incentive-motivational value to cues in the environment associated with reward (Berridge, 2004; Bindra, 1974). Although it is often difficult to dissociate the predictive properties of cues from their incentive-motivational properties, PCA procedures allow one to do so by separating in space the cue that predicts an impending reward from the location of reward delivery. For example, in the procedure used here, extension of a retractable lever, situated a few centimeters away from a pellet magazine, response-independently predicts the delivery of food pellets. Thus, on any given trial the rat may choose to interact with the lever (sign-tracking) or enter the magazine (goal-tracking) when the lever is extended, even though neither action influences reward delivery (Flagel et al., 2009). For STs, the reward-related cue acquires incentive-motivational value, evidenced by their propensity to approach and interact with it as well as their willingness to work for it during a conditioned reinforcement test (Robinson & Flagel, 2009). In contrast, for GTs, the reward-related cue acquires predictive value, but it does not appear to become particularly attractive or rewarding for them (i.e., the cue itself does not acquire incentive-motivational value).

In the present study, we assessed the effects of single prolonged stress (SPS) on the ability of food-related reward cues to acquire incentive-motivational value. SPS is the serial application of three stressors (restraint, forced swim, and ether exposure), which has been reliably used to model PTSD-like behaviors (Knox et al., 2012) and depression-like behaviors, such as "despair" in the forced swim test (Serova et al., 2013a; Serova et al., 2013b). We used a PCA procedure to test the ability of reward-related cues to elicit sign-tracking (or goal-tracking) as well as conditioned reinforcement in rats exposed to SPS. In addition, because decreased DA transmission has been proposed to mediate stress-induced reductions in reward-seeking behavior (Bekris et al., 2005; Cabib & Puglisi-Allegra, 2012; Pascucci et al., 2007; Puglisi-Allegra et al., 1991) and DA release within the NAc underlies the attribution of incentive-motivational value to reward-related cues (Berridge & Robinson, 1998; Flagel et al., 2011; Saunders & Robinson, 2012), we used *in vivo* microdialysis to measure baseline and evoked levels of DA in the NAc of SPS-exposed rats.

Materials and Methods

Animals

Adult male Sprague Dawley rats (275-300 g) were purchased from Charles River and Harlan Laboratories. Rats were selected from these two vendors to maximize variability in sign- and goal-tracking behaviors (Fitzpatrick et al., 2013). Animals were maintained on a 12:12-hr light/dark cycle and housed individually with food and water available *ad libitum* for the duration of experimentation. All procedures were approved by the University Committee on the Use and Care of Animals (University of Michigan; Ann Arbor, MI).

Experimental timeline

In Experiment 1, rats were exposed to SPS followed by five daily PCA training sessions. In Experiment 2, rats underwent five daily PCA training sessions followed by SPS. Following the seven-day quiescent period, a single PCA training session was used to measure the expression of PCA behavior. Twenty-four hours later, rats underwent a test for conditioned reinforcement. Rats were counterbalanced into SPS and control groups as equally as possible from both vendors in Experiment 1 (Harlan: SPS = 12, Control = 12; Charles River: SPS = 12, Control = 12) and Experiment 2 (Harlan: SPS = 10, Control = 11; Charles River: SPS = 6, Control = 8). In Experiment 3, rats were exposed to SPS, implanted with *in vivo* microdialysis probes, then tested one week later (approximately two weeks after the administration of SPS) for baseline and evoked DA levels in the NAc. In Experiment 3, rats were only purchased from Charles River (SPS = 5, Control = 6).

Pavlovian conditioned approach (PCA): Apparatus and procedure

Sixteen conditioning chambers (24.1 cm width × 20.5 cm depth x 29.2 cm height; MED Associates, Inc.; St. Albans, VT) were used for Pavlovian conditioning. Each chamber was situated in a sound-attenuating cubicle equipped with a ventilation fan to provide ambient background noise. Each chamber was equipped with a pellet magazine, a retractable lever (counterbalanced on the left or right side of the magazine), and a red house light on the wall opposite the magazine. The magazine contained an infrared sensor to detect magazine entries, and the lever was calibrated to detect lever deflections in response to 10 g of applied weight. Whenever the lever was extended into the chamber, an LED mounted inside the lever mechanism illuminated the slot through which the lever protruded.

For two days prior to the start of training, rats were familiarized with banana-flavored pellets (45 mg; Bioserv; Frenchtown, NJ) in their home cages. Rats were then placed into the test chambers for one pretraining session during which the red house light remained on, but the lever was retracted. Twenty-five food pellets were delivered on a VT 30-s schedule (i.e., one pellet was delivered on average every 30 s, but varied 0-60 s). Each trial during a test session consisted of presentation of the illuminated lever (CS) into the chamber for 8 s on a VT 90-s schedule (i.e., the lever was presented on average every 90 s, but varied 30-150 s between CS presentations). Retraction of the lever was immediately followed by the response-independent delivery of one food pellet (US) into the magazine. The beginning of the next inter-trial interval commenced immediately after pellet delivery. Each test session consisted of 25 trials of a CS-US pairing. All rats consumed all pellets that were delivered. Rats were not food deprived at any point during experimentation.

Conditioned Reinforcement: Procedure

For the conditioned reinforcement test, which lasted 40 min, each chamber was equipped with two nose-poke ports adjacent to a lever located in the center of the front wall of the chamber. Nose-poke responses in the active nose-poke port resulted in presentation of the lever-CS for 2 s on a fixed ratio 1 (FR1) schedule, whereas nose pokes of the inactive nose-poke port did not result in presentation of the lever-CS.

Single prolonged stress (SPS)

Rats were exposed to the SPS procedure as previously described (Liberzon et al., 1997), and an equal number of control rats were placed in a novel room and left undisturbed for an

equivalent time (~3 h). SPS consisted of the serial application of restraint, forced swim, and ether exposure until general anesthesia. Rats were restrained for two hours, followed immediately by a 20-min forced swim in room temperature (20-25°C) water. Forced swim occurred with eight rats at a time in an 18-gal plastic tub, filled two-thirds from the bottom with water. After the forced swim, rats were dried with towels and allowed to recuperate for 15 min on heating pads. Next, rats were exposed to ether (75 mL) in a container within a fume hood until the loss of consciousness. Serial exposure to all three stressors[33] is critical to the effects of SPS, because partial exposure to some stressors (e.g., restraint and forced swim) or substitution of stressors (e.g., isoflurane for ether) abolishes stress-induced behavioral alterations (Knox et al., 2012b). Following SPS, rats were returned to the housing colony and left undisturbed in their home cages for seven days prior to PCA training.

In vivo microdialysis

Rats were surgically prepared for *in vivo* microdialysis as previously described (Becker & Rudick, 1999) to measure baseline and evoked DA levels in the NAc. Commercially available microdialysis probes were used for the experiment (MAB 6.14.2; 2 mm, 15 kDa cut-off PES membrane; SciPro, Inc.; Sanborn, NY). All probes were tested for *in vitro* recovery less than one week before the day of the experiment. During recovery testing, a Ringer's solution (145 mM NaCl; 2.7 mM KCl; 1 mM $MgSO_4$; 1.2 mM $CaCl_2$; 1.55 mM Na_2HPO_4 and 0.445 mM NaH_2PO_4; pH = 7.3) was pumped through the probes at a flow rate of 1.5 µL/min. The probes were immersed in a DA standard solution warmed to 37 ± 1°C. Samples were collected every 5 min,

[33] Previously, it has been shown that SPS is a stressor capable of promoting stress responses in the hypothalamic-pituitary-adrenal axis, such as fast-negative feedback (Liberzon et al., 1997) and glucocorticoid receptor activation (Kohda et al., 2007). In the present experiment, assays were not performed to directly measure if SPS activated the hypothalamic-pituitary-adrenal axis.

and the DA recovery percentage was determined relative to the standard concentration. Only probes that had greater than 10% recovery were used.

Rats were surgically implanted with a unilateral microdialysis guide cannula (MAB 6.14 G; SciPro, Inc.) aimed at the NAc core-medial shell boundary (AP: +1.7 mm, measured from bregma; ML: ± 1.4 mm; DV: -6.8 mm, measured from the skull surface), and a dummy probe extending 2 mm below the guide was inserted. Rats recovered for one week before the microdialysis experiment. The dummy probe was removed one day before microdialysis, and the microdialysis probe was inserted and secured in place. Rats were placed in the microdialysis chamber (Med Associates, Inc.) with food and water provided *ad libitum*. Ringer's solution was perfused through the probe at a constant rate of 0.4 µL/min overnight and 1.5 µL/min on the day of testing using a Harvard Apparatus pump (Instech Laboratories, Inc.; Plymouth Meeting, MA). On the day of testing, samples were collected every 10 min. After the collection of three consistent baseline samples with stable DA concentration, the perfusion fluid was switched to a high-K^+ Ringer's solution with a KCl concentration of 75 mM for 10 min. High-K^+ perfusion is an extraphysiological stimulus that reliably releases DA, which is believed to occur primarily through depolarization-induced exocytosis of synaptic vesicles (Arbuthnott et al., 1990a; b). Next, the perfusate was switched back to the original Ringer's solution, and samples were collected for an additional 30 min.

DA content of the dialysate was determined using high-performance liquid chromatography with electrochemical detection. A C-18 ESA (ESA Biosciences, Inc.; Chelmsford, MA) column (HR-80X3.2; 3 µm particle size, 80 mm length) was used to separate DA in the samples at 27°C by pumping a mobile phase consisting of 75 mM NaH_2PO_4, 0.2 mM EDTA, 1.4 mM OSA (1-ocatanesulfonic acid sodium salt monohydrate), and 19% methanol (pH

= 4.7) at a flow rate of 0.7 mL/min. Potentials of -75 mV and 100 mV were applied to a dual coulometric analytical cell (ESA Model #5014B; ESA Biosciences, Inc.), and the latter potential was used to determine DA content. Current in the analytical cell was detected by a Coulochem II/III detector (ESA Biosciences, Inc.).

Statistical analysis

PCA behavior was scored using an index that combines the number, latency, and probability of lever presses and magazine entries during CS presentations (Meyer et al., 2012). Briefly, we averaged the response bias (i.e., number of lever presses and magazine entries for a session; [lever presses – magazine entries] / [lever presses + magazine entries]), latency score (i.e., average latency to perform a lever press or magazine entry during a session; [magazine entry latency – lever press latency]/8), and probability difference (i.e., proportion of lever presses or magazine entries; lever press probability – magazine entry probability) for each session. The index scores behavior from +1.0 (absolute sign-tracking) to -1.0 (absolute goal-tracking) with 0 representing no bias. In addition, latency of pellet retrieval was measured as the time elapsed between the retraction of the lever-CS and the first magazine entry during the non-CS period.

SPSS (Version 24; IBM, Inc.) was used for all statistical analysis. Repeated measures were analyzed using a linear mixed model with a covariance structure selected using Akaike's information criterion (i.e., the lowest criterion value represents the highest quality statistical model using a given covariance structure; Akaike, 1974). Group differences were analyzed using independent samples t-test or two-way analysis of variance (ANOVA) when appropriate. With significant effects or interactions, post hoc comparisons were performed using the Sidak correction.

Results

SPS decreases sign-tracking and increases goal-tracking during the acquisition of PCA behavior

In Experiment 1, SPS was administered one week prior to the start of PCA training. During PCA training, rats were presented with a lever-CS for 8 s followed by the response-independent delivery of a food pellet for 25 trials over five daily sessions. SPS decreased PCA index scores over the five training sessions (Figure 4.1; effect of Stress: $F_{(1,57.82)} = 9.84$, $p = 0.003$; effect of Session: $F_{(1,177.3)} = 9.19$, $p = 9.02 \times 10^{-7}$; interaction of Stress x Session: $F_{(1,177.3)} = 0.94$, $p = 0.44$), indicating a bias towards goal-tracking. Post-hoc comparisons revealed that differences in PCA index scores between SPS-exposed and control rats presented during Sessions 3-5 ($p < 0.05$). Averaged PCA index scores from Session 4-5 are routinely used to phenotype rats as sign-trackers (score ≥ 0.5), intermediate-responders ($0.5 >$ score > -0.5), and goal-trackers (score ≤ -0.5). Compared to the control group, the SPS-exposed group had less rats that would normally be classified as sign-trackers and more rats that would be classified as intermediate-responders and goal-trackers (Figure S4.1).

Figure 4.2 shows that the decrease in PCA index scores in SPS-exposed rats resulted from decreased sign-tracking behavior and increased goal-tracking behavior. SPS decreased the number (effect of Stress: $t_{(1,46)} = 2.28$, $p = 0.027$), latency (effect of Stress: $t_{(1,46)} = -2.92$, $p = 0.005$), and probability (effect of Stress: $t_{(1,46)} = 2.28$, $p = 0.026$) of lever presses and increased the number (effect of Stress: $t_{(1,46)} = -2.35$, $p = 0.023$), latency (effect of Stress: $t_{(1,46)} = 2.59$, $p = 0.013$), and probability (effect of Stress: $t_{(1,46)} = -2.77$, $p = 0.008$) of magazine entries. In contrast to the effects of SPS on behavior while the lever was extended just before food delivery, SPS had no effect on the latency of rats to enter the magazine after the lever retracted, and all rats ate all pellets during every session (Figure S4.2*A*; effect of Stress: $F_{(1,63.58)} = 6.73 \times 10^{-5}$, $p = 0.99$; effect

of Session: $F_{(4,150.3)} = 8.21$, $p = 5.16 \times 10^{-6}$; interaction of Stress x Session: $F_{(4,150.27)} = 1.06$, $p = 0.38$). Thus, SPS decreased cue-directed (sign-tracking) behavior and shifted behavior toward reward-directed (goal-tracking) behavior. In addition, magazine entries outside the CS-period decreased over time (Figure S4.2*B*; effect of Session: $F_{(4,170.24)} = 25.9$, $p = 8.9 \times 10^{-17}$), but SPS had no effect on them (effect of Stress: $F_{(1,49.30)} = 0.001$, $p = 0.98$). Moreover, omissions (i.e., failures to perform any CR during CS-periods) decreased over time (Figure S4.3; effect of Session: $F_{(4,170.24)} = 25.9$, $p = 8.9 \times 10^{-17}$), but SPS had no effect on them (effect of Stress: $F_{(1,49.30)} = 0.001$, $p = 0.98$. These latter findings further suggest that SPS did not produce non-specific motor deficits or decrease discrimination between conditioned responding during CS and non-CS periods. Moreover, the food itself maintained its motivational value.

SPS does not affect the expression of PCA behavior or conditioned reinforcement

In Experiment 2, rats underwent five daily PCA training sessions, SPS (or control treatment), then a single PCA session to test for the expression of PCA behavior. Figure 4.3 shows that SPS did not affect the expression of lever press number (interaction of Session x Stress: $F_{(1,40)} = 0.13$, $p = 0.72$), latency (interaction of Session x Stress: $F_{(1,40)} = 0.48$, $p = 0.83$), or probability (interaction of Session x Stress: $F_{(1,40)} = 0.06$, $p = 0.80$) or magazine entry number (interaction of Session x Stress: $F_{(1,40)} = 3.22 \times 10^{-6}$, $p = 0.99$), latency (interaction of Session x Stress: $F_{(1,40)} = 0.03$, $p = 0.87$), or probability (interaction of Session x Stress: $F_{(1,40)} = 0.19$, $p = 0.67$). Similarly, PCA index scores did not change between Session 5 and 6 (data not shown; interaction of Session x Stress: $F_{(1,40)} = 0.44$, $p = 0.83$). In other words, after PCA behavior had been acquired, rats were unaffected by subsequent exposure to SPS.

During the conditioned reinforcement test, all rats performed more nose pokes into the active relative to the inactive port (effect of Port: $F_{(1,40)} = 37.81$, $p = 2.92 \times 10^{-7}$). SPS, however, did not affect conditioned reinforcement (Figure 4.4*A*; effect of Stress; $F_{(1,40)} = 2.50$, $p = 0.12$; interaction of Stress x Port; $F_{(1,40)} = 2.22$, $p = 0.14$). Similarly, SPS did not affect the number of lever presses performed following presentation of the lever-CS (Figure 4.4*B*; effect of Stress: $t_{(1,20)} = 0.076$, $p = 0.12$).

SPS decreases evoked but not baseline DA levels in the NAc

In Experiment 3, a separate cohort of rats were exposed to SPS followed by intracranial implantation of microdialysis probes targeted unilaterally at the NAc (core/medial shell boundary). Following a postsurgical recovery period of seven days (for a total of 14 days after the SPS procedure), DA levels in the NAc of both SPS-exposed and control rats were measured during seven 10-min sampling periods: baseline (Periods 1-3), K^+ stimulation (Period 4), and post-stimulation recovery (Periods 5-7). SPS decreased DA release in response to K^+ stimulation (Figure 4.5; interaction of Stress x Session: $F_{(5,49.65)} = 4.22$; $p = 0.003$). Post-hoc comparisons revealed that SPS did not affect DA levels during baseline ($p > 0.05$) or post-stimulation recovery ($p > 0.05$); however, SPS decreased DA during the K^+-stimulation sampling period ($p < 0.001$). In addition, control rats had significantly higher DA release during K^+ stimulation compared to baseline ($p < 0.001$), and DA levels decreased during post-stimulation recovery ($p < 0.001$). On the other hand, DA release during K^+ stimulation in SPS-exposed rats was not significantly different from DA levels during baseline ($p > 0.05$) or post-stimulation recovery ($p > 0.05$).

Discussion

In the present study, a single exposure to prolonged, uncontrollable stress in rats reduced sign-tracking behavior. In Experiment 1, SPS decreased sign-tracking and increased goal-tracking during the acquisition of PCA behavior; however, SPS did not affect the expression of either sign- or goal-tracking behavior or conditioned reinforcement. In Experiment 3, SPS decreased DA release[34] within the NAc at a time point comparable to when sign-tracking behavior decreased during the acquisition of PCA behavior in Experiment 1. Because blocking dopaminergic signaling in the NAc decreases sign-tracking behavior, these results suggest that reduced dopaminergic activity in the NAc may contribute to SPS-induced reductions in cue-directed behavior (Saunders & Robinson, 2012; Saunders et al., 2013).

Interestingly, SPS (1) increased the acquisition of goal-tracking behavior (and decreased the acquisition of sign-tracking behavior) and (2) did not affect the expression of either sign- or goal-tracking behaviors. Regarding the acquisition of PCA behavior, one possibility is that altered dopaminergic tone within the NAc shifted conditioned responding away from reward-distal cues (e.g., the lever) to reward-proximal cues (e.g., the pellet magazine; Holden & Peoples, 2010; Simon et al., 2009). In addition, SPS may not have affected the expression of PCA behavior, because conditioned approach becomes DA-independent after sufficient training (Clark et al., 2013). Although it is possible that SPS-induced changes in the expression of PCA behavior could have been observed with more training sessions, previous studies have demonstrated that the expression of PCA behavior is very resistant to manipulation (Fitzpatrick et al., 2016).

The fact that SPS decreased sign-tracking behavior and increased goal-tracking behavior during acquisition has important implications for anhedonia in patients, because “liking” the

[34] The failure of K^+-stimulation to induce DA release in SPS-exposed rats suggests that these rats did not have DA in vesicles to release upon stimulation of synaptic terminals in the NAc.

reward may be triggered by reward-proximal cues (e.g., the smell of food) while "wanting" the reward may not be triggered by reward-distal cues (e.g., advertisements on television for a grocery store or friends associated with eating out at restaurants). Indeed, many patients with anhedonia enjoy primary rewards, but complain bitterly about not wanting to obtain them (Klein, 1987). The long-standing definition of anhedonia as the inability to experience pleasure has recently been challenged as accumulating evidence suggests that pursuing rewards involves aspects of "wanting", "liking", and learning (Berridge & Robinson, 2003; Thomsen, 2015). Interest has arisen in the heterogeneity of anhedonia symptoms, leading to sub-classifications such as consummatory anhedonia (i.e., deficits in hedonic responses to reward) and motivational anhedonia (i.e., diminished motivation to pursue reward; Treadway & Zald, 2011). In the present study, it appears that SPS impairs motivational but not consummatory aspects of reward-seeking behaviors. One limitation of the current study, however, is that pleasure and "liking" cannot be directly measured using PCA and cocaine-self administration procedures. Future studies can explore "liking" by investigating, for instance, how SPS affects behavioral responses to oral sucrose administration (Pecina & Berridge, 2000).

One striking feature of the SPS-induced decreases in sign-tracking behavior is the persistence of the effects over time[35]. The reductions in sign-tracking behavior and evoked dopaminergic activity within the NAc were observed approximately two weeks after SPS. Although studies of neurochemical changes after a single stressor tend to focus on acute responses, our findings agree with previous studies reporting long-term effects of "chronic" exposure to repeated stressors over multiple days (Gambarana et al., 1999; Mangiavacchi et al.,

[35] Previously, it has been demonstrated that SPS-induced molecular alterations last up to 14 days (Ding et al., 2014). Our results support these findings that SPS-induced changes in behavior can last up to 14 days. Although it has not explicitly studied, it is possible that SPS-induced molecular and behavioral changes last more than 14 days, at which point the changes subside or compensation begins.

2001; Shimamoto et al., 2011). In support of this, neither acute restraint stress (Puglisi-Allegra et al., 1991) nor ether-exposure (Schwarting & Huston, 1987) alter DA concentrations in the NAc. On the other hand, although acute swim stress increases DA concentrations in the NAc 150 min after exposure, DA concentrations return to baseline levels at 210 min (Yadid et al., 2001). Therefore, it seems that the acute, serial application of all three stressors is critical to producing the observed chronic-like state of reduced dopaminergic activity in the NAc.

The effects of SPS on sign-tracking behavior and DA release within the NAc complement a long line of research demonstrating that reduced DA transmission in the NAc is related to motivational deficits (Berridge & Robinson, 1998; Ikemoto & Panksepp, 1999; Roberts et al., 1977; Salamone & Correa, 2002). The effects of stress on DA within the NAc are dependent on the nature and timing of the stress: brief or controllable stressors increase DA release in the NAc, while prolonged, uncontrollable stressors typically decrease DA release in the NAc (Cabib & Puglisi-Allegra, 2012). Our finding that SPS falls into the latter category agrees with other reports that SPS reduces behavioral sensitization to methamphetamine, cocaine-conditioned place preference and sucrose preference, and striatal dopamine content (Eagle & Perrine, 2013; Enman et al., 2015), though there may be heterogeneity in individual responses to SPS (Toledano et al., 2013). However, it should be noted that the temporal and spatial resolution of *in vivo* microdialysis does not permit isolation of DA release on sub-second timescales. In future studies, fast-scan cyclic voltammetry can be used to more precisely measure DA release in the NAc, for instance, surrounding CS and US presentations during PCA training.

Similar to our results, patients experiencing anhedonia have reduced activity in the ventral striatum during reward conditioning (Kumar et al., 2008). Even after recovery from neuropsychiatric disorders characterized by anhedonia, many patients still have reduced activity

in the ventral striatum in response to reward-related sensory cues, despite subjectively rating the pleasantness, intensity, and desirability of the rewarding stimulus the same as control subjects (McCabe et al., 2009). Clinically, increasing neural activity in the NAc through deep brain stimulation (Bewernick et al., 2010) or administering DA agonists (Lemke et al., 2006; Reichmann et al., 2006) has been shown to have pro-motivational effects and reduce anhedonia in patients. Our results provide insight into behavioral and neurobiological mechanisms explaining the utility of these treatments and suggest that future investigations of anhedonia should focus on restoring cue-directed incentive motivation in patients suffering from PTSD and other neuropsychiatric disorders.

In conclusion, we demonstrated that SPS decreases the acquisition of sign-tracking (and increases the acquisition of goal-tracking) behavior during a PCA procedure. SPS, however, did not affect the expression or conditioned reinforcing properties of incentive stimuli. Importantly, reward-directed behaviors, such as the consumption of food pellets were unaffected following exposure to SPS. In addition, we demonstrated that SPS decreases DA release in the NAc, which may underlie the observed reductions in sign-tracking, because the behavior is DA-dependent in the NAc. These findings have important implications for classifying and treating anhedonia observed in PTSD and other neuropsychiatric disorders by suggesting uncontrollable stress impairs motivational, but not consummatory, aspects of reward-seeking behavior.

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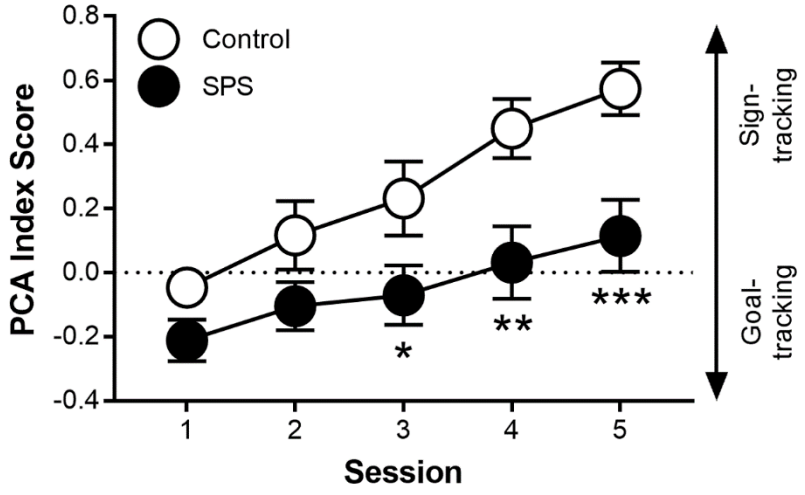

Figure 4.1. Single prolonged stress (SPS) decreases Pavlovian conditioned approach (PCA) index scores. PCA index scores incorporate both lever press and magazine entry number, latency, and probability into a single value. On the PCA index, +1.0 represents absolute sign-tracking and -1.0 represents absolute goal-tracking. Data are mean and S.E.M. * — $p < 0.05$, ** — $p < 0.01$, *** — $p < 0.001$.

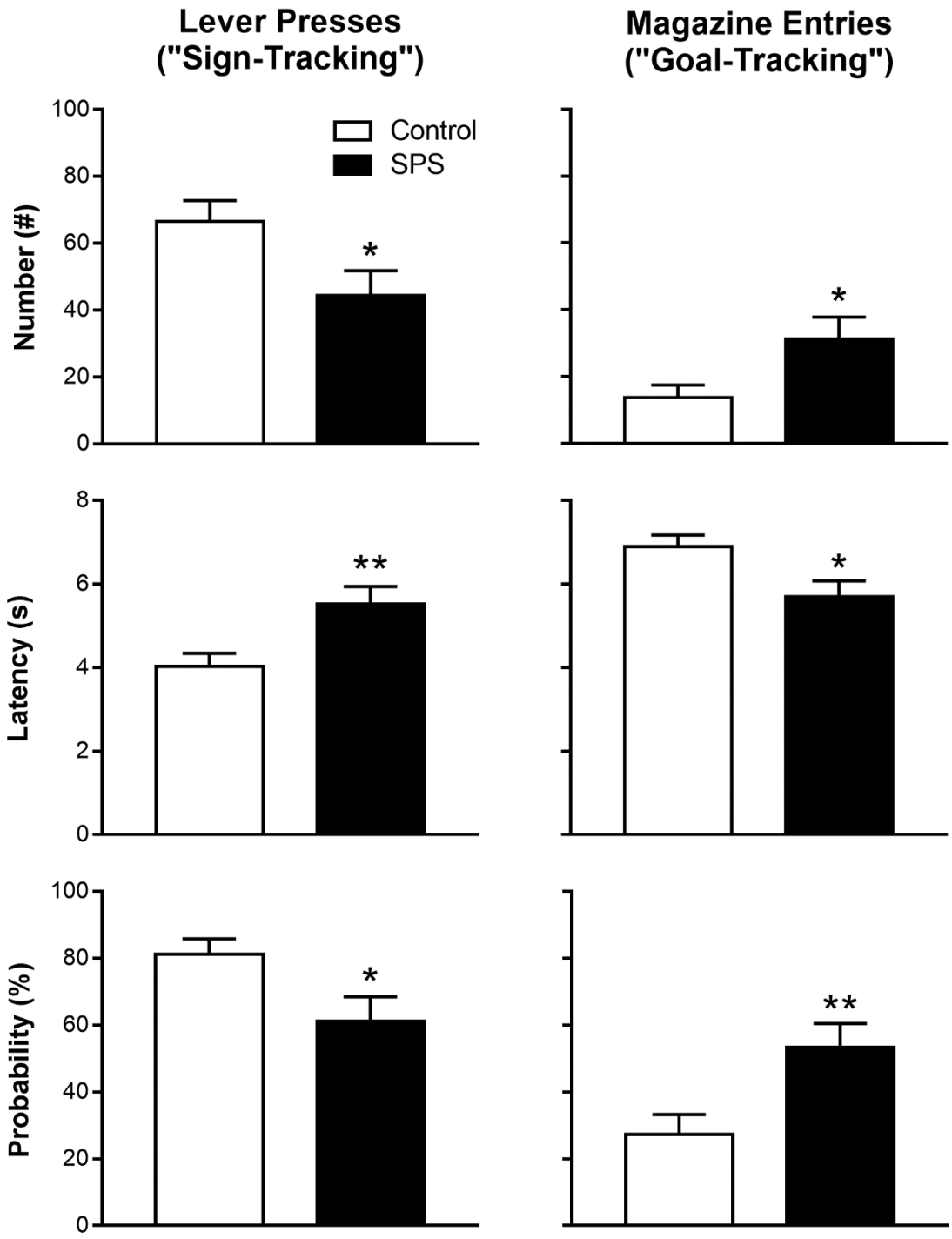

Figure 4.2. Single prolonged stress (SPS) decreases the acquisition of Pavlovian conditioned approach (PCA) behavior. SPS decreased lever press and increased magazine entry number, latency, and probability on the final two session of PCA training (Sessions 4 and 5). Data are mean and S.E.M. * — $p < 0.05$, ** — $p < 0.01$.

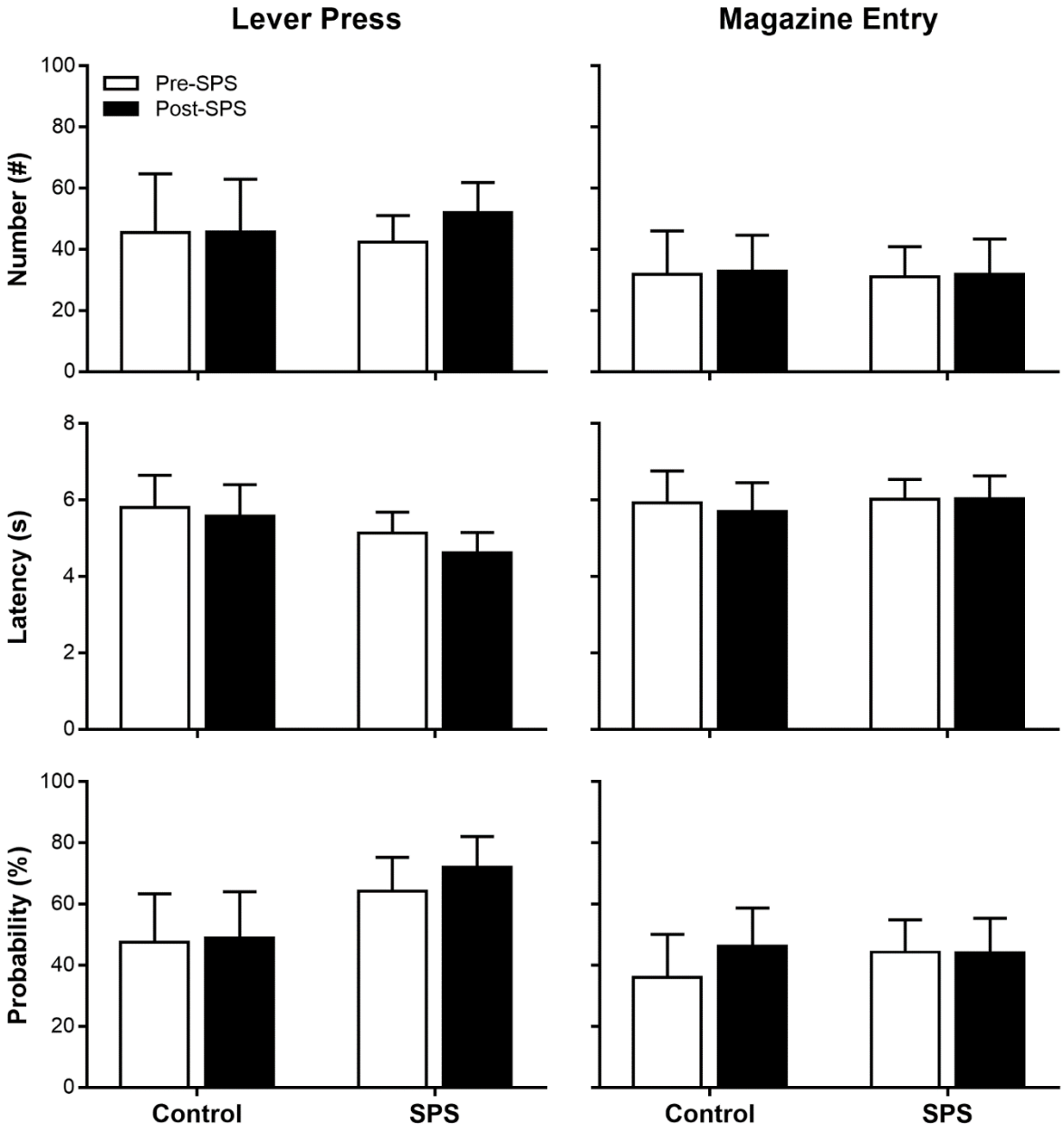

Figure 4.3. Single prolonged stress (SPS) does not affect the expression of Pavlovian conditioned approach (PCA) behavior. SPS did not decrease lever press or magazine entry number, latency, and probability on a post-SPS test session (Sessions 6). Data are mean and S.E.M.

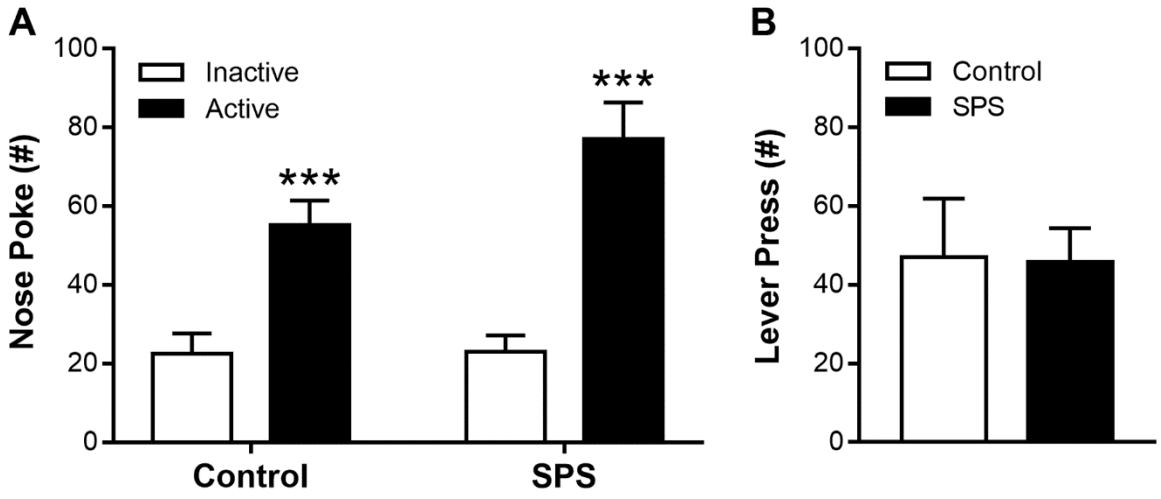

Figure 4.4. Single prolonged stress (SPS) does not affect conditioned reinforcement. Sign-trackers (STs) and goal-trackers (GTs) underwent a conditioned reinforcement test following exposure to SPS and the expression test for Pavlovian conditioned approach behavior. Data are mean and S.E.M. *** — $p < 0.001$.

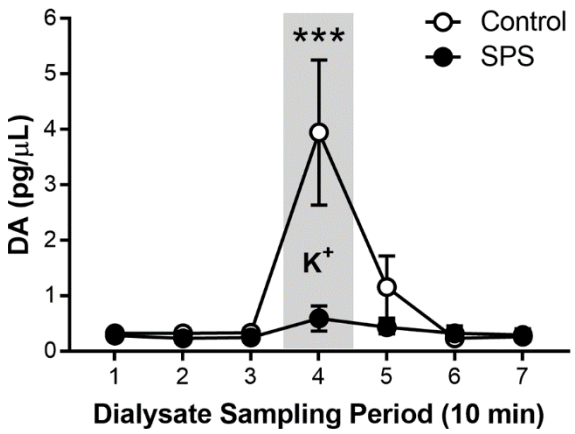

Figure 4.5. Single prolonged stress (SPS) decrease dopamine (DA) release in the nucleus accumbens. Two weeks following SPS exposure, DA levels in the nucleus accumbens were measured using *in vivo* microdialysis. Samples were collected over seven 10-minute periods: baseline (Period 1-3), K^+-evoked stimulation (75 mM; Period 4), and post-stimulation recovery (Periods 5-7. Data are mean and S.E.M. *** — $p < 0.001$.

Appendix C: Chapter IV Supplemental Information

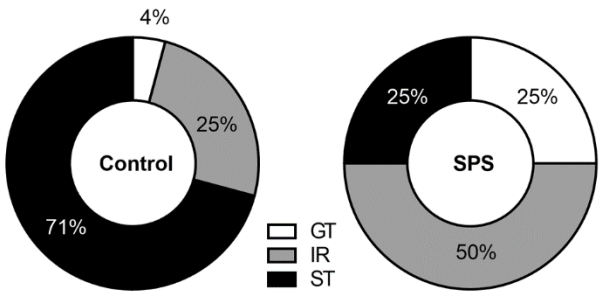

Figure S4.1. Sample distribution of sign-trackers (STs), intermediate-responders (IRs), and goal-trackers (GTs). Compared to the population of control rats, the population of SPS-exposed rats contained less STs and more IRs and GTs. Phenotypes were determined using the average Pavlovian conditioned approach index scores during Sessions 4 and 5: STs (score $\geq$ 0.5), IRs (0.5 > score > -0.5), and GTs (score $\leq$ -0.5).

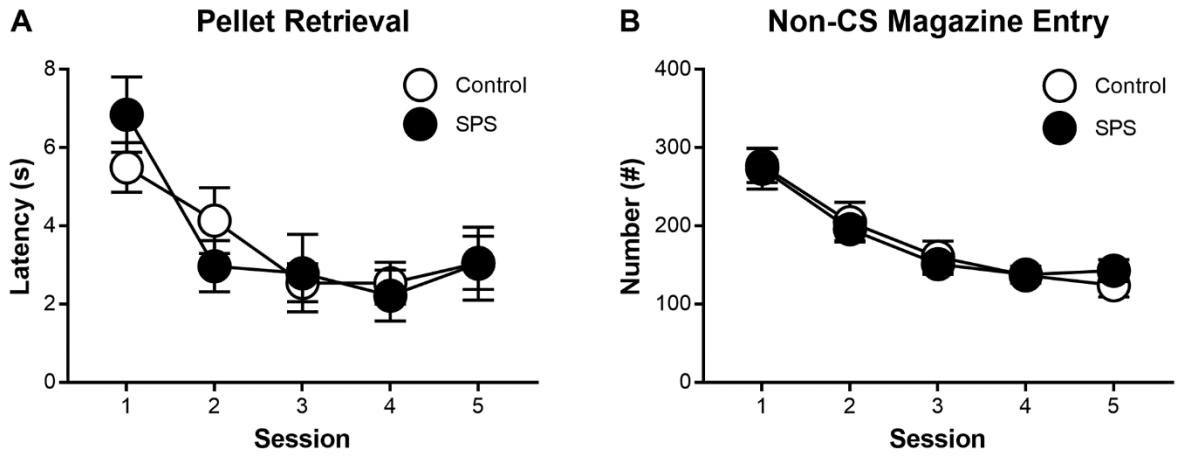

Figure S4.2. Single prolonged stress (SPS) does not affect consummatory or exploratory behaviors. During the acquisition of Pavlovian conditioned training, single prolonged stress (SPS) did not affect the (A) latency of pellet retrieval nor (B) the number of non-conditioned stimulus (CS) magazine entries (i.e., the number of magazine entries performed outside presentation of the lever-CS) in sign-trackers (STs) and goal-trackers (GTs). Data are mean and S.E.M.

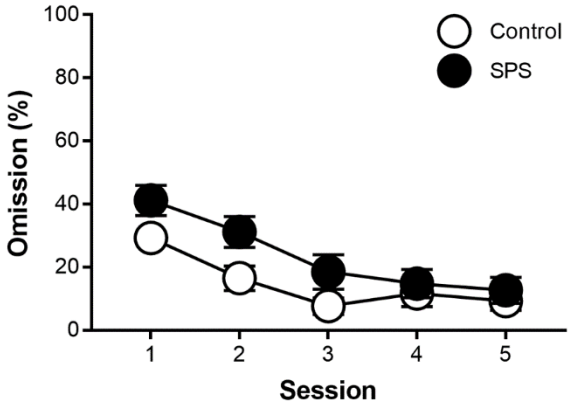

Figure S4.3. Single prolonged stress (SPS) does not affect omissions. Omissions (i.e., failures to perform any conditioned response during a conditioned stimulus period) were measured during the acquisition of Pavlovian conditioned approach behavior. Data are mean and S.E.M.

CHAPTER V

Subanesthetic ketamine decreases sign-tracking behavior

Note: *Some of the text and figures have appeared previously in print in Journal of Psychopharmacology (Fitzpatrick and Morrow, 2017) and are used with the permission of the publisher, SAGE Publishing.*

Abstract

The attribution of incentive-motivational value to reward-related cues contributes to cue-induced craving and relapse in addicted patients. Recently, it was demonstrated that subanesthetic ketamine increases motivation to quit and decreases cue-induced craving in cocaine-dependent individuals. Although the underlying mechanism of this effect is currently unknown, one possibility is that subanesthetic ketamine decreases the incentive-motivational value of reward-related cues. In the present study, we used a Pavlovian conditioned approach procedure to identify sign-trackers (STs), rats that bias their conditioned responding toward reward-related cues, and goal-trackers (GTs), rats that bias their conditioned responding toward the location of reward delivery. This model is of interest because STs are more vulnerable to cue-induced reinstatement of drug-seeking behavior and will persist in this drug-seeking behavior despite adverse consequences. We tested the effect of subanesthetic ketamine on the expression of PCA behavior and the conditioned reinforcing properties of a reward-related cue in STs and GTs. We found that subanesthetic ketamine decreased sign-tracking and increased goal-

tracking behaviors in STs, though it had no effect on conditioned reinforcement. These results suggest that subanesthetic ketamine may be a promising pharmacotherapy for addiction that acts by decreasing the incentive-motivational value of reward-related cues.

Introduction

The attribution of incentive-motivational value to reward-related cues is believed to contribute to relapse and cue-induced craving in addiction (Robinson & Berridge, 1993; Sinha & Li, 2007). In support of this, drug-related cues can acquire incentive-motivational value (Wolfling et al., 2008), bias attention (Attwood et al., 2008; Waters et al., 2003), and rapidly induce craving (Charboneau et al., 2013; Michalowski & Erblich, 2014) in addicted patients. In addition, relapse is associated with increased cue-induced neural activity within the motive circuit of addicted patients (Li et al., 2015). Even during prolonged periods of drug abstinence, drug-related cues can maintain sustained incentive-motivational value in both humans (Preller et al., 2013) and rodents (Ciccocioppo et al., 2001; Weiss et al., 2001).

NMDA receptor signaling is critical for reward-cue associations (Vengeliene et al., 2015), and glutamatergic synaptic plasticity in the motive circuit is believed to underlie addiction pathophysiology (Kalivas et al., 2009; Kalivas & Volkow, 2011; van Huijstee & Mansvelder, 2014). Targeting NMDA receptor signaling using subanesthetic doses of ketamine, a noncompetitive NMDA receptor antagonist, has been investigated previously for the treatment of major depressive disorder, where it has been found to produce a rapid reduction in symptomology that endures long after drug clearance (aan het Rot et al., 2010; Price et al., 2009). Based upon the results of these and other studies, subanesthetic ketamine has been investigated for the treatment of addiction, and Dakwar et al. (2014) showed that subanesthetic

ketamine administration increases motivation to quit and reduces cue-induced craving in cocaine-dependent subjects twenty-four hours after infusion. In an earlier study, Krupitsky et al. (2002) also demonstrated that subanesthetic ketamine reduces cravings and increases abstinence for up to two years in heroin-dependent individuals. Currently, it is unknown how subanesthetic ketamine affects reward processing to increase motivation to quit and reduce cue-induced craving; however, one possibility is that it decreases the incentive-motivational value of reward-related cues.

To investigate this possibility, we used a PCA procedure in rats. During PCA training, rats are presented with a CS (e.g., a lever) that response-independently predicts the delivery of an US (e.g., a food pellet). Over the course of training, three patterns of CRs typically develop: sign-tracking (CS-directed CRs), goal-tracking (US-directed CRs), and an intermediate-response (both CRs). Previously, it has been demonstrated that STs, compared to GTs and IRs, attribute incentive-motivational value to reward-related cues, which become attractive, powerful motivators of behavior in and of themselves (Robinson & Flagel, 2009). It has also been shown that STs have increased cue-induced reinstatement of drug-seeking and continue to seek drugs despite adverse consequences, two hallmarks of addiction (Saunders & Robinson, 2010; Saunders et al., 2013). PCA procedures are useful in determining how pharmacological manipulations can alter the incentive-motivational value of reward-related cues without the confounds inherently associated with long-term exposure to drugs of abuse. In the current study, we investigated how subanesthetic ketamine influences the incentive-motivational value and conditioned reinforcing properties of reward-related cues in rats. In Experiment 1, rats underwent PCA training to phenotype rats as STs and GTs, and then subanesthetic ketamine was administered systemically to determine its effect on PCA behavior. In Experiment 2, rats

underwent PCA training sessions to phenotype rats as STs and GTs, and then subanesthetic ketamine was administered systemically before a conditioned reinforcement test.

Materials and Methods

Animals

Fifty-three, adult male Sprague Dawley rats (275-300g) were purchased from Harlan Laboratories and Charles River Laboratories to obtain a relatively equal distribution of sign- and goal-trackers (Fitzpatrick et al., 2013). Although it is not always necessary to purchase rats from different barriers, it oftentimes provides behavioral heterogeneity and phenotypic diversity. In Experiment 1, 28 rats were used (Harlan = 16; Charles River = 12), in Experiment 2, 25 rats were used (Charles River, n = 25). Rats were maintained on a 12:12-hr light/dark cycle, and food and water were available *ad libitum* for the duration of the study. Rats were acclimatized to the housing colony for two days prior to handling. All procedures were approved by the University Committee on the Use and Care of Animals (University of Michigan; Ann Arbor, MI).

Drugs

Ketamine hydrochloride was used (racemic mixture; Hospira, Inc.; Lake Forest, IL). Ketamine (100 mg/kg) was diluted in sterile saline to make a subanesthetic dose of ketamine (32 mg/kg; 1 mL/kg; pH = 7.34-7.36). This dose was selected based upon previous studies showing that subanesthetic ketamine (30-35 mg/kg) increases brain metabolism and glutamatergic transmission in rats (Duncan et al., 1998b; Kim et al., 2011). Sterile saline was used as the vehicle control.

Pavlovian Conditioned Approach: Apparatus

Conditioning chambers (24.1 cm width × 20.5 cm depth x 29.2 cm height; MED Associates, Inc.; St. Albans, VT) were used for Pavlovian conditioning. Each chamber was located in a sound-attenuating cabinet equipped with a ventilation fan to provide ambient white noise. Each chamber was equipped with a pellet magazine, an illuminated, retractable lever (counterbalanced on the left or right of the pellet magazine), and a red house light on the wall opposite of the pellet magazine. When inserted into the chamber, the retractable lever was illuminated by an LED light within the lever housing. A pellet dispenser delivered banana-flavored food pellets into the pellet magazine. An infrared sensor measured head entries into the pellet magazine.

Pavlovian Conditioned Approach: Procedure

For two days prior to pretraining, rats were familiarized with banana-flavored food pellets (45 mg; Bioserv; Frenchtown, NJ) in their home cages. Twenty-four hours later, rats were placed into the operant chambers and underwent one pretraining session during which the red house-light remained on, but the lever was retracted. Fifty food pellets were delivered on a VT 30-s schedule (i.e., one food pellet was delivered on average every 30 s, but actual delivery varied between 0-60 s). All rats consumed all the food pellets by the end of the pretraining session. Each trial during a test session consisted of extension of the illuminated lever (conditioned stimulus; CS) into the chamber for 8 s on a VT 90-s schedule (i.e., one food pellet was delivered on average every 90 s, but actual delivery varied between 60-120 s). Retraction of the lever was immediately followed by the response-independent delivery of one food pellet (unconditioned stimulus; US) into the pellet magazine. Each test session consisted of 25 trials of

CS-US pairings, resulting in a total session length of approximately 40 min. Each rat consumed all the food pellets that were delivered.

Conditioned Reinforcement: Procedure

For the conditioned reinforcement test, which lasted 40 min, each chamber was equipped with two nose-poke ports adjacent to a lever located in the center of the front wall of the chamber. Nose-poke responses in the active nose-poke port resulted in presentation of the lever-CS for 2 s on a FR1 schedule, whereas nose pokes of the inactive nose-poke port did not result in presentation of the lever-CS.

Experimental Procedure

In Experiment 1, rats underwent a total of ten daily PCA training sessions. The eighth PCA training session served as a baseline to ensure that rats within each phenotype, which would be divided into drug conditions, did not differ in their conditioned responding. During the ninth PCA training session, rats were administered subanesthetic ketamine (32 mg/kg) or vehicle 30 min before testing[36]. During the tenth PCA training session, rats were tested without any drug or vehicle injection to determine whether acute, subanesthetic ketamine administration had enduring effects. In Experiment 2, rats underwent seven daily PCA training sessions followed twenty-four hours later by a test of conditioned reinforcement. Similar to Experiment 1, rats were administered subanesthetic ketamine (32 mg/kg)[37] or vehicle 30 min before testing.

[36] The half-life of ketamine in young Sprague Dawley rats (8-10 wk; approximately the age of the one used in the present experiment) is 1.26 h (Veilleux-Lemieux et al., 2013). This timepoint was used, because it has been previously used in the literature; however, it is possible that peak concentrations of subanesthetic ketamine wane partially through the test session of PCA behavior following administration.

[37] Subanesthetic doses of ketamine range from 3-32 mg/kg. On the other hand, 90-100 mg/kg is considered an anesthetic dose of ketamine.

Statistical Analysis

PCA behavior was scored using an index that incorporates the number, latency, and probability of lever presses (sign-tracking CR) and magazine entries (goal-tracking CR) during CS presentations within a session. Briefly, we averaged the response bias (i.e., number of lever presses and magazine entries for a session; [lever presses – magazine entries] / [lever presses + magazine entries]), latency score (i.e., average latency to perform a lever press or magazine entry during a session; [magazine entry latency – lever press latency]/8), and probability difference (i.e., proportion of lever presses or magazine entries; lever press probability – magazine entry probability). The PCA index score ranges from +1.0 (absolute sign-tracking) to -1.0 (absolute goal-tracking), with 0 representing no bias. PCA index scores were used to classify rats as STs (score ≥ 0.5), GTs (score ≤ -0.5), and IRs ($-0.5 <$ score < 0.5). For conditioned reinforcement, inactive and active nose-poke port responses were quantified and compared between groups.

SPSS (Version 22; IBM, Inc.) was used for all statistical analysis. For all linear mixed models, the covariance structure was selected based upon Akaike's information criterion (i.e., the lowest number criterion represents the highest quality statistical model using a given covariance structure; Akaike, 1974). PCA behavior across training sessions was analyzed using a linear mixed model with Phenotype (GT and ST) and Drug (Ketamine and Vehicle) as between-subject factors when appropriate. In Experiment 1, latency of pellet retrieval and non-CS magazine entries during PCA training were analyzed using a two-way analysis of variance (ANOVA) with Phenotype (GT and ST) and Drug (Ketamine and Vehicle) as factors. In Experiment 2, conditioned reinforcement was analyzed using a three-way ANOVA with Phenotype (GT and ST), Drug (Ketamine and Vehicle), and Port (Active and Inactive) as between-subject factors.

When significant effects or interactions were revealed, multiple comparisons were performed using Fisher's LSD post hoc test.

Results

Experiment 1: Subanesthetic ketamine administration decreases sign-tracking behavior and does not affect goal-tracking behavior

Rats underwent PCA training and were classified as STs, GT, and IRs; however, only STs (n = 12) and GTs (n = 16) were used for further experimental testing. Figure 5.1 shows that during eight daily PCA training sessions STs and GTs differed in their lever press number (effect of Phenotype: $F_{(1,28.83)} = 41.88$, $p = 4.53 \times 10^{-7}$), latency (effect of Phenotype: $F_{(1,30.53)} = 46.17$, $p = 1.41 \times 10^{-7}$), and probability (effect of Phenotype: $F_{(1,32.44)} = 61.78$, $p = 5.21 \times 10^{-9}$) as well as their magazine entry number (effect of Phenotype: $F_{(1,31.69)} = 25.63$, $p = 1.7 \times 10^{-5}$), latency (effect of Phenotype: $F_{(1,37.04)} = 38.65$, $p = 3.18 \times 10^{-7}$), and probability (effect of Phenotype: $F_{(1,34.7)} = 33.48$, $p = 2.0 \times 10^{-6}$). STs and GTs differed on their PCA index scores over the eight daily PCA training sessions, (effect of Phenotype: $F_{(1,32.44)} = 61.78$, $p = 5.21 \times 10^{-9}$), and the PCA index score of Session 8, which also served as the baseline session for subanesthetic ketamine administration, was used to determine PCA phenotypes.

Figure 5.2 shows the PCA behavior of rats during baseline, test (drug-on), and post-test (drug-off) sessions. During the baseline session, STs continued to lever press more than GTs across CS trials (effect of Phenotype: $F_{(1,123.8)} = 367.99$, $p = 6.83 \times 10^{-39}$), and there was no difference in the respective conditioned responding of GTs (effect of Drug: $F_{(1,75.42)} = 0.15$, $p = 0.7$) or STs (effect of Drug: $F_{(1,52.28)} = 1.31$, $p = 0.26$) that would later receive subanesthetic ketamine (ST, n = 5; GT, n = 8) or vehicle (ST, n = 7; GT, n = 8). Likewise, GTs continued to

enter the magazine more than STs across CS trials (effect of Phenotype: $F_{(1,131.53)} = 237.04$, $p = 3.21 \times 10^{-31}$). Subanesthetic ketamine decreased sign-tracking (effect of Drug: $F_{(1,55.74)} = 21.44$, $p = 2.12 \times 10^{-5}$) and increased goal-tracking (effect of Drug: $F_{(1,73.22)} = 19.01$, $p = 4.19 \times 10^{-5}$) in STs; however, subanesthetic ketamine did not affect sign-tracking (effect of Drug: $F_{(1,63.81)} = 1.68$, $p = 0.2$) or goal-tracking (effect of Drug: $F_{(1,67.91)} = 3.19$, $p = 0.078$) in GTs. During the post-test (drug-off) session, sign-tracking behavior in STs previously treated with subanesthetic ketamine was still decreased compared to saline-treated STs (effect of Drug: $F_{(1,54.87)} = 3.98$, $p = 0.05$), but goal-tracking was no longer different between ketamine- and saline-treated STs (effect of Drug: $F_{(1,89.63)} = 1.24$, $p = 0.27$). In addition, GTs that were previously administered saline or subanesthetic ketamine continued to show no within-session differences in sign-tracking (effect of Drug: $F_{(1,67.91)} = 3.19$, $p = 0.08$) or goal-tracking (effect of Drug: $F_{(1,57.91)} = 0.48$, $p = 0.49$) during the post-test (drug-off) session. During the test session, subanesthetic ketamine administration did not alter the latency to retrieve food pellets from the magazine following CS presentation (Figure 5.3*A*; effect of Drug: $F_{(1,23)} = 0.048$, $p = 0.83$; interaction of Phenotype x Drug: $F_{(1,23)} = 0.03$, $p = 0.86$), and as previously mentioned, all rats consumed all food pellets that were delivered. Subanesthetic ketamine did, however, increase non-CS magazine entries (i.e., increased overall activity; Figure 5.3*B*; effect of Drug: $F_{(1,24)} = 7.44$, $p = 0.012$) and omissions (i.e., failures to perform any CR during CS-periods; Figure S5.1; interaction of Phenotype x Drug: $F_{(1,24)} = 8.11$, $p = 0.009$). Post hoc comparisons revealed that vehicle-treated GTs had higher omissions than vehicle-treated STs ($p = 0.048$), and subanesthetic ketamine, compared to vehicle, increased omissions in STs ($p = 0.022$), but not GTs ($p = 0.14$).

Experiment 2: Subanesthetic ketamine administration does not affect conditioned reinforcement

Rats underwent PCA training and were classified as STs, GT, and IRs; however, only STs (n = 14) and GTs (n = 11) were used for further experimental testing. During seven daily PCA training sessions, STs and GTs differed in their lever press number (Figure S5.2; effect of Phenotype: $F_{(1,23.57)} = 32.61$, $p = 7.0 \times 10^{-6}$), latency (effect of Phenotype: $F_{(1,24.78)} = 50.38$, $p = 2.05 \times 10^{-7}$), and probability (effect of Phenotype: $F_{(1,25.02)} = 63.84$, $p = 2.39 \times 10^{-8}$) as well as their magazine entry number (effect of Phenotype: $F_{(1,28.88)} = 41.06$, $p = 5.34 \times 10^{-7}$), latency (effect of Phenotype: $F_{(1,31.62)} = 60.16$, $p = 8.26 \times 10^{-9}$), and probability (effect of Phenotype: $F_{(1,28.11)} = 51.06$, $p = 8.73 \times 10^{-8}$). STs and GTs differed on their PCA index scores over the seven daily PCA training sessions, (effect of Phenotype: $F_{(1,25.41)} = 97.5$, $p = 3.5 \times 10^{-10}$), and the average PCA index score of Sessions 6 and 7 were used to determine PCA phenotypes.

Following PCA training, rats were administered ketamine (ST, n = 7; GT, n = 6) or vehicle (ST, n = 7; GT, n = 5) before undergoing a conditioned reinforcement test. Figure 5.4*A* shows that all rats performed more nose pokes into the active relative to the inactive port (effect of Port: $F_{(1,42)} = 15.65$, $p = 2.87 \times 10^{-4}$). Consistent with previous findings, STs performed more active nose-poke responses than GTs (effect of Phenotype: $F_{(1,21)} = 16.97$, $p = 4.88 \times 10^{-4}$). Subanesthetic ketamine did not affect conditioned reinforcement (Figure 5.4*A*; interaction of Drug x Port: $F_{(1,42)} = 0.34$, $p = 0.56$; interaction of Phenotype x Drug x Port: $F_{(1,42)} = 0.13$, $p = 0.72$) or discrimination (i.e., ratio of active/inactive nose-pokes) between ports (data not shown; interaction of Phenotype x Drug: $F_{(1,21)} = 1.28$, $p = 0.27$); however, it did decrease the number of lever presses per CS presentation as a result of active nose-poke responding (data not shown; interaction of Phenotype x Drug: $F_{(1,21)} = 4.67$, $p = 0.042$), ultimately decreasing total conditioned approach to the lever-CS (i.e., lever presses over all lever-CS presentations; Figure 5.4*B*; interaction of Phenotype x Drug: $F_{(1,21)} = 6.0$, $p = 0.023$). Post-hoc comparisons revealed

that vehicle-treated STs had higher lever presses than vehicle-treated GTs ($p = 2.51 \times 10^{-6}$) and that ketamine decreased the number of lever presses in STs ($p = 0.0012$) but not GTs ($p = 0.97$).

Discussion

In Experiment 1, we demonstrated that a subanesthetic dose of ketamine (32 mg/kg) decreases the expression of sign-tracking behavior in STs without affecting goal-tracking behavior in GTs. Interestingly, this effect was still detectable twenty-four hours after administration during a post-test (drug-off) PCA test session. In addition, subanesthetic ketamine increased goal-tracking behavior in STs, although the effect was not detectable during the post-test (drug-off) session. During the test session, subanesthetic ketamine did not influence food pellet consumption (i.e., all rats ate all food pellets during the test session) or latency to retrieve food pellets following lever retraction. Subanesthetic ketamine did, however, increase non-CS magazine entries (a measure of general exploratory activity), which is in accordance with previous findings that subanesthetic ketamine increases locomotor activity (Littlewood et al., 2006b). We do not believe that this influenced the interpretation of our results, however, because locomotor hyperactivity, in the absence of effects on the incentive-motivational value of the lever-CS, would have increased the likelihood of STs approaching and interacting with the lever-CS, which it did not. Moreover, if subanesthetic ketamine-induced alterations in PCA behavior resulted from locomotor effects, both phenotypes would have presumably been affected equally, which they were not. In Experiment 2, subanesthetic ketamine did not affect conditioned reinforcement (i.e., the number of times a rat performed a nose-poke response for presentation of the lever-CS), however, it reduced conditioned approach (i.e., number of lever presses during lever-CS presentation) in STs, but not GTs, during the conditioned reinforcement test.

In both rats and humans, subanesthetic doses of ketamine produce global increases in neural activity, as compared to anesthetic doses of ketamine, which produce global suppression of neural activity (Duncan et al., 1998b). In humans, subanesthetic ketamine increases cerebral glucose metabolism (Breier et al., 1997; Duncan et al., 1998a; Langsjo et al., 2004; Vollenweider et al., 1997), cerebral blood perfusion (Holcomb et al., 2001; Langsjo et al., 2003) and blood oxygen level-dependent contrast (De Simoni et al., 2013) in brain regions such as the frontal cortex, thalamus, HPC, and striatum; and, similar findings have been reported in rats using glucose metabolism (Duncan et al., 1998b) and blood oxygen level-dependent contrast (Littlewood et al., 2006a; Littlewood et al., 2006b). It has been suggested that this differential regulation of neural activity involves a dose-dependent bias between antagonizing NMDA receptors on inhibitory GABAergic interneurons (low-dose, subanesthetic ketamine) and excitatory pyramidal neurons (high-dose, anesthetic ketamine; Miller et al., 2016). Therefore, subanesthetic doses of ketamine are believed to increase neural activity in brain regions by inhibiting GABAergic interneurons and disinhibiting glutamatergic neurons. In support of this, subanesthetic ketamine decreases extracellular GABA and increases extracellular Glu concentrations within the rat PFC (Moghaddam et al., 1997; Perrine et al., 2014). Because sign-tracking behavior has been suggested to result from low "top-down" inhibition of subcortical structures (Haight & Flagel, 2014), it is possible that subanesthetic ketamine decreases the expression of sign-tracking behavior in STs by increasing activity of glutamatergic projection neurons innervating the NAc and originating from the PFC. Moreover, subanesthetic ketamine may decrease sign-tracking behavior by increasing synaptic plasticity between afferents originating in the PFC and terminating in limbic structures of the motive circuit. For example, subanesthetic ketamine administration has been shown to increase thalamocortical connectivity

in humans (Dawson et al., 2014; Rivolta et al., 2015), and it has been previously shown that GTs, but not STs, have increased functional connectivity between the thalamus and PFC in their neural responses to lever-CS presentations (Flagel et al., 2011a; Haight & Flagel, 2014).

Increased glutamatergic activity in the PFC may also explain the enduring effect of subanesthetic ketamine on the expression of sign-tracking behavior in STs twenty-four hours following administration. Ketamine can have a half-life up to 2.5 h (Wieber et al., 1975) and subanesthetic ketamine alters Glu release only up to two hours following administration (Moghaddam et al., 1997). The enduring behavioral effects of subanesthetic ketamine have been hypothesized to result from an increased α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-to-NMDA receptor ratio (Du et al., 2006; Maeng et al., 2008) with AMPA receptors in the PFC facilitating brain-derived neurotrophic factor (BDNF) release (Lepack et al., 2015; Zhou et al., 2014). Moreover, infusion of anti-BDNF antibodies into the PFC blocks the behavioral effects of subanesthetic ketamine (Lepack et al., 2015). Previously, it has been shown that STs have lower levels of BDNF in the PFC compared to GTs (Morrow et al., 2015). Therefore, it is plausible that subanesthetic ketamine decreases sign-tracking behavior in STs by normalizing low levels of BDNF in the PFC of STs.

In addition to its effects in the PFC, subanesthetic ketamine increases DA release in the NAc of rats (Littlewood et al., 2006b; Moghaddam et al., 1997). This action presumably arises from local NMDA receptor inhibition in the NAc, as subanesthetic ketamine does not alter DA metabolism nor tyrosine hydroxylase levels in the rat VTA, the primary source of DA afferents to the NAc (Baptista et al., 2015). It is known that lever-CS presentations result in discrete cue-associated increases in DA in the NAc core of STs, but not GTs, which underlie the attribution of incentive salience to reward-related cues (Flagel et al., 2011b), and administration of

flupenthixol, a nonselective D1/D2 receptor antagonist, into the NAc core impairs the expression of sign-tracking (Di Ciano et al., 2001; Flagel et al., 2011b; Saunders & Robinson, 2012). Acute amphetamine administration, however, also decreases sign-tracking behavior and increases goal-tracking behavior, similar to our results with subanesthetic ketamine (Holden & Peoples, 2010; Simon et al., 2009). These results suggest that indiscriminately increasing DA levels may interfere with the cue-evoked DA release that imbues reward-related cues with incentive-motivational value. One possibility is that ketamine-induced DA release shifts conditioned responding from the reward-distal lever-CS (i.e., sign-tracking) to the reward-proximal pellet magazine (i.e., goal-tracking; Simon et al., 2009; Tindell et al., 2012). This would explain why sign-tracking behavior decreased and goal-tracking behavior increased, rather than sign-tracking behavior being exclusively affected.

The subanesthetic ketamine-induced shift from sign- to goal-tracking behavior has important implications from a therapeutic angle given that sign-tracking and goal-tracking behaviors are believed to represent model-free and model-based reinforcement learning, respectively (Huys et al., 2014). Clinically, a departure from model-free to model-based reinforcement learning would represent a transition from habitual, stimulus-driven responses to goal-directed cognitive control (Otto et al., 2015). One possibility is that subanesthetic ketamine could produce this shift through a combination of increased prefrontal cortical activation and altered striatal DA homeostasis (Deserno et al., 2015; Doll et al., 2016).

During the conditioned reinforcement test, subanesthetic ketamine did not influence conditioned reinforcement (i.e., the number of times a rat performed an active nose-poke response for presentation of the lever-CS); however, it decreased conditioned approach to the lever-CS in STs, but not GTs. These results confirm that, while PCA and conditioned

reinforcement measure closely related incentive-motivational processes, the two are dissociable and depend on neural substrates that do not completely overlap (Hitchcott & Phillips, 1998). Because NMDA receptor antagonism (i.e., AP-5) has previously been shown to decrease conditioned reinforcement, these results also suggest that subanesthetic ketamine has different pharmacological actions than other NMDA receptor antagonists (Wickham et al., 2015).

Although only two clinical studies have investigated the effects of subanesthetic ketamine in addicted patients, interest in the use of subanesthetic ketamine as a treatment for neuropsychiatric disorders has surged over the past decade, and many studies have already been performed to optimize its use as a pharmacotherapy. For example, a sublingual preparation of subanesthetic ketamine was recently reported to produce rapid and enduring antidepressant effects in refractory depression with no euphoric or dissociative effects (Lara et al., 2013). In addition, ketamine stereoisomers have been investigated to maximize therapeutic potential while minimizing side effects. For example, R-ketamine is more potent, longer lasting, produces less psychotomimetic effects, and more robustly increases BDNF signaling in the PFC than S-ketamine (Yang et al., 2015; Zhang et al., 2014). Alongside these pharmacological advances, it is also important to understand how a potential pharmacotherapy affects the underlying behaviors of a particular neuropsychiatric disorder. Currently, it is unknown how subanesthetic ketamine decreases craving in addicted patients, and our results provide insight into a potential mechanism, suggesting that subanesthetic ketamine decreases the incentive-motivational properties of reward-related cues in subjects vulnerable to addiction-like behaviors.

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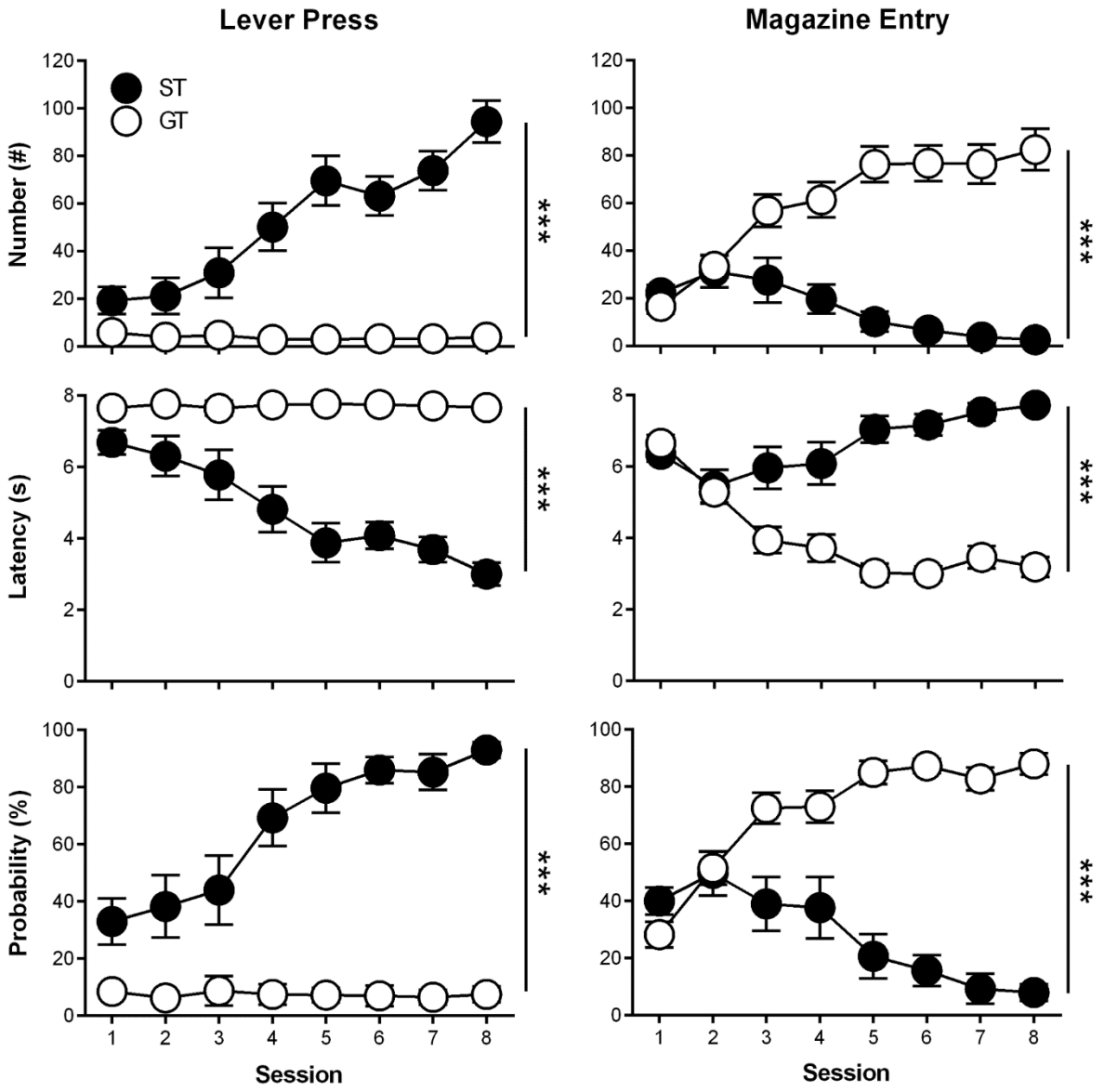

Figure 5.1. Pavlovian conditioned approach (PCA) training. Rats underwent Pavlovian conditioned approach training over eight daily sessions and were classified as sign-trackers (STs) or goal-trackers (GTs) based on their lever press and magazine entry number, latency, and probability during Session 8. Data are mean and S.E.M. *** — $p < 0.001$.

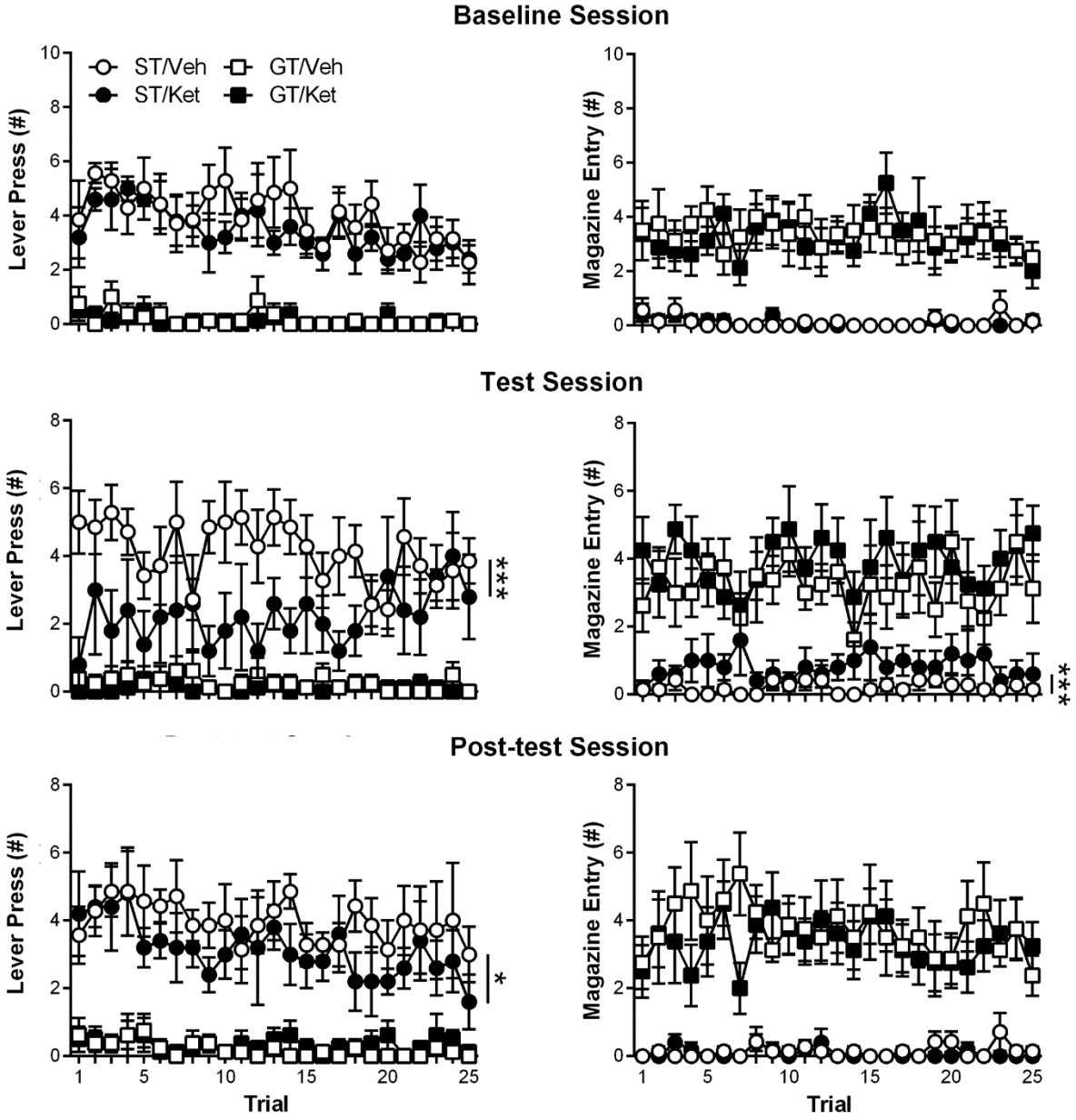

Figure 5.2. Subanesthetic ketamine decreases sig-tracking and increases goal-tracking in sign-trackers (STs). In Experiment 1, sign- and goal-tracking behavior was measured in STs and goal-trackers (GTs) during three additional Pavlovian conditioned approach sessions: baseline, test, and post-test. During the Pavlovian conditioned approach test session, subanesthetic ketamine (Ket; 32 mg/kg) or vehicle (Veh; saline) were administered 30 min prior to testing. Data are mean and S.E.M. * — $p < 0.05$, *** — $p < 0.001$.

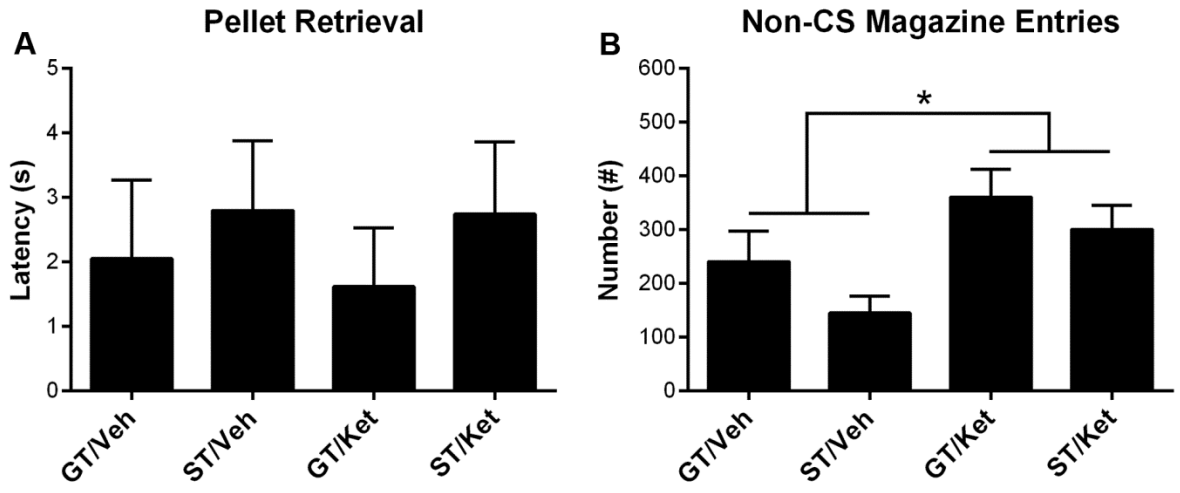

Figure 5.3. Subanesthetic ketamine does not influence pellet retrieval, but it increases magazine entries outside the conditioned stimulus (CS) period. During the Pavlovian conditioned approach test session, the latency of pellet retrieval and number of non-CS magazine entries (i.e., the number of magazine entries performed outside presentation of the lever-CS) were measured in sign-trackers (STs) and goal-trackers (GTs) that were administered subanesthetic ketamine (Ket; 32 mg/kg) or vehicle (Veh; saline). Data are mean and S.E.M. * — $p < 0.05$.

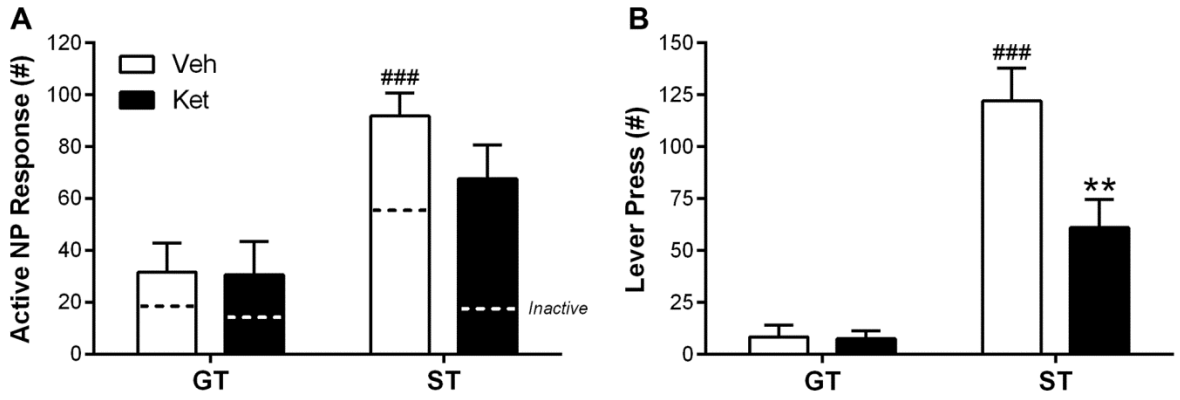

Figure 5.4. Subanesthetic ketamine does not affect conditioned reinforcement. In Experiment 2, sign-trackers (STs) and goal-trackers (GTs) were administered subanesthetic ketamine (Ket; 32 mg/kg) or vehicle (Veh; saline) before undergoing a conditioned reinforcement test during which (a) nose-poke responses and (b) lever presses were measured. Data are mean and S.E.M. ** — $p < 0.01$, within-subjects comparison; ### — $p < 0.001$, between-subjects comparison.

Appendix D: Chapter V Supplemental Information

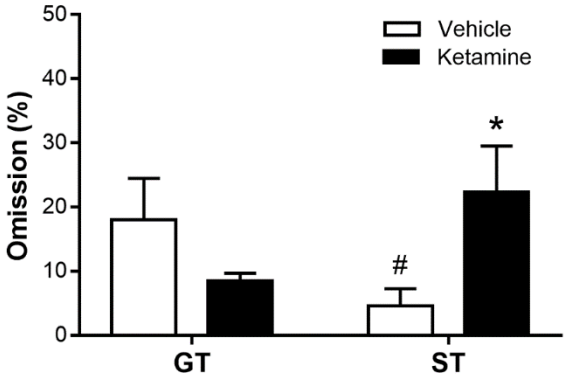

Figure S5.1. Subanesthetic ketamine increases omissions in sign-trackers (STs). During the test session, STs had fewer omissions than goal-trackers (GTs); in addition, subanesthetic ketamine (32 mg/kg) increased omissions in STs, but not GTs. Data are mean and S.E.M. * — $p < 0.05$, within-subjects comparison; # — $p < 0.05$, between-subjects comparison.

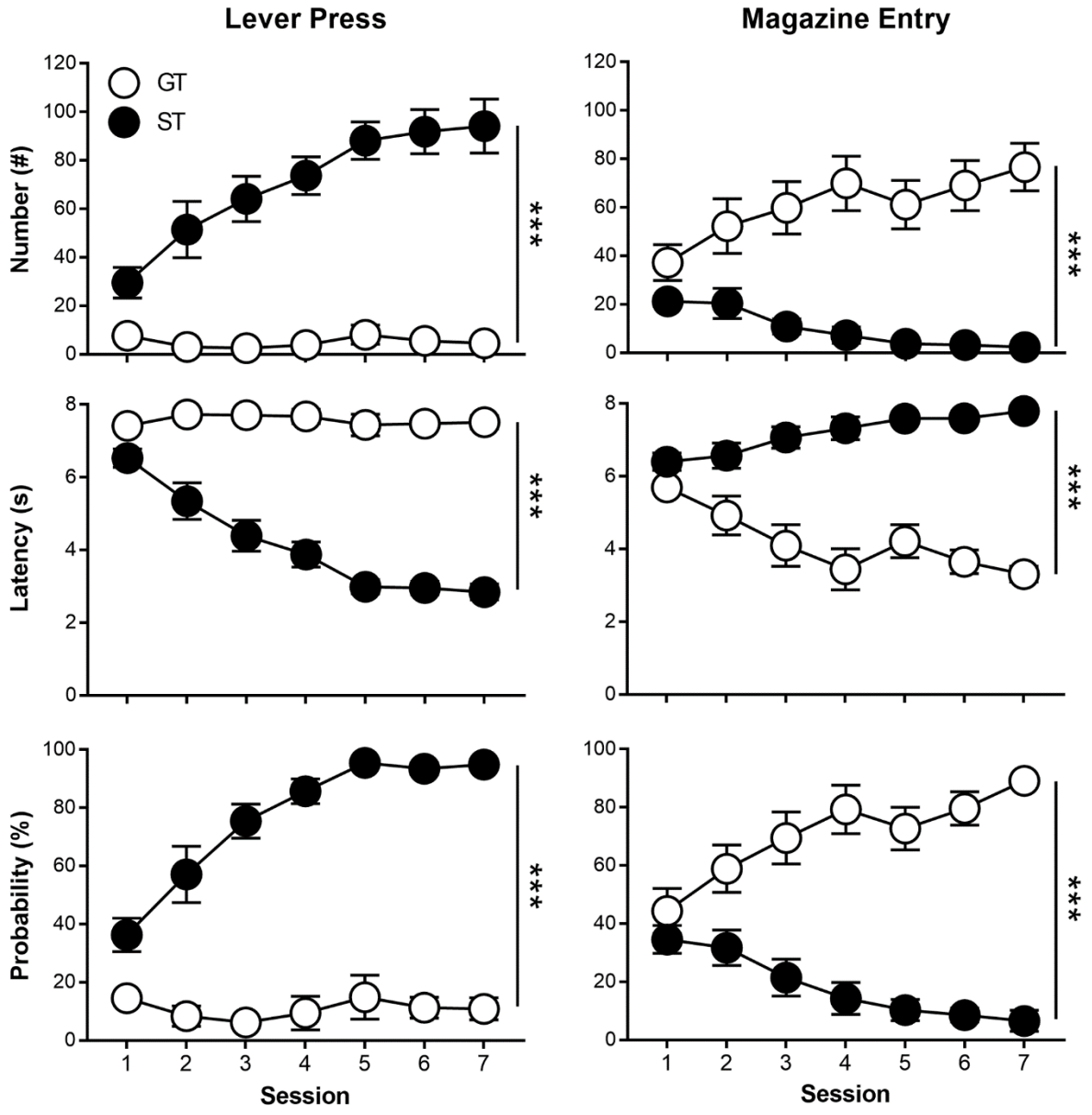

Figure S5.2. Acquisition of Pavlovian conditioned approach (PCA) behavior. Rats underwent Pavlovian conditioned approach training over seven daily sessions and were classified as sign-trackers (STs) or goal-trackers (GTs) based on their average lever press and magazine entry number, latency, and probability during Sessions 6 and 7. Data are mean and S.E.M. *** — $p < 0.001$.

Chapter VI

Conclusion and Future Directions

This thesis had four main aims. The first aim was to determine whether basal levels of Glu/GABA-Gln (the major metabolic cycle for excitatory/inhibitory neurotransmission), NAA (a marker of neuronal integrity), or Ins (a marker of astrocyte activity/proliferation) within the motive circuit (mPFC, NAC, and HPC) were different between PCA phenotypes and related to PCA behavior. In this aim, we demonstrated that Ins is elevated in STs compared to GTs and IRs in the NAc and vHPC, and it is positively correlated with sign-tracking behavior. The second aim demonstrated that the ventral hippocampus has a causal role in sign-tracking behavior and DA activity in the NAc and regulates the acquisition and expression of sign-tracking behavior. The third aim demonstrated that the motive circuit can be manipulated environmentally to decrease sign-tracking behavior and DA release in the NAc. Finally, the fourth aim demonstrated that the motive circuit can be manipulated pharmacologically using a subanesthetic dose of ketamine to decrease sign-tracking behavior and the conditioned reinforcing properties of incentive stimuli.

Neurochemistry in the nucleus accumbens and ventral hippocampus: Role of *myo*-inositol in sign-tracking behavior

The role of the NAc in regulating sign-tracking behavior is well-established. Dopaminergic signaling in the NAc contributes to the attribution of incentive-motivational value to reward-related cues (Flagel et al., 2011; Saunders & Robinson, 2012). In addition, this DA signaling is speculated to be modulated through inhibitory top-down control by the corticothalamic system and an excitatory drive from the limbic system (Haight & Flagel, 2014). The identity and involvement of these afferent regions and the neurochemical changes underlying individual variation in the attribution of incentive salience, however, is poorly understood.

In Chapter II, we used ^{1}H-MRS to measure baseline levels of 19 neurochemicals within the motive circuit (mPFC, NAc, vHPC, dHPC) of STs, IRs, and GTs. We hypothesized that neurochemicals in the Glu/Gln-GABA cycle, NAA (marker of neuronal integrity), or Ins (marker of astrocyte activity/proliferation) would be different between STs, IRs, and GTs in the NAc as well as the mPFC and HPC, both of which regulate NAc activity. The hypothesis was based on clinical studies using ^{1}H-MRS in addicted patients demonstrating differences in these neurochemicals. There were no differences between PCA phenotypes in basal levels of Gln or GABA; however, Glu was elevated in STs compared to IRs. A recent study by Batten et al. (2018) demonstrated that presentation of the reward-related cue during PCA training increases Glu in the prelimbic cortex of the mPFC in STs, but not GTs. Therefore, it is possible that baseline glutamatergic tone in STs subtly contributes to differentiating STs from IRs, whereas Glu signaling during a PCA procedure discriminates STs from IRs *and* GTs. Similarly, GABA signaling may be critical only during the acquisition and expression of PCA behavior (Fraser & Janak, 2017; Stringfield et al., 2017). Although NAA was not different between PCA phenotypes, basal levels of Ins in the mPFC and vHPC were increased in STs compared to IRs

and GTs, and PCA index scores on the last PCA training session positively correlated with Ins in both regions. Importantly, our results are the first to demonstrate neurochemical differences between STs and GTs, which correlate with PCA behavior, in the vHPC.

In ^{1}H-MRS studies, Ins is believed to represent astrocyte activity and proliferation, because it is detectable in the spectra of glia, but not neurons, in primary cultures (Brand et al., 1993) and increases in parallel with astrocytic markers (e.g., S100B and glial fibrillary acidic protein; Hammen et al., 2008; Rothermundt et al., 2007). In astrocytes, Ins contributes to a variety of processes involving osmosis and volume regulation (Hijab et al., 2011) as well as regulating cytoplasmic dynamics (e.g., protein folding; Fan et al., 2012). In addition, Ins is a precursor for phosphoinositides that participate in intracellular signaling cascades (e.g., Gq-coupled and inositol triphosphate receptors; Fiacco & McCarthy, 2006; Rizzuto, 2001) and synaptic plasticity (Baker et al., 2013). Increased astrocyte proliferation/activity during baseline would therefore prime tripartite synapses[38] in the local environment for Glu signaling/recycling and synaptic remodeling (Kim et al., 2017; Rose et al., 2017).

In future studies, the contribution of astrocytes to sign- and goal-tracking behavior should be more explicitly investigated. For example, immunohistochemistry of astrocytic markers (e.g., GFAP) can be used to visualize astrocyte number and arborization (Barros et al., 2006) and determine whether our Ins signal truly reflects astrocyte proliferation, hypertrophy, or neither (Song & Zhao, 2001). Because astrocyte-neuron communication is highly complex and synapse-, cell-, and circuit-specific, it is unclear whether results in the NAc and vHPC would be more similar or dissimilar (Durkee & Araque, 2018). Fluorocitrate, an inhibitor of glial activity with selectivity for astrocytes (Hassel et al., 1992; Paulsen et al., 1987), could be infused into the NAc

[38] The tripartite synapse refers to the proximity, integration, and contribution to synaptic activity of the neuronal presynapse, neuronal postsynapse, and glial processes (Araque et al., 1999; Heller & Rusakov, 2017).

or vHPC to inhibit activity during the acquisition or expression of PCA behavior (Murakami et al., 2015)[39]. By inhibiting astrocyte activity in these regions, a causal relationship between astrocyte activity and the acquisition or expression of PCA behavior can be established.

The ventral hippocampus and the motive circuit: Influence on sign-tracking behavior and dopamine turnover in the nucleus accumbens

In Chapter III, we used a combination of permanent lesions, temporary inactivation, and HPLC to investigate how the vHPC contributes to sign-tracking behavior and DA turnover in the NAc. We demonstrated that permanent lesions of the ventral, but not dorsal or total HPC, blocks sign-tracking behavior while increasing the acquisition of goal-tracking behavior[40]. During the expression of PCA behavior, however, permanent lesions of the vHPC or temporary inactivation of the vSUB had no effect on sign- and goal-tracking behaviors or conditioned reinforcement. Finally, vHPC lesions decreased dopamine turnover (HVA/DA) in STs, but not GTs, in the NAc, suggesting phenotype-specific differences in connectivity in this pathway. Taken together, we showed that the vHPC critically contributes to the acquisition of sign-tracking behavior and modulates DA turnover in the NAc of STs, but not GTs.

Despite mounting evidence that the vHPC is involved in approach behavior (Schumacher et al. 2018; Schumacher et al., 2016), motivation (Kanoski, 2013), and DA signaling in the NAc (Perez, 2018), we are the first to connect these findings to cue-directed behavior toward reward-related cues and individual variation in the attribution of incentive salience. For all intents and

[39] Flourocitrate cannot be used systemically as a glial inhibitor, because it requires a lethal dose (Fonnum et al., 1997).
[40] Lesions of the dHPC increased goal-tracking behavior without affecting sign-tracking behavior. See Chapter III for a more detailed discussion.

purposes, the vHPC is a part of the motive circuit; however, it should be more definitively incorporated into the conceptual framework of the motive circuit in future studies and reviews.

In future studies, a pharmacological[41] or optogenetic disconnection procedure, depending on the temporal specificity required, could be performed between the vHPC/VTA and/or vHPC/NAc, to determine whether the vHPC promotes sign-tracking behavior through modulation of VTA DA cell bodies, VTA DA afferents in the NAc, or a combination of both. Based on the results, FLEX-DREADDs (designer receptors exclusively activated by designer drugs) could be injected into the vHPC and NAc or VTA to investigate how the pathway affects the acquisition of sign-tracking behavior[42]. Specifically, adeno-associated viral constructs containing hM3Dq and hM4Di can be used to excite or inhibit this pathway, respectively. In addition, specific promoters can be incorporated into viral constructs to allow cell-specific expression. In future studies, a VGLUT1 (or CAMKII-alpha) promoter could be incorporated into a viral construct to specifically target the glutamatergic projection neurons in the vHPC that project to the NAc (Egashira et al., 2018). Furthermore, microdialysis or fast-scan cyclic voltammetry probes, depending on the desired temporal specificity, could be implanted in the NAc as well to measure DA release during PCA training sessions.

Environmentally manipulating the motive circuit: Prolonged stress decreases sign-tracking behavior and dopamine release in the nucleus accumbens

Stress is a psychosocial factor that contributes to the pathophysiology of addiction[43]. Specifically, stress exposure can potentiate reactivity to reward-related cues and promote craving

[41] The pharmacological disconnection procedure would involve the contralateral injection of lidocaine or muscimol/baclofen into the two brain regions.
[42] For a review of the FLEX-DREADDs technique, please see Roth (2016).
[43] In some theories of addiction, stress (and subsequent negative reinforcement and dysphoria) is conceptualized as the major contributor to the development of addiction (Koob et al., 2014).

and relapse (Koob, 2008; Sinha, 2008). Little is known, however, how stress affects the attribution of incentive salience during PCA procedures.

In Chapter IV, we demonstrated that SPS decreases the acquisition of sign-tracking behavior and increases goal-tracking behavior. Interestingly, these results were similar to our results with vHPC lesions during the acquisition of PCA behavior. Previously, it has been demonstrated that SPS affects the motive circuit (Enman et al., 2015) and causes apoptosis in the HPC (Han et al., 2013; Li et al., 2010). Therefore, it is possible that SPS-induced alterations (e.g., decreased DA release in Chapter IV and decreased DA turnover in Chapter III) are caused by a similar mechanism (i.e., decreased vHPC activity)[44].

In future studies, apoptosis in the vHPC of SPS-exposed rats could be investigated using immunohistochemistry to label caspase-3, one of the primary molecular switches for apoptosis (Cohen, 1997), to determine whether apoptosis is increased in SPS-exposed rats and whether the number of apoptotic neurons correlates with PCA behavior. Currently, it is unknown if particular cell types are more vulnerable to SPS-induced apoptosis. Future studies can utilize dual-labeling immunohistochemistry to simultaneously label caspase-3 and cell-type markers, such as specific interneurons (e.g., calbindin and calretinin) or dopaminergic neurons (e.g., tyrosine hydroxylase). Alternatively, an acute stressor could be investigated before and during PCA training to investigate whether acute stress (e.g., foot shock or restraint) can potentiate the acquisition and/or expression of sign-tracking behavior. If one of these procedures potentiates sign-tracking behavior, systemic administration of pharmacological antagonists against glucocorticoid or beta-adrenergic receptors during acute stress could determine whether

[44] It is also possible that SPS causes apoptosis in the VTA, decreasing K^+-induced DA release by reducing the number of dopaminergic cell bodies.

potentiation is via corticosterone signaling[45] or NE/epinephrine signaling, two stress systems that positively modulate sign-tracking behavior (Cogan et al., 2018; Rice et al., 2018).

Pharmacologically manipulating the motive circuit: Subanesthetic ketamine decreases sign-tracking behavior

Subanesthetic ketamine has primarily been investigated as a novel therapeutic treatment for depression (Duman, 2018; Krystal et al., 2013). Recently, however, a small study of eight cocaine-dependent individuals demonstrated that a single infusion of subanesthetic ketamine decreases cue-induced cravings and increases motivation to quit (Dakwar et al., 2014). Most preclinical studies of subanesthetic ketamine have focused on depression-like behavior (Chu, 2018; Mishra et al., 2018; Salat et al., 2015), and no studies have investigated a subanesthetic dose of ketamine on the attribution of incentive salience.

In Chapter V, a subanesthetic dose of ketamine administered systemically decreased sign-tracking behavior and increased goal-tracking behavior in STs, but had no effect in GTs. Additionally, subanesthetic ketamine had enduring effects in decreasing sign-tracking 24 hours later in STs. Moreover, subanesthetic ketamine decreased the conditioned reinforcing properties of a reward-related cue in STs, but not GTs.

Because subanesthetic doses of ketamine do not alter DA release in the NAc (Can et al., 2016; Irifune et al., 1997), the effects of subanesthetic ketamine on sign-tracking behavior are likely mediated through increased synaptic plasticity between brain regions in the motive circuit (vHPC → mPFC and/or mPFC → NAc), including vHPC-mediated stimulation of the mPFC (Carreno et al., 2016; Jett et al., 2015), increased top-down control of the NAc by the mPFC

[45] Plasma corticosterone levels are increased following a PCA procedure (Tomie et al., 2000; Tomie et al., 2002; Tomie et al., 2004), and plasma levels are higher in STs than IRs and GTs (Flagel et al., 2009).

(Dandash et al., 2015; Razoux et al., 2007), and decreased synaptic plasticity of the NAc (Yao et al., 2018).

In future studies, low micromolar concentrations of ketamine could be delivered into the vHPC, mPFC, and NAc to determine what brain region underlies the effect of systemic administration of subanesthetic ketamine on sign-tracking behavior. Specifically, a pharmacological disconnection approach could be used with ketamine infused in the mPFC and NBQX/CNQX (AMPA receptor antagonists) or MK-801 (NMDA receptor antagonist) infused in the NAc to determine whether subanesthetic ketamine modulates sign-tracking behavior by altering glutamatergic signaling to the NAc[46]. Alternatively, because it has been suggested that release of BDNF underlies the behavioral effects of subanesthetic ketamine (Bjorkholm & Monteggia, 2016; Lepack et al., 2014), BDNF antibodies, siRNA, or oligonucleotides could be infused into the NAc following either systemic or intra-mPFC administration of subanesthetic doses of ketamine during PCA training sessions.

Conclusion

In conclusion, the studies presented in this thesis advance our knowledge regarding the neurochemistry in the motive circuit underlying sign-tracking behavior as well as how sign-tracking behavior is influenced by ventral hippocampal activity, environmental stress, and pharmacological manipulation. I propose that decreased ventral hippocampal activity due to lesions or exposure to prolonged stress result in decreased vHPC-stimulated DA signaling in the NAc (Figure 6.1). In addition, I propose that subanesthetic ketamine activates ventral hippocampal afferents to the mPFC, which promotes cognitive flexibility, alters strategy

[46] For an example of a pharmacological disconnection procedure, see McGlinchey et al. (2016).

selection of CRs, and increases top-down control of NAc DA activity, decreasing expression of sign-tracking behavior and increasing expression of goal-tracking behavior (Figure 6.2). Taken together, this thesis demonstrates that the vHPC is a part of the motive circuit that regulates NAc DA activity and suggests its involvement in medial prefrontal modulation of NAc DA activity (Figure 6.3). Moreover, the studies presented in this thesis provide a framework from which to expand our knowledge of the motive circuit and investigate connectivity between nodes within it. Identification of novel brain regions and signaling pathways related to the acquisition and expression of sign-tracking behavior will hopefully lead to the development of novel therapeutic targets to treat craving, relapse, and the pathophysiology of addiction.

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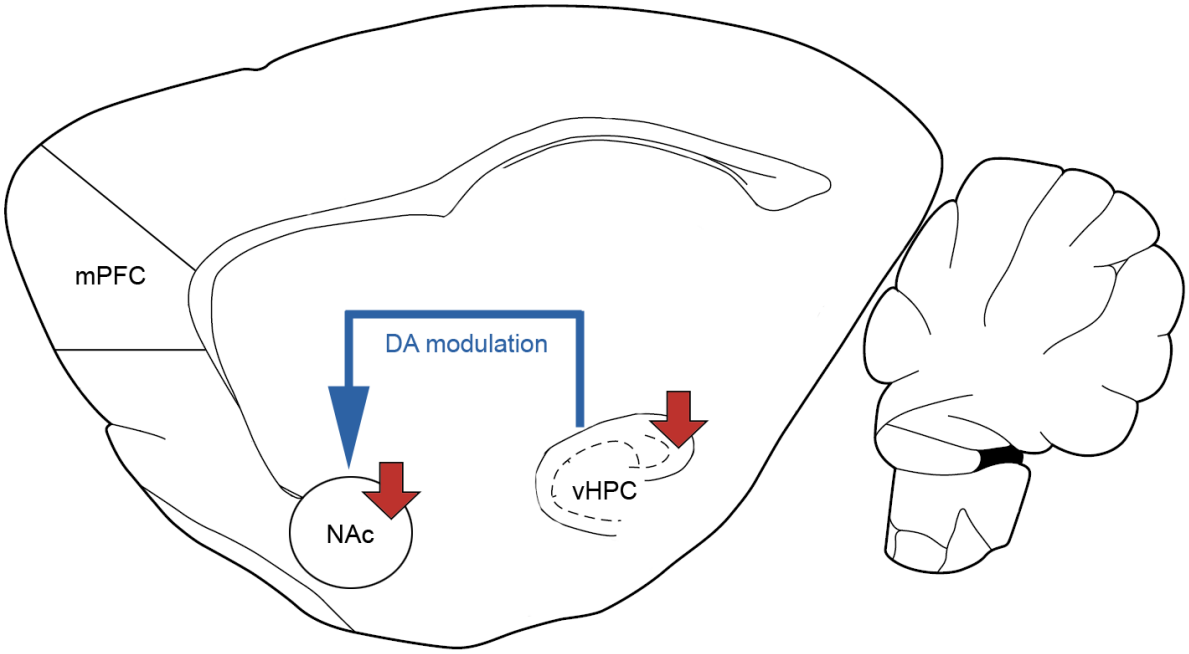

Figure 6.1. Schematic of the proposed effect of ventral hippocampal lesions and single prolonged stress on the motive circuit. Ventral hippocampal lesions decrease excitatory input to ventral tegmental afferents in the nucleus accumbens (NAc), lowering dopamine (DA) release. Similarly, stress-induced alterations in ventral hippocampal activity decreases DA release within the NAc. *Image adapted from Paxinos & Watson (2007).*

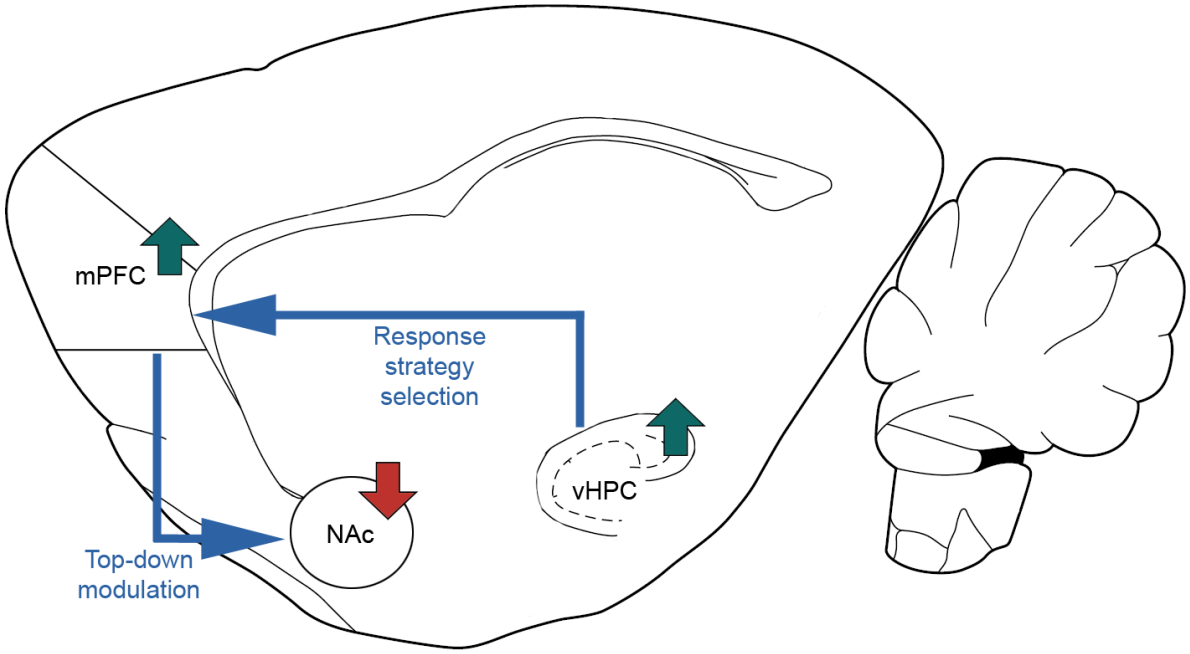

Figure 6.2. Schematic of the effect of subanesthetic ketamine on the motive circuit. **Increased** connectivity between the vHPC and mPFC alters response strategy selection, and increased top-down modulation of the NAc by the mPFC results in altered synaptic plasticity. As a consequence, sign-tracking behavior decreases, and goal-tracking behavior increases. mPFC – medial prefrontal cortex, NAc – nucleus accumbens, vHPC – ventral hippocampus. *Image adapted from Paxinos & Watson (2007).*

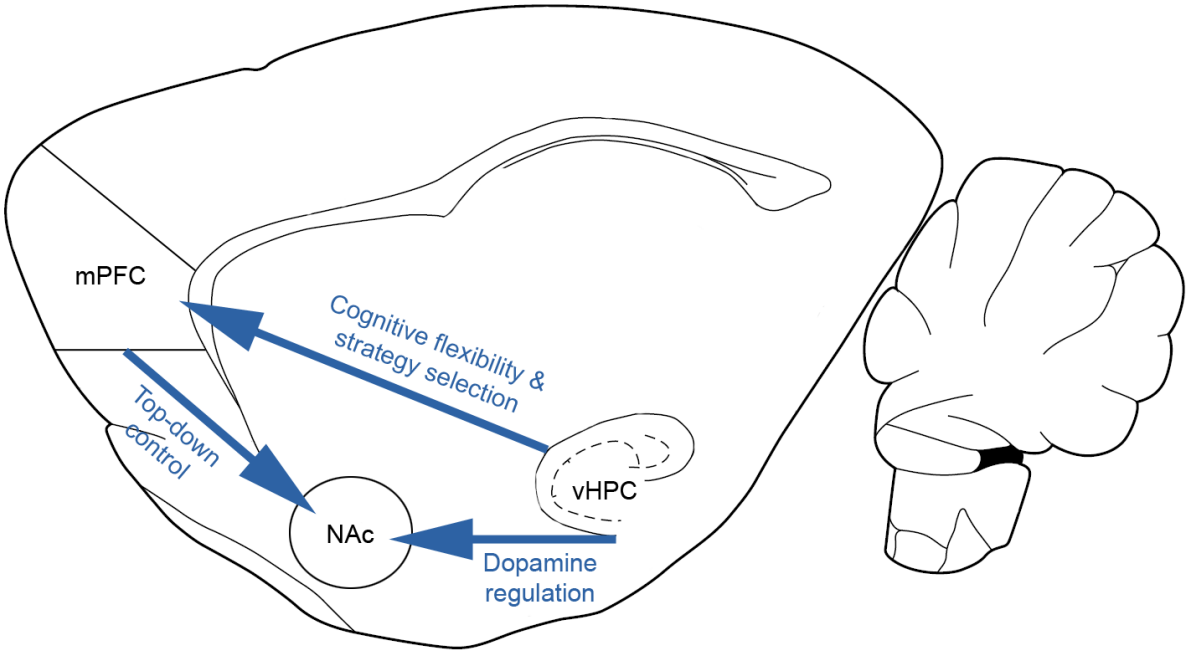

Figure 6.3. Schematic of a revised portion of the motive circuit with the inclusion of the ventral hippocampus (vHPC) with the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC), including proposed roles of the pathways. (This schematic does not include brain regions in the motive circuit that were not investigated in the present thesis, such as the ventral tegmental area, amygdala, and thalamic subregions. *Image adapted from Paxinos & Watson (2007).*