Diametric Changes in Ventral Striatal Dopamine Release Regulate Drug-Taking and Drug-Seeking Behavior

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Abstract

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The overall goal of this dissertation work was to determine how drug-cue elicited phasic dopamine neurotransmission changes over prolonged drug use, in both drug-taking and drug-seeking contexts. My initial work, done in collaboration with Dr. Ingo Willuhn, illustrated differences in dopamine dynamics between striatal subregions during active drug taking periods, and demonstrated a causal role for ventral striatal cue-elicited dopamine signals in regulating drug intake. Through these studies, we found that decreases in ventral striatal phasic dopamine release evoked by drug cues drive the escalation of drug intake observed in rats given protracted drug access (Chapter 2). These results are consistent with one of the preeminent theories of drug abuse, which implicates ventral striatal dopamine in producing drug satiety and regulating drug taking behavior. Though altered dopamine transmission is implicated in most

contemporary theories of drug abuse, the timing, context, and directionality of these changes remain a matter of debate. In contrast to the satiety theory, another large body of work suggests that ventral striatal dopamine mediates craving and promotes cue driven drug-seeking. Do these theoretical changes in dopamine actually co-exist? This is the question that has driven the bulk of my dissertation work.

Drug cues serve different purposes in different situations. During drug taking, cues confirm the success of drug seeking actions and indicate imminent drug delivery, signaling that drug seeking can be terminated. In contrast, during reinstatement paradigms, the same cues, presented unexpectedly during abstinence, signal possible drug availability nearby and promote initiation of drug seeking. The objective of my work was to understand how the dynamics of ventral striatal phasic dopamine signals, evoked by drug cues that are presented unexpectedly during abstinence, differ from those observed during drug taking. To study this, I used fast-scan cyclic voltammetry, an electrochemical detection method that allows for real time monitoring of dopamine release in situ, in awake behaving animals, to measure cue-elicited phasic dopamine signals throughout cocaine self-administration and following long periods of abstinence. The main findings are summarized below:

Unexpected drug cues elicit larger dopamine responses over drug taking history. I found that over the course of drug history, the directionality of changes in cue elicited signals obtained in drug-taking and drug-seeking contexts oppose one another. Specifically, cue elicited

signals during drug-taking decrease over time, whereas, at the same time point unexpected presentation of the same cues in a drug seeking context produces larger dopamine signals over time (Chapter 3).

Dopamine responses elicited by unexpected drug cues increase during abstinence. Studies by Grimm *et al*. (2001), and others[1-4], have demonstrated a positive correlation between the duration of abstinence, and resistance to extinguish responding for drug cues, a phenomenon that has been termed the "incubation of craving". We hypothesized that this resistance to extinction after prolonged abstinence was mediated by an increase in cue elicited dopamine release. I measured unexpected cue elicited phasic dopamine release after one day or one month of abstinence and observed striking increases in dopamine release evoked by cues after longer periods of abstinence (Chapter 4). These increases in dopamine paralleled the incubation of craving, and increases in cue elicited drug seeking as assessed by conditioned approach behavior. These data are consistent with ventral striatal dopamine mediating craving and promoting drug seeking, an idea with empirical support from many previous studies.

Collectively, these studies suggest that the dynamics of cue elicited phasic dopamine transmission depend upon the context in which cues are presented, and explain how dopamine in the ventral striatum might contribute to different, but equally important, core symptoms of substance use disorders.

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ACSF	artificial cerebrospinal fluid
ANOVA	Analysis of Variance
CS	conditioned stimulus
DLS	dorsolateral striatum
FSCV	fast-scan cyclic voltammetry
KOR	kappa opioid receptors
kg	kilogram
L-DOPA	l-3,4-dihydroxyphenylalanine, dopamine precursor
LgA	long-access self-administration (6 hours access / day)
nA	nanoamps
NAcc	nucleus accumbens core (used synonymously with VMS in this work)
nor-BNI	norbinaltorphimine, long-acting KOR antagonist
μm	micrometer
μL	microliter
mg	milligrams
mL	milliliter
s.e.m.	standard error of the mean
ShA	short-access self-administration (1-hour access / day)
V	volts
VMS	ventromedial striatum (used synonymously with NAcc in this work)

Acknowledgements

I would first like to thank Dr. Paul Phillips for his guidance and mentorship throughout graduate school. I am grateful to have had the opportunity to work on such an exciting project with your full support. You have taught me a great deal about how to think as a scientist, and have helped me to develop the skills necessary to communicate my work with the greater scientific community. I am particularly thankful for your support in traveling to conferences to present my work and network with other scientists. This has helped me expand my mentorship team well beyond those at the University of Washington and this will be an invaluable resource as I progress in my career. I would also like to thank the rest of my dissertation committee, Drs. Charles Chavkin, Sheri Mizumori, Larry Zweifel, John Neumaier. I have benefitted greatly from the advice and guidance that I received from each of you. Also, a special thank you to Drs. Paul Phillips, Charles Chavkin, and John Neumaier for reading my thesis and giving me helpful feedback.

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Finally, thank you to my family for supporting me in pursuing in everything that I do!

CHAPTER 1

Introduction

Drug addiction imposes a large social and economic burden on our society. Current reports estimate that the addiction related economic loss stemming from crime, loss of work related productivity, and health care costs exceeds $700 million annually in the United States (NIDA, 2017). Treatment for this disorder has proven difficult as those with addiction exhibit a high rate of relapse following periods of both self-imposed and forced abstinence, at time points far beyond those at which drugs maintain their pharmacological effect. Despite major advances in our understanding of the neurobiology of addiction over the last several decades, many questions remain about the biological underpinnings responsible for regulating drug-intake, drug-seeking behavior, and craving. We must continue to pursue the answers to these questions so that we can provide a framework to inform the development of more efficacious pharmacotherapies to help those with substance use disorders mitigate ongoing drug use, and prevent relapse following periods of abstinence.

Defining Addiction

The hallmarks of addiction used for diagnosis, as currently defined in the *Diagnostic and Statistical Manual of Mental Disorders* (fifth edition; Am. Psychiatric Assoc., 2013)[5], fall into four major categories:

1) Impaired Control of Substance Use: Those afflicted with addiction often exhibit a loss of control over the amounts of drug used, and the duration of time spent using drug. Significant amounts of time are spent obtaining, using, and recovering from the effects of drug use. Cravings for drug are very strong, and as a result desires to terminate or regulate drug use are unsuccessful.
2) Social Impairment: Recurrent use of drug may lead to a failure to fulfill obligations at work, school, or home. Important social or occupational recreational activities may be given up, and drug use continued despite it causing recurrent interpersonal problems.
3) Risky Use: To obtain and use drug, many patients with substance use disorders will repeatedly put themselves in situations that are physically hazardous. In addition, many

will continue to use drug despite the persistent physical or psychological problems that are caused or exacerbated by the substance of abuse.

4) Pharmacological Effects: Following repeated substance use tolerance is developed, thus the dose required to achieve the desired effect increases significantly. In addition, those with substance use disorders will often experience a withdrawal syndrome when concentrations of the substance become too low.

Animal Models of Addiction

Preclinical studies of the neurobiological mechanisms that underlie addiction utilize numerous animal models to investigate distinct features of the addiction process. Behavioral paradigms have been developed for modeling different stages of the addiction cycle, psychological constructs, such as positive and negative reinforcement, and other characteristics or symptoms observed in humans with substance use disorders[6]. The drug-abuse cycle can be divided into three major phases: a binge/intoxication phase, a withdrawal phase, and a preoccupation/anticipation phase (for detailed review see Koob and Le Moal, 2008[6]). Studies of the intoxication phase are typically focused on understanding the mechanisms that mediate the positive reinforcing properties of drugs, which support their self-administration, and regulate drug-intake levels[7-9]. Following the termination of drug taking, physical and psychological withdrawal symptoms emerge. In humans, the desire to mitigate the negative withdrawal symptoms often triggers the resumption of drug taking[10,11]. The severity of many withdrawal symptoms can be studied in animals and thus therapeutic strategies for minimizing withdrawal can be tested[10,12-18]. Following the withdrawal phase, during periods of abstinence, subjects become consumed by preoccupation/anticipation of seeking and taking drug[19-22]. Enhanced drug-craving and drug-seeking during this phase lead to relapse and reinitiate the drug-abuse cycle. Many different behavioral paradigms have been developed to study drug seeking and craving during periods of abstinence[3,23-26] with the hopes of better understanding the neural

mechanisms that underlie relapse. The studies presented in this dissertation are focused on understanding the neurobiological mechanisms that underlie the regulation of drug intake during the binge/intoxication phase, and the drug-seeking and craving which precipitate relapse. A brief discussion of the models used for the study of these phases of the drug-abuse cycle follows.

Modeling the regulation of drug intake

Drugs of abuse serve as strong reinforcers in both humans and animals [8,19,27]. In self-administration paradigms, animals are allowed access to a chamber in which their performance of an operant response will trigger the delivery of drug. Once the relationship between the operant response and the outcome is learned animals will respond in an intermittent but consistent manner, so as to maintain drug levels above a desired threshold [28-32]. Drug-intake during self-administration is stable across days in animals given limited access to self-administer drug (short-access, 1-hour daily sessions), however when given longer daily drug access periods (long-access, 6-hour daily sessions), drug intake will begin to escalate over days[33]. Allowing protracted daily access to self-administer drugs better models the escalation of drug intake observed in humans with drug abuse disorders. Animals allowed long-access also exhibit other traits reminiscent of the human addiction criteria that animals allowed short-access do not. Animals given long-access exhibit an increased motivation to obtain drug[33-35] and an enhanced propensity for drug seeking [36,37].

Relapse Models

Relapse to drug use following periods of abstinence is one of the biggest challenges for the treatment of addiction[38]. In those afflicted with addiction, drug craving and relapse are often precipitated by acute re-exposure to the abused drug[39], cues associate with the drug[38,40], or stress[41-43]. The most common animal model used to study relapse is the reinstatement model [3,23-26]. In reinstatement studies, animals with a history of drug self-administration undergo a series of operant extinction sessions in which the operant response that was previously paired

with drug is no longer reinforced. Once animals extinguish responding they undergo a period of abstinence, and then reinstatement of responding is measured following a challenge in the form of drug delivery, drug cue presentation or stress exposure[44-46].

Evidence from humans suggests that drug craving is dynamic, increasing over weeks to months of abstinence[47]. This time dependent increase in drug seeking during abstinence, a phenomenon which has been termed the 'incubation of craving', has also been observed in animals[1]. Initial studies of the incubation of craving used similar methods to those in reinstatement studies, measuring animals' resistance to extinction and reinstatement of drug seeking following different periods of abstinence. In this work, increases in the resistance to extinguish responding for drug cues and in the reinstatement of cue-induced drug-seeking were observed over the course of 60 days of abstinence[1]. However, the face validity of studies utilizing operant extinction prior to reinstatement has been called into question, as human addicts do not undergo extinction prior to relapse. As such, recent adaptations of the incubation of craving model have employed only brief cue-induced drug-seeking sessions in which animals are brought back to the drug taking environment following differing periods of abstinence and allowed to respond for presentation of the drug paired cue alone[3].

Conflicting Theories of the Mesolimbic Dopamine System's Role in Addiction

Though most contemporary theories of drug abuse agree that alterations in dopamine neurotransmission underlie addiction, these theories propose conflicting ideas of the timing, context, and directionality of these changes in dopaminergic activity. The mesolimbic dopamine pathway, which projects from the ventral tegmental area to the nucleus accumbens (NAc, ventral region of the striatum), is implicated in mediating different aspects of addiction related behaviors during multiple stages of the drug-abuse process.

Mesolimbic Dopamine Serves as a Satiety Signal

Enhanced mesolimbic dopamine neurotransmission mediates the acute reinforcing effects of drugs of abuse[48-51]. Drugs of abuse increase mesolimbic dopamine release in both humans and animals [52-62], and these increases in dopamine correlate with the intense feelings of euphoria and pleasure induced by these drugs in humans[52-58].

One theory of dopamine's role in drug abuse is that mesolimbic dopamine neurotransmission signals drug satiety and thus, serves as a key regulator of drug intake[61]. This theory was derived from decades of work which established that animals will adjust their response rates across a wide range of drug doses and work requirements[28,63-66] in order to maintain NAc dopamine levels above some satiety threshold [67]. This work was also corroborated by studies demonstrating that animals will adjust their drug intake patterns to compensate for pharmacological manipulation of dopamine receptors or neurotoxic lesion of the mesolimbic dopamine pathway[51,68]. Overall this work suggests that drug administration leads to a rapid increase in NAc dopamine release which serves as a satiety signal and prevents further responding. Eventually the elimination of the drug will cause dopamine levels to decrease, and once below a certain threshold further responding for drug is initiated.

Mesolimbic Dopamine Promotes Drug Seeking and Enhances Craving

Drug-associated cues (environmental stimuli associated with drug use) can also evoke mesolimbic dopamine neurotransmission[69-71]. Through associative learning processes, previously neutral stimuli that are repeatedly paired with delivery of natural or drug rewards gain the ability to evoke NAc dopamine release when presented alone[69,71-74]. This enables drug-associated cues to exert powerful control over behavior in both drug-taking and drug-seeking contexts[69,72]. Indeed, when experienced in the absence of drug, or following long periods of abstinence, drug-associated cues alone can precipitate drug craving in humans[40,75-77], and promote drug seeking in rodents[23,24,44,78,79]. Increases in dopamine parallel, and are thought to mediate these feelings of craving in humans, and promote drug seeking during abstinence in animal models of

relapse[52,74,80-86]. The incentive sensitization theory of addiction, proposed by Robinson & Berridge (1993)[87], posits that during drug use, progressive and persistent neuroadaptations develop which sensitize the mesolimbic dopamine system. This leads to progressive enhancement of the incentive salience, or "wanting", attributed to drug-cues and this desire is what drives drug seeking [87,88].

Dissertation Summary

While the satiety theory implicates enhanced mesolimbic dopamine neurotransmission in regulating drug intake during the intoxication phase of the drug abuse cycle, another equally well substantiated body of work implicates mesolimbic dopamine in regulating drug seeking during abstinence in the preoccupation/anticipation phase of the drug abuse cycle.

At face value, these theories implicate mesolimbic dopamine in the conflicting roles of both suppressing drug intake and promoting drug seeking. **Are there actual changes in dopamine neurotransmission that are concordant with these ideas?**

These theories were derived from studies of two different phases of the drug abuse cycle in which drug cues serve different purposes. During drug taking, cues confirm the success of drug seeking actions and indicate drug delivery is imminent, signaling that drug seeking actions can be terminated and suppressing further drug intake. In contrast, during reinstatement paradigms, the same cues, presented unexpectedly during abstinence, signal that drug may be available nearby, thereby promoting the initiation of drug seeking. **The main objective of the studies presented in this dissertation was to test the hypothesis that diametric changes in cue-elicited NAc dopamine release mediate drug-taking and drug-seeking behavior.**

In order to measure changes in dopamine neurotransmission elicited by drug cues throughout the course of long-term behavior we need to be able to monitor dopamine release with high temporal resolution, and do so over long periods of time. As such, we used fast-scan cyclic voltammetry, an electrochemical detection method which allows for the measurement of

rapid changes of dopamine release *in situ*[89,90], at chronically implantable carbon fiber micro electrodes [89] to measure cue-elicited phasic dopamine release over weeks of cocaine self-administration and following prolonged periods of abstinence. My initial work, done in collaboration with Dr. Ingo Willuhn, illustrated differences in dopamine dynamics between striatal subregions during active drug-taking periods, and demonstrated a causal role for NAc cue-elicited dopamine signals in regulating drug intake (Chapter 2). Through these studies, we found that decreases in ventral striatal phasic dopamine release evoked by drug-cues drive the escalation of drug intake observed in rats given protracted drug access (Chapter 2). These results were consistent with the satiety theory of drug abuse, which implicates NAc dopamine in producing drug satiety and regulating drug taking behavior. The bulk of my thesis work was focused on understanding how these drug-cue elicited signals differed in drug seeking contexts. I first measured responses to unexpected drug cue presentations during the drug taking (intoxication) phase, but prior to self-administration sessions when animals were not intoxicated. I found that rather than decreasing over long-access drug-taking, these signals increased (Chapter 3). I also measured drug-cue elicited dopamine responses during short and long periods of abstinence and found that these signals robustly increase during long periods of abstinence, and that these increases in dopamine parallel enhancements in drug seeking (Chapter 4). These data were consistent with the incentive sensitization theory of addiction. Thus, together these data support our hypothesis that diametric changes in cue-elicited NAc dopamine release mediate drug-taking and drug-seeking behavior, and unify two of the competing contemporary theories describing dopamine's role in addiction.

I have also included an overview of preliminary data from ongoing studies looking at the mechanisms that govern these changes in cue-elicited dopamine release, and therapeutic approaches for combatting addiction (Chapter 5).

CHAPTER 2

Characterization of the Dynamics of Cue-Elicited

*Striatal Phasic Dopamine Release During Drug Taking**

*The bulk of this chapter was originally published in two manuscripts in which I was the second author. The full references to the publications and my specific contributions to this work are listed below and components that are reproduced are indicated within the chapter as well.

Willuhn I, Burgeno LM, Everitt BJ, Phillips PEM (2012). Hierarchical recruitment of phasic dopamine signaling in the striatum during the progression of cocaine use. *Proc. Nat. Acad. Sci.*, 109(50), 20703-8.
I carried out all behavioral pharmacology studies.

Willuhn I, Burgeno LM, Groblewski P, Phillips PEM (2014). Excessive cocaine use results from decreased phasic dopamine signaling in the striatum. *Nature Neuroscience*, 17(5), 704-9.
I performed neurochemistry recordings in these studies.

Introduction

My early graduate work, carried out in collaboration with Dr. Ingo Willuhn resulted in the publication of two manuscripts; I am the second author on both studies. The bulk of my thesis work, presented in Chapters 3-5, has built directly upon this work. Before presenting the reformatted manuscripts as published, I have included a brief overview of each of these manuscripts and outlined my contributions to the work.

Hierarchical recruitment of phasic dopamine signaling in the striatum during the progression of cocaine use[91].

Drug addiction is the final stage of a process beginning with recreational drug use and ending in habitual and compulsive drug use. During this progression, multiple brain areas play roles in mediating this progression at different stages. Dopamine signaling in the ventral striatum is strongly implicated in the acute reinforcing effects of drugs of abuse. In addition, there is growing evidence suggesting that as drug taking becomes more habitual, dopamine signaling in the dorsolateral striatum becomes increasingly important in mediating this behavior[21,92-94]. Our work, presented in Willuhn *et* al., 2012[91], illustrated differences in dopamine dynamics between striatal subregions during active drug-taking periods. Phasic dopamine signaling within the dorsolateral striatum developed only after multiple weeks of cocaine self-administration, whereas phasic dopamine signaling in the ventral striatum was present from the initiation of drug taking. In addition, serial disconnection studies showed that the development of this signal in the dorsolateral striatum is reliant upon prior activity within the ventral striatum. Furthermore, blocking dopamine receptors in the dorsolateral striatum during late time points in cocaine self-administration decreases the animal's ability to discriminate actions that yield drug from those they previously learned do not yield drug. This work demonstrates the importance of phasic dopamine signaling in different striatal subregions in the control of drug-taking behavior during different epochs of drug-history. I carried out the behavioral pharmacology studies which resulted in the data presented in **Figure 4**[91].

Excessive cocaine use results from decreased phasic dopamine signaling in the striatum.
Willuhn I, Burgeno LM, Groblewski P, Phillips PEM (2014). *Nature Neuroscience*, 17(5), 704-9.

In order to investigate the role that changes in cue-elicited phasic dopamine signaling in the ventral and dorsolateral striatum might play in later stages of drug abuse, we studied animals in a long-access (6-hour session/day) self-administration paradigm in which they escalate their drug intake relative to short-access controls (1-hour session/day). Through these studies, we found that decreases in ventral striatal phasic dopamine release evoked by drug cues drive the escalation of drug intake observed in rats given protracted drug access. These results are consistent with one of the preeminent theories of drug abuse, which implicates ventral striatal dopamine in producing drug satiety and regulating drug-taking behavior. Most of my dissertation work has been a direct follow up to this study (Chapters 3-5). Importantly, I have replicated the main result of this study (data presented in Chapter 3). My contribution to this work was running self-administration studies and carrying out neurochemical recordings.

*notes:
1. Figure numbering is separate for each manuscript to preserve the same figure numbers as the published versions.
2. All recordings of phasic dopamine release throughout all experiments in this dissertation were performed in the nucleus accumbens core. The following papers refer to the nucleus accumbens core as the ventromedial striatum.

Hierarchical recruitment of phasic dopamine signaling in the striatum during the progression of cocaine use

Ingo Willuhn[a], Lauren M. Burgeno[a], Barry J. Everitt[b] and Paul E. M. Phillips[a] *Proc. Nat. Acad. Sci.*, 109(50), 20703-8.

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Abstract

Drug addiction is a neuropsychiatric disorder that marks the end stage of a progression beginning with recreational drug taking but culminating in habitual and compulsive drug use. This progression is considered to reflect transitions among multiple neural loci. Dopamine neurotransmission in the ventromedial striatum (VMS) is pivotal in the control of initial drug use, but emerging evidence indicates that once drug use is well-established, its control is dominated by the dorsolateral striatum (DLS). In the current work, we conducted longitudinal neurochemical recordings to establish the spatiotemporal profile of striatal dopamine release and investigate how it changes during the period from initial to established drug use. Dopamine release was detected using fast-scan cyclic voltammetry simultaneously in the VMS and DLS of rats bearing indwelling intravenous catheters over the course of three weeks of cocaine self-administration. We found that phasic dopamine release in DLS emerged progressively during drug taking over the course of weeks, a period during which VMS dopamine signaling declined. This emergent dopamine signaling in DLS mediated discriminated behavior to obtain drug but did not promote escalated or compulsive drug use. We also demonstrate that this recruitment of dopamine signaling in the DLS is dependent upon antecedent activity in VMS circuitry. Thus, the current findings identify a striatal hierarchy that is instantiated during the expression of established responding for cocaine.

Introduction

Drug use often begins as a recreational behavior driven by the rewarding properties of the abused drug. However, addiction is characterized by habitual and compulsive drug use where other factors such as withdrawal symptoms, stress, and drug-associated conditioned stimuli (CS) also contribute to the motivation to consume drugs[95], and drug taking has become increasingly prioritized over other behaviors[5]. A wealth of evidence shows that the mesolimbic dopamine projection from the ventral tegmental area to the ventromedial striatum (VMS) is central to drug reinforcement[61,95-97].

The ambient concentration of dopamine in VMS is increased when animals self-administer drugs of abuse including cocaine[98,99], and animals maintain this elevated dopamine level by regulating their rate of responding for drug[67]. In addition, with repeated pairing of environmental stimuli with the drug, these conditioned stimuli also gain the propensity to elicit changes in dopamine concentration in VMS[69,100-102]; and even though these phasic neurochemical responses last just a few seconds, they are capable of controlling drug seeking and taking behavior[103]. Together, these results implicate dopamine release in the VMS as a critical substrate in the control of drug use.

However, the progression of drug taking beyond recreational use is considered to reflect the engagement of different psychological processes within several neural loci[21,92], with a particular emphasis on the incorporation of the sensorimotor (dorsolateral) striatum in the control of established drug seeking behavior[21,93,104]. Specifically, dopamine transmission in the dorsolateral striatum (DLS) has been linked to *habitual* CS-elicited reward seeking[94] and therefore it has been posited that it may play an important role in the development of habitual and compulsive seeking of drugs[7,22,105].

However, it is not known whether encoding of drug-related actions or stimuli by phasic dopamine changes as drug-taking behavior advances from recreational drug use or whether this coding extends beyond VMS to other parts of the striatum. In support of generalized signaling properties of dopamine across striatal regions, reward-associated cues produce transient increases in the firing rate of dopamine neurons throughout midbrain nuclei where the

projection targets collectively encompass the entire striatum[73,106]. However, evidence for this "global" signaling scheme from neurochemical recordings within the striatum itself is lacking. In fact, recent studies with natural rewards have challenged the concept of uniform phasic dopamine signaling throughout the striatum, instead reporting dopamine release in VMS in response to natural rewards and associated cues, but little or no dopamine release in DLS[107,108].

Therefore, to gain a fuller comprehension of the neural substrates underlying the development of drug abuse, we assessed the spatiotemporal dynamics of phasic dopamine release across the striatum over the progression of the early stages of drug taking by conducting neurochemical recordings in the VMS and DLS simultaneously and repeatedly over multiple cocaine self-administration sessions (three weeks) in rats. We complemented these measurements with pharmacological and lesion approaches to investigate the behavioral function of DLS dopamine signaling and its relationship to that in the VMS, respectively.

Methods

**For more detailed methods see the Supplemental Methods published online with this manuscript*[91]

Surgical Procedures

Stereotaxic surgery was performed as described previously[89]. The target coordinates for DLS were 1.2-mm anterior, 3.1-mm lateral, and 4.8-mm ventral to Bregma[109] and 1.3-mm, 1.3-mm, and 7.2-mm for the nucleus accumbens core of the VMS. For the pharmacological experiment, guide cannulas were implanted bilaterally into DLS. For the lesion experiment, quinolinic acid (0.09 M; 0.5 µl) was infused unilaterally into VMS to induce an excitotoxic lesion[110]. Intravenous catheters were implanted in a separate surgery.

Cocaine self-administration

Rats were trained to obtain cocaine following an operant response on continuous reinforcement (FR-1) schedule in an operant chamber equipped with two nose-poke response devices. Nose-poking in the active port resulted in an intravenous infusion of cocaine (0.5 mg/kg) paired with a 20-second presentation of an audiovisual stimulus (CS). During CS presentation, a 20-second time out was imposed during which nose poking did not result in any programmed consequences. To control for response specificity, nose-poking of the second (inactive) port was monitored. Rats were given access to cocaine for one hour per day for three weeks (six days per week).

Infusion of Flupenthixol into DLS

The effects of the dopamine receptor antagonist flupenthixol (5 µg dissolved in 0.5 µl vehicle into each side; 0.5 µl/min) or vehicle on drug-taking behavior were examined in single sessions during the first or third weeks of self-administration. One group of rats received flupenthixol or vehicle in the first week of cocaine self-administration, counterbalanced on two days, and a separate group received counterbalanced infusions in the third week.

Voltammetric Measurements and Analysis

Electrochemical recordings using chronically implanted carbon-fiber microsensors (two days per week) and data analysis were carried out as described previously[89] and are described in more detail in the supplemental information. In brief, during each voltammetric scan (every 100 ms), the potential at the carbon-fiber electrode was linearly ramped from -0.4 V versus Ag/AgCl to +1.3 V and back at 400 V/s (8.5-ms total scan time). Dopamine at the surface of the electrode is oxidized during the anodic sweep to form dopamine-o-quinone which is reduced back to dopamine in the cathodic sweep. The ensuing flux of electrons is measured as current and is directly proportional to the number of molecules that undergo electrolysis. The background-subtracted, time-resolved current obtained provided a chemical signature characteristic of the analyte, allowing resolution of dopamine from other substances[146]. Dopamine was isolated from the voltammetric signal using chemometric analysis using a standard training set[89] based upon electrically stimulated dopamine release detected at chronically implanted electrodes. Dopamine concentration was estimated based upon the average post-implantation sensitivity of electrodes[89], averaged over the seven seconds following the operant response (post-response) or non-contingent presentation of the CS, and compared to the average concentration over the two seconds prior (baseline).

Statistical Analysis

Individual voltammetric recordings were averaged across session, animals, and weeks. These means were then compared using one-, two- and three-way ANOVAs with post-response, brain region, and week as factors. For comparison with voltammetric data, behavioral data was also binned into weeks. For the flupenthixol-infusion experiment, mean "baseline" values for weeks (one and three) during which flupenthixol was infused were computed by averaging the data over three days in the respective week during which no infusions were administered. Behavioral data were analyzed using one- and two-way ANOVAs with drug and weeks as factors. When appropriate, post-hoc analyses were conducted and p values were adjusted according to the

Holm-Bonferroni correction method for multiple testing[111]. Plots were made using Prism (GraphPad Software, La Jolla, CA, USA). All statistical analyses were carried out using SPSS, version 17.0 (Chicago , IL, USA). All data are presented as mean + s.e.m.

Histological Verification of Recording Sites

On completion of experimentation, recording sites were marked with an electrolytic lesion and verified using cresyl violet staining.

Results

Male Wistar rats with chronically implanted microsensors[89] in VMS and DLS (see **Supplementary Figure 1** for histological verification of electrode placement), and indwelling intravenous catheters were trained to self-administer cocaine during daily one-hour sessions in a chamber equipped with two nose-poke ports (**Figure 1a**). A nose poke into the active port elicited an infusion of cocaine (0.5 mg/kg/infusion) and 20-s presentation of a light-tone conditioned stimulus (CS) on a FR-1 schedule of reinforcement (**Figure 1b**). Responses in the second (inactive) nose-poke port, or in the active port during CS presentation (time-out), were without programmed consequence. Cocaine-reinforced responding remained relatively stable over three weeks with only a modest increase in intake which did not reach significance (n = 18; $F_{(2, 34)}$ = 1.682, p = 0.201; **Figures 1c ,d**), whereas inactive and time-out responding (i.e., non-reinforced responding) significantly diminished ($F_{(2, 34)}$ = 5.075, p = 0.012; **Figures 1c,e**). Consequently, the ratio of reinforced to total responses (efficiency of responding) was significantly greater in the second and third weeks compared to the first ($F_{(2, 34)}$ = 16.803, p < 0.001; **Figure 1f**).

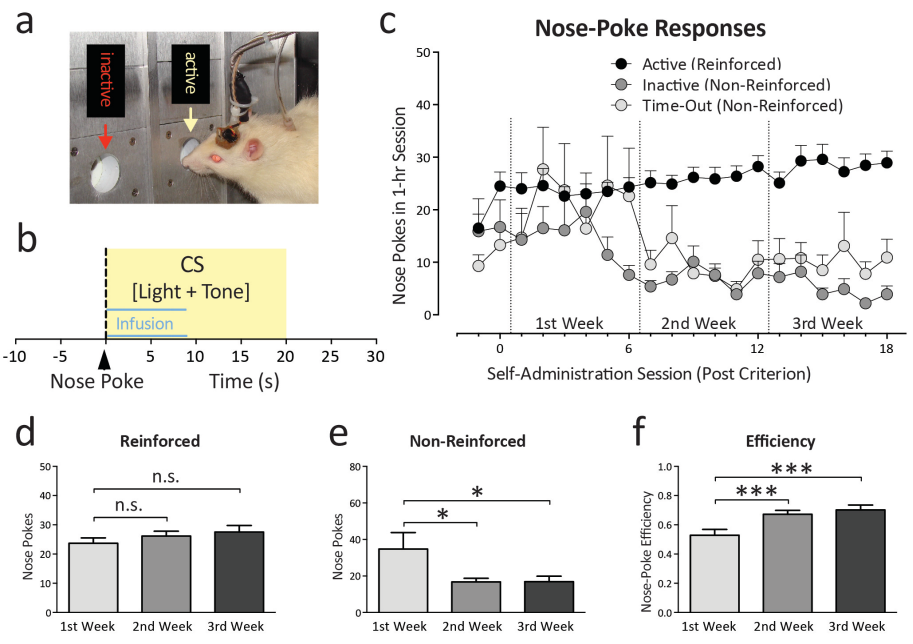

Figure 1.
Drug-taking behavior over the course of weeks.
a) Depiction of a rat connected to voltammetric recording equipment and infusion pump for i.v. delivery of cocaine during an approach to the active nose-poke port in the operant chamber. **b)** A nose poke (dashed line) into the active port elicits an infusion of cocaine (0.5 mg/kg per infusion) and the presentation of a CS (yellow box) during a 20-s time-out. **c)** Nose pokes into the active port, inactive port, and during the time-out period over 20 d of self-administration (n = 18). **d)** The number of reinforced nose pokes did not change significantly across weeks, whereas **e)** the number of nonreinforced responses decreased, and **f)** the ratio of reinforced over total number of nose pokes (efficiency) increased in the second and third weeks compared with the first week. *p < 0.05, ***p < 0.001; n.s., not significant.

Drug cue-induced phasic dopamine release in VMS is present early in cocaine self-administration.

To characterize the long-term dynamics of dopamine transmission, longitudinal neurochemical recordings were carried out using fast-scan cyclic voltammetry. In the first week of self-administration, there was a significant phasic increase in extracellular dopamine concentration in VMS following active responses ($p = 0.002$; **Figure 2a and Supplementary Figure 2**) which produced an average change in dopamine concentration over the 7-s following the response of 7.77 ± 1.69 nM with a mean peak of 13.47 ± 2.16 nM, 2.45 ± 0.26 s after the response and returning to baseline at 7.41 ± 0.28 s. These kinetics are similar to those reported in previous studies following a comparable amount of training[69,100-102] and the concentration matches those from recordings in the VMS with unbiased recording site selection[102] as in the current study (**Supplementary Figure 3**, for further discussion of this topic see Supplementary Discussion published online with this manuscript). This pattern of activation continued into the second and third weeks ($p < 0.01$; **Figure 2a and Supplementary Figure 2a**) but diminished in amplitude with an average change in dopamine concentration of 5.96 ± 0.84 and 2.99 ± 0.85 nM, respectively (main effect of week: $F(2,44) = 5.176$, $p = 0.010$; **Figure 2b**). In contrast, no significant change in dopamine concentration was detected following inactive nose pokes, in either the first or the second and third week (main effect of inactive poke: $F(1,160) = 1.392$, $p = 0.240$; **Figure 2c**), indicating that the neurochemical signal was not simply a result of the motor response. However, non-contingent CS presentation alone was sufficient to elicit a significant VMS dopamine signal ($t(17) = -2.361$, $p = 0.030$; **Figure 2d**) that was similar in magnitude and duration to the signal following contingent CS presentation ($R = 0.92$; $p < 0.001$).

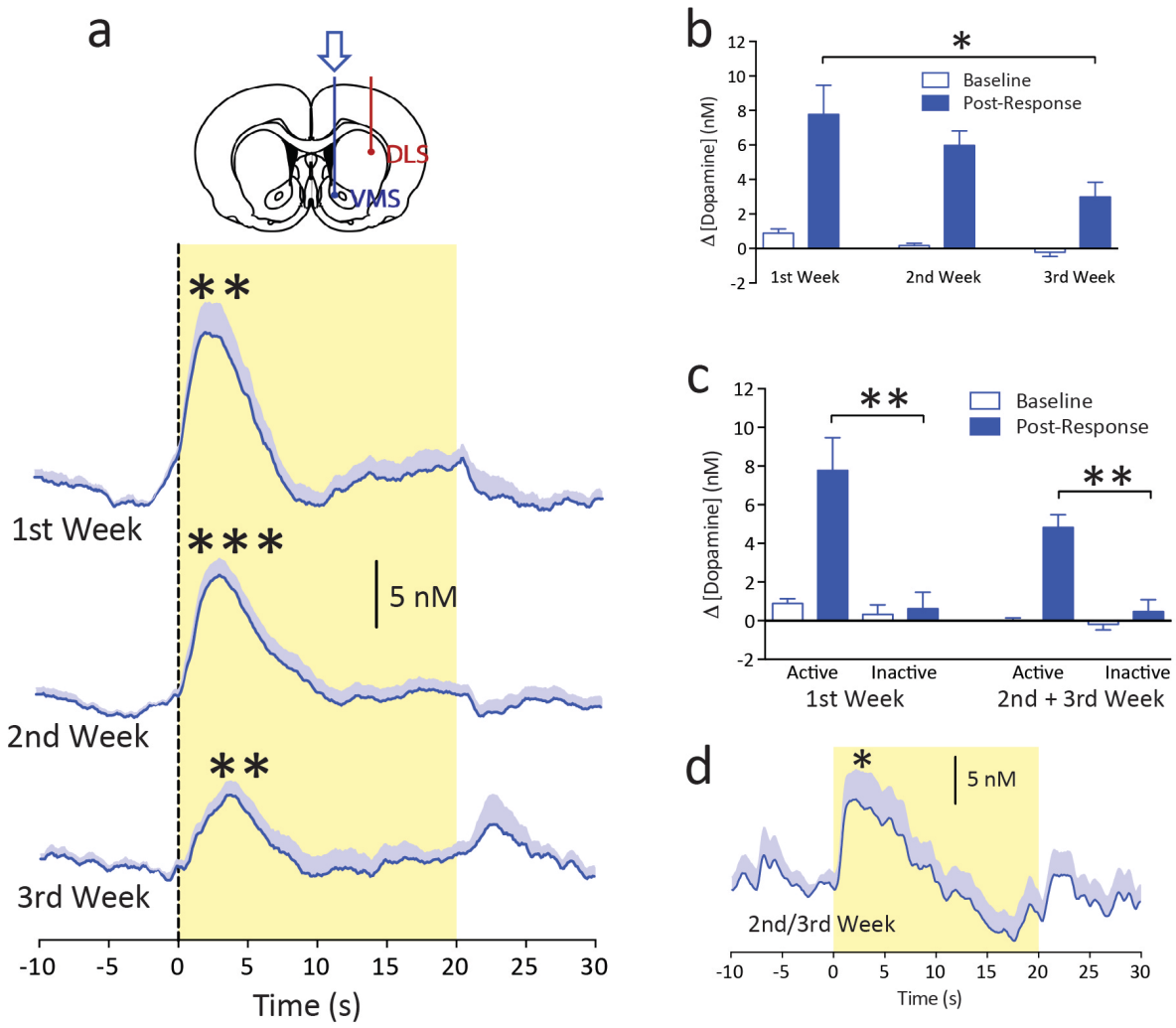

Figure 2. Dopamine signaling in the VMS over the course of weeks.

a) Phasic dopamine release in the VMS following responses into the active nose-poke port was observed during all 3 wk of cocaine self-administration (n = 10). **b)** Dopamine signals decreased in amplitude over the course of 3 wk. **c)** Dopamine signals following responses into the active nose-poke port were larger than signals following inactive responses. **d)** Noncontingent delivery of the CS induced dopamine release. *p < 0.05, **p < 0.01, ***p < 0.001.

Phasic dopamine signaling in DLS develops over the course of weeks.

Measurements in DLS revealed phasic dopamine release, similar to that in VMS, in the second and third weeks of self-administration with an average change in dopamine concentration of 3.10 ± 0.70 and 2.24 ± 0.38 nM, respectively ($p < 0.001$; **Figure 3a**). However, such signaling was absent in DLS during the first week (0.14 ± 0.50 nM; $p = 0.298$; **Figure 3a**), demonstrating that phasic dopamine release in DLS emerges over the course of drug taking (main effect of week: $F_{(2,62)} = 8.843$, $p < 0.001$; active poke x week interaction: $F_{(2,62)} = 6.468$, $p = 0.003$; **Figure 3b and Supplementary Figure 2b**), that is, its long-term dynamics are in the opposite direction of those in VMS (nose poke x week x region interaction: $F_{(2, 106)} = 5.505$, $p = 0.005$; see **Supplementary Figures 2 and 4**). Nonetheless, like in VMS, the signal in DLS was not elicited by the motor response (main effect of inactive poke: $F_{(1,193)} = 2.238$, $p = 0.136$; **Figure 3c**), but increased following CS presentation ($t_{(17)} = -3.083$, $p = 0.007$; **Figure 3d**; $R = 0.91$; $p < 0.001$). These data demonstrate that phasic dopamine signals are elicited by the same drug-associated stimuli in DLS and VMS, but they emerge at a later stage of drug taking in DLS, at a time when the VMS dopamine signal is actually decreasing.

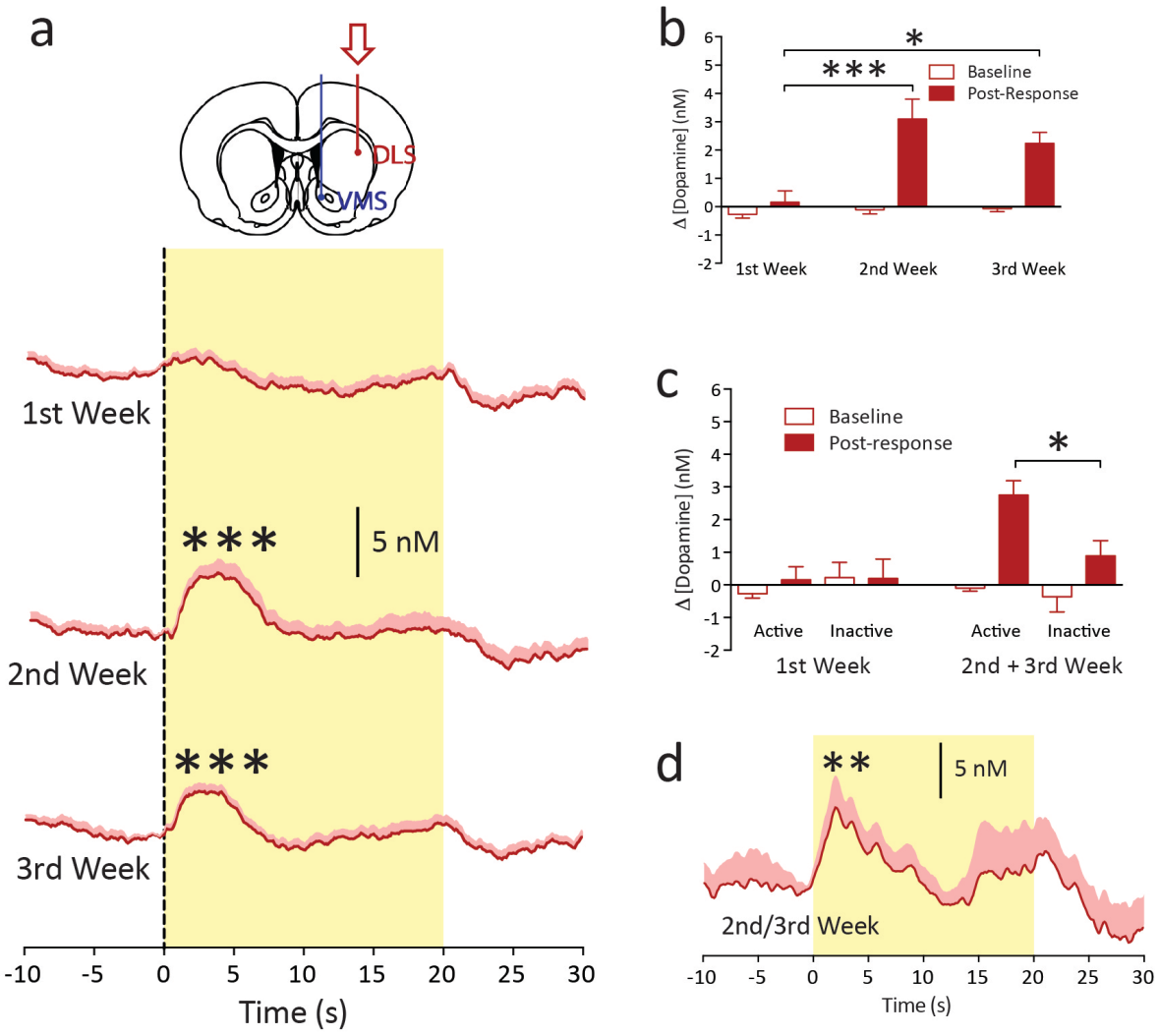

Figure 3. Dopamine signaling in the DLS over the course of weeks.

a) Phasic dopamine release in the DLS following responses into the active nose-poke port was observed during the second and third weeks of cocaine self-ad- ministration (n = 15). **b)** Dopamine signals in the second and third weeks were greater in amplitude than those in the first week. **c)** Dopamine signals following responses into the active nose-poke port were larger than signals following inactive responses during the second and third weeks but not during the first week. **d)** Noncontingent delivery of the CS induced dopamine release. *p < 0.05, **p < 0.01, ***p < 0.001.

Dopamine receptors in DLS are necessary for discriminated responding for cocaine.

To test the causal relationship between these neurochemical and behavioral observations, dopamine signaling was manipulated by bilateral infusion (see **Supplementary Figure 5** for histological verification of cannula placement) of the non-specific dopamine receptor antagonist alpha-flupenthixol into DLS of additional groups of animals (n = 32; **Figure 4**). In one group, flupenthixol and vehicle were infused on counterbalanced days in the first week of cocaine self-administration, corresponding to an early time point prior to the onset of CS-associated DLS signaling. A second group of animals was infused in the third week, corresponding to the later time point when DLS dopamine signals were present. In these animals, during self-administration sessions where no intracranial infusions were made, the temporal pattern of the assessed responses was similar to that in the previous cohort of animals (**Figures 1d-f**). Specifically, the rate of reinforced nose pokes remained stable over time ($F_{(2,\ 141)}$ = 1.092, p = 0.338; **Figure 4a**), but the rate of non-reinforced nose pokes decreased significantly over the weeks of self-administration ($F_{(2,\ 141)}$ = 4.155, p = 0.018; Figure 4B), producing an increase in response efficiency across this period ($F_{(2,\ 141)}$ = 7.843, p < 0.001; **Figure 4c**). Intra-DLS infusion of flupenthixol resulted in an increase in cocaine intake (reinforced nose pokes) at both the early and late time points (p < 0.05 vs vehicle; **Figure 4d**), suggesting that DLS dopamine may contribute to the reinforcing properties of cocaine, consistent with previous reports[112,113]. Importantly, this effect is therefore not attributable to the CS-associated phasic dopamine signal, which was present at the late time point but not the early time point. In contrast to the effect on reinforced responding at both time points, the average number of non-reinforced responses was increased after the late infusion (p = 0.024; **Figure 4e**), but not the early infusion (p = 0.970). Accordingly, the nose-poke efficiency was decreased after the intracerebral administration of flupenthixol at the late (p = 0.004; **Figure 4f**), but not the early (p = 0.762) time point (drug x time-point interaction: $F_{(1,\ 27)}$ = 7.482, p = 0.011). These data show that the gain in efficiency as measured by discriminated drug-taking responses between the first and third weeks of cocaine self-administration was reversed by the infusion of flupenthixol into DLS, indicating that

emergent dopamine signaling in DLS is necessary for the improved action selection of drug-taking behavior.

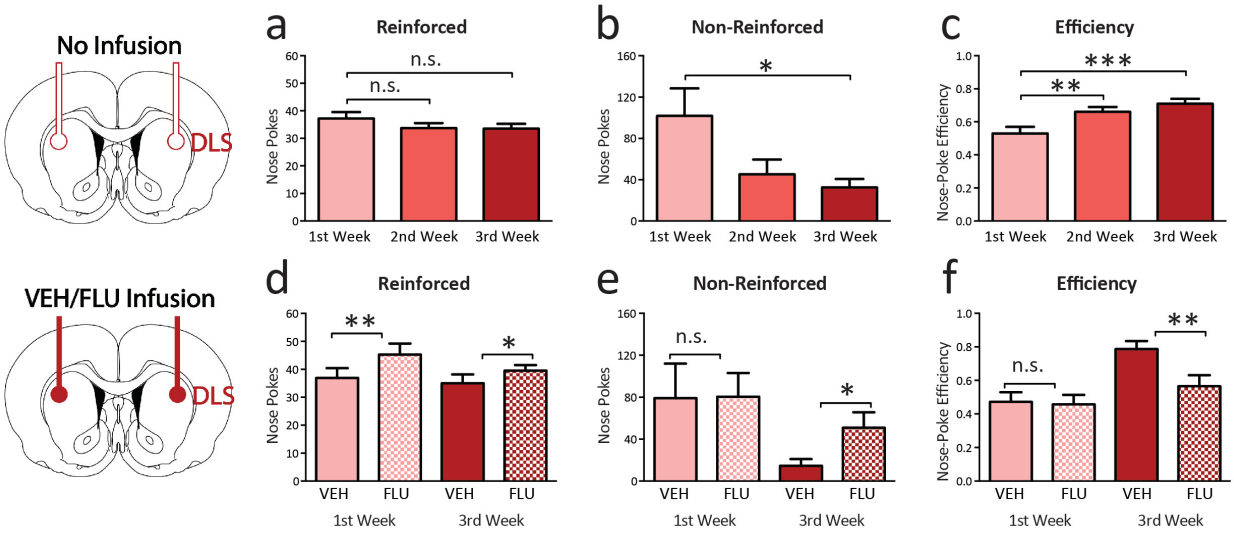

Figure 4. Blockade of dopamine receptors in the DLS disrupts discriminated drug-taking behavior.
a) The rate of reinforced nose pokes remained stable across weeks , **b)** but the rate of nonreinforced nose pokes was decreased, and **c)** response efficiency increased during the second and third weeks compared with the first week. **d)** Infusion of flupenthixol (FLU) into the DLS produced an increase in reinforced nose pokes in both the first (n = 16) and the third weeks (n = 16). **e)** The average number of nonreinforced responses was increased after flupenthixol only during the third but not during the first week. **f)** Consequently, response efficiency was decreased after flupenthixol at the late but not at the early time point. *p < 0.05, **p < 0.01, ***p < 0.001; VEH, vehicle.

Development of phasic dopamine signaling in DLS depends on VMS.

A salient feature of the current findings and others[112] is the progressive onset of function in DLS during drug use. This progressive involvement of DLS in drug seeking has been linked to circuitry that connects VMS to DLS by the results of a serial disconnection study that demonstrated the dependence of the development of advanced cue-controlled drug seeking behavior on intact VMS circuitry[110]. Therefore, to test whether the later-emerging phasic dopamine signal in DLS reported in the present study was dependent upon antecedent activity in VMS circuitry, we mimicked such a disconnection of VMS from DLS on one side of the brain with a unilateral excitotoxic lesion of the nucleus accumbens core (VMS) by infusing quinolinic acid prior to training[110], leaving the other side intact. Voltammetric microsensors were bilaterally implanted in DLS (n = 17) which permitted within-subject comparison of emergent DLS dopamine transmission between hemispheres, one having an intact, and the other a lesioned, VMS (see **Supplementary Figure 6** for histological verification of lesion and electrode placement). Cocaine intake was similar to non-lesioned animals (**Supplementary Figure 7**), consistent with previous findings[110]. Also, similar to non-lesioned animals (**Figure 3**), active nose-poke responses evoked significant dopamine release in DLS contralateral to the lesion in the second and third weeks (1.81 ± 0.23 and 1.77 ± 0.22 nM; $p < 0.01$; **Figure 5a**), but not the first week (-0.19 ± 0.49; $p = 0.778$; **Figure 5a**). However, in the hemisphere ipsilateral to the VMS lesion, there were no significant changes in dopamine release compared to baseline at any time point of cocaine self-administration with an average change in dopamine concentration of 0.85 ± 0.38, 0.84 ± 0.22, and 0.92 ± 0.23 nM in weeks one to three, respectively ($p > 0.05$; **Figure 5b**). Thus, phasic dopamine signals evolved over the three weeks of self-administration contralateral (main effect of active poke: $F_{(1, 63)} = 19.386$, $p < 0.001$; main effect of week: $F_{(2, 63)} = 15.294$, $p < 0.001$; active poke x week interaction: $F_{(2, 63)} = 19.386$, $p = 0.048$; **Figure 5c and Supplementary Figure 8**) but not ipsilateral (main effect of week: $F_{(2, 43)} = 0.001$, $p = 0.999$; **Figure 5c**) to the lesion conferring significantly different patterns of dopamine release between hemispheres (brain region x week interaction: $F_{(2, 106)} = 7.204$, $p < 0.001$; **Figure 5c**). Similarly, non-contingent delivery of the CS induced significant dopamine release ($p = 0.040$; **Figure 5d**) contralateral, but not ipsilateral to

the VMS lesion (p = 0.761; **Figure 53 and Supplementary Figure 9**). Importantly, during periods in the recording sessions that were free of operant behavior and CS presentations, the magnitude of "spontaneous" dopamine release in DLS was similar ipsilateral and contralateral to VMS lesion (**Supplementary Figure 9**). Furthermore, magnitude of DLS signals measured in the first study (**Figure 3a**) and contralateral DLS (**Figure 5a**) were not significantly different (main effect of brain region = $F_{(1,125)}$ = 0.851 ; p = 0.358).Therefore, the VMS lesion did not produce a general suppression of dopamine transmission in DLS, but had a selective effect on task-related signaling. These results demonstrate that neural activity in VMS is required for the development of CS-elicited dopamine signaling in DLS that regulates the efficiency, or automaticity, of drug taking responses.

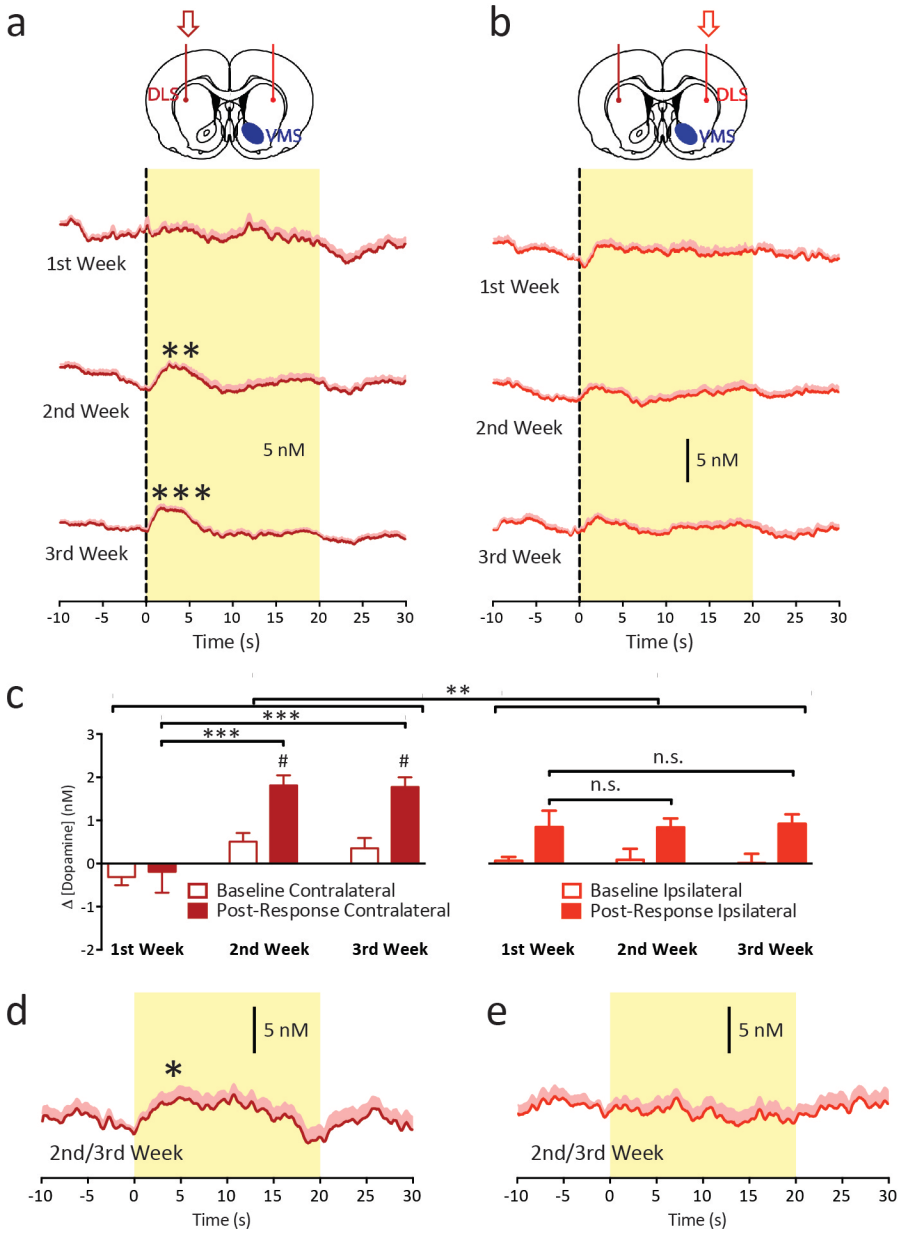

Figure 5. VMS lesion prevents development of phasic signaling in the DLS.

a) Phasic dopamine release was observed in the DLS contralateral to the unilateral lesion of the VMS following responses into the active nose- poke port during the second and third weeks of cocaine self-administration (n = 17). **b)** Dopamine release in the ipsilateral DLS was not significantly increased in any week (n = 11). **c)** In the contralateral DLS, phasic signaling in the second and third weeks was larger in amplitude than signals detected in the first week (Left), whereas signals did not change in amplitude across weeks in the ipsilateral DLS (Right). Emergence of such signaling had significantly different patterns of dopamine release between hemispheres. A direct post hoc comparison between ipsilateral and contralateral hemispheres showed greater dopamine release in the contralateral hemisphere in the second and third weeks of training but not in the first week ($p < 0.05$). (**d** and **e**) Noncontingent delivery of the CS consistently induced DLS dopamine release contralateral (**d**) but not ipsilateral (**e**) to the VMS lesion. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Discussion

Spatiotemporal changes in striatal dopamine signaling.

Drug self-administration studies in animals have revealed neuroadaptations in functional markers that progress from VMS to encompass DLS over the course of drug use[114]. To test whether there are complimentary changes in phasic dopamine transmission, we carried out longitudinal sub-second dopamine measurements simultaneously in the VMS and DLS during the establishment of drug taking in rats. We observed phasic dopamine release in both VMS and DLS following the operant response for drug during the course of our study, where the VMS signal declined and the DLS signal emerged during the progression of drug taking. Despite these differences in temporal profiles, phasic dopamine release encoded similar information in the VMS and DLS. In both regions, it was selectively elicited by active and not inactive nose pokes, indicating that the signal was not simply related to the motoric action of making a response. Instead, we hypothesized that dopamine release was a result of successful completion of the response to obtain cocaine (signaled by the CS). This notion was supported, as non-contingent presentation of the CS alone was sufficient to recapitulate dopamine release following an active response in both regions, similar to that reported previously[69] for a time point equivalent to the first week of training in the present study. Drug-associated conditioned stimuli are integral to drug use, guide the acquisition and maintenance of drug taking, and increasingly assume control over behavior to the extent of triggering the resumption of drug taking, even after long periods of abstinence[20,115,116]. Thus, the current findings reveal a process by which drug-associated stimuli gain access sensorimotor circuitry with repeated drug use. Interestingly, the emergent sensorimotor signal was generally smaller than that in the VMS, even when drug use was established. This observation is notable since the dopamine terminal density[117], tissue content[118] and capacity for release[118-120] are greater in the DLS compared to the VMS, and suggest that the phasic dopamine responses in the DLS use less of the available "bandwidth" for the encoding of drug cues than the VMS. Similarly, the long-term effect of prior cocaine

exposure on the processing of stimuli associated with natural reinforcers across these two regions is not uniform either. Instead of increasing processing in both the VMS and DLS, cocaine reduces the degree and flexibility of cue-evoked neuronal firing in VMS while enhancing firing in DLS with effects in the DLS being relatively weak compared to those in the VMS[121].

Overall, our data identify the spatiotemporal pattern of phasic dopamine release in the striatum during the establishment of drug-taking behavior. The gradual decline in VMS dopamine signaling is somewhat surprising in the context of models postulating that the amount of dopamine release to drug cues, specifically in the nucleus accumbens, increases over repeated drug administration as they undergo incentive sensitization[88]. In contrast, the emergence of phasic dopamine signaling in DLS provides further empirical support for current theories postulating an engagement of an increasing number of brain regions with prolonged drug use[21,92,93,104,105].

Dopamine signaling in the sensorimotor striatum emerges prior to compulsive drug abuse.

The observed spatiotemporal dynamics of striatal dopamine signaling illustrate the progressive engagement of brain systems with persistent drug self-administration. It has been suggested that each of the stages in the series of transitions from goal-directed to habitual, and eventually to compulsive responding for drug, is associated with specific brain systems that are recruited progressively[21]. Indeed, DLS comes to exert more dominant control over drug seeking over the course of drug use[122,123] as it becomes maintained by drug-associated stimuli[21,93,105]. While, we have demonstrated that phasic dopamine release does indeed develop at a later stage of drug use in DLS compared to VMS, the training regimen utilized is typically not sufficient to produce compulsive responding or the significant escalation of drug intake that emerges following extended or long-access drug self-administration training[32,124,125]. Thus, our data demonstrate that the engagement of DLS dopamine, which is thought to be closely linked to stimulus-response processing[94], is not sufficient to account for the loss of control over drug intake characteristic of addiction, underlining the important dissociation between habitual and

compulsive stages of drug taking and their neural substrates[126]. In fact, the behavioral measure that most closely correlated with the emergence of phasic dopamine release in the DLS was the response efficiency, that is, the number of active nose-poke responses as a proportion of the total number of responses (including time-out responses and responses in the inactive port). This increase in response efficiency between the first and third weeks of self-administration was reversed by dopamine-receptor antagonism in the DLS, whereas this treatment had no effect on efficiency in the first week, before phasic dopamine signaling in the DLS had emerged. In contrast, cocaine intake (reinforced nose pokes) was increased by the antagonist in both the first and third weeks, suggesting that this effect is likely not associated with the phasic modality of dopamine signaling time-locked to drug taking, and therefore "tonic" dopamine signaling may be implicated. This notion is consistent with the work of others indicating a role for DLS dopamine in mediating the reinforcing properties of cocaine[112,113]. Therefore, rather than contributing to escalated or compulsive responding, the progressive recruitment of DLS phasic dopamine promotes the refinement of behavior towards reinforced actions, as operant responding for the drug becomes more reliably discriminated over the course of weeks in the absence of escalated drug intake.

While DLS dopamine appears to suppress non-reinforced responses, it was not observed around these actions. Instead, it seems that the feedback collected from reinforced responses promotes exclusivity (i.e., actions that are not associated with a DLS dopamine signal are not maintained). While this inference may appear elaborate, it is consistent with the idea that the striatum does not generate movement itself but rather promotes focused selection of available actions by simultaneously and focally removing the inhibition of specific actions and acting broadly by inhibiting rivaling/conflicting motor mechanisms that would otherwise interfere with the desired action[127]. Consistent with our findings, dorsal striatal circuits serve to evaluate behavior and exploit optimal behaviors following initial behavioral variability during trial-and-error learning (exploration)[128] as an integral part of the sensorimotor domain of the basal-ganglia network mediating action sequencing as well as selection/inhibition of competing motor programs[127,129]. Thus, our findings suggest that the observed changes DLS dopamine signaling

(i.e., task representation in DLS circuits) might facilitate a switch from exploring the availability of drug rewards present in the environment to exploiting this environment.

Addiction is often described as a disorder of brain memory systems. The DLS is considered to be a critical locus for procedural learning[130,131] with dopamine acting as a neurotransmitter that induces plasticity to enable the formation of long-lasting network changes. Brain regions that mediate the evolving discrimination of drug cues and drug taking are potentially of great interest in the identification of neural systems underlying addiction. Our data suggests that the observed behavioral refinement may represent an amplified focus on drug-related behaviors that causes the prioritization of drug taking over behavior not reinforced by drug, a development also observed in drug addiction[5]. Thus, although "efficiency" of drug taking does not itself imply compulsive or addiction-like behavior, monitoring response discrimination may prove useful in the investigation of abuse-related behaviors comparable to a period when drug abusers narrow their behavioral repertoire to actions that prioritize the intake of drugs over other actions. Taken together, these data demonstrate a mechanism involving sequential recruitment of phasic dopamine transmission in the striatum in the dynamically changing neural control over drug intake even before compulsive use emerges.

A hierarchy for recruiting dopamine in different striatal modules.

Limbic circuits that converge on VMS have been hypothesized to affect and enable sensorimotor circuits, thus functioning as a gateway for limbic structures to reach motor systems[132,133]. Sensorimotor aspects of the striatum are thought to contribute to the facilitation of automatic execution of motor acts or the implementation of habits by building up individual motor acts to coherent chunks of performance units[129]. We investigated interactions between motivational and sensorimotor networks within the striatum during drug self-administration using the combination of unilateral VMS lesion and bilateral electrochemical recordings in DLS. This approach enabled the study of dopamine neurotransmission simultaneously in intact and disrupted basal ganglia circuits in the same trial of the same animal, and thus in the same

motivational state. Our data provide functional evidence supporting an interaction between limbic and motor networks in the development of discriminated responding for cocaine, where VMS that receives limbic inputs enables dopamine signaling in sensorimotor DLS.

Previous support for a role of serial circuitry that connects VMS and DLS, comes from a study where the VMS was lesioned on one side of the brain and combined with antagonism of dopamine receptors in the contralateral DLS, thereby functionally disconnecting serial interactions between these striatal domains on both sides of the brain[110]. While either manipulation on its own was without effect, combined, the procedures selectively decreased cocaine seeking in extensively trained rats, but not in rats having only undergone moderate training[110]. Together with our study, these findings underline the functional significance of the network interaction between VMS and DLS in drug-related behavior. Specifically, they indicate that this circuit is utilized for multiple related processes in the procurement of drugs, both in prioritization of drug-taking behavior and the exploitation of a drug environment in animals with a moderate drug history (present study), and energizing and driving drug-seeking behavior in an environment where the drug is not readily available in animals with an extended drug taking history[126]. Therefore, the hierarchical recruitment of striatal subregions for dopamine-mediated control of behavior may signify an overarching organizing principle throughout stages of drug use to enable representation of drug cues in DLS.

There has been a long-standing debate on how interactions between limbic and motor systems are implemented. On the level of basal ganglia circuitry, a potential anatomical substrate for this interaction of striatal modules is the interconnectivity between striatal projection neurons and the dopaminergic midbrain. Nauta and colleagues discovered that VMS neurons (that receive dopaminergic afferents from the VTA) send axons to the substantia nigra, which provides a dopaminergic projection to the dorsal striatum[134]. This connectivity was later found to display an elaborate spiraling organization with several striato-nigro-striatal loops spanning from the limbic VMS to sensorimotor DLS[135]. However, there are also other pathways channeling information from VMS to DLS via the midbrain[133,136-138]. Irrespective of anatomical pathway, the demonstration of a striatal hierarchy in the control of dopamine transmission

provides important insight into how neurotransmission within neural circuits regulating behavior is shaped over prolonged drug use. In addition to informing about pathological processes, these data offer mechanistic information for functional anatomically-based therapies targeting VMS for addiction such as deep-brain stimulation[139], as VMS acts as a node which regulates other circuit components.

Conclusions

Overall, the present data offer insight into neurobiological processes that establish drug-taking behavior. It demonstrates that phasic dopamine signaling in the striatum is dynamic and region specific, emerging in the VMS then DLS sequentially in the early stages of drug use. We ascertained that the progression from limbic to sensorimotor regions of the striatum requires intact VMS circuitry. This hierarchical control enables drug-associated stimuli to access brain systems implicated in the development of a drug-taking habit.

Acknowledgements

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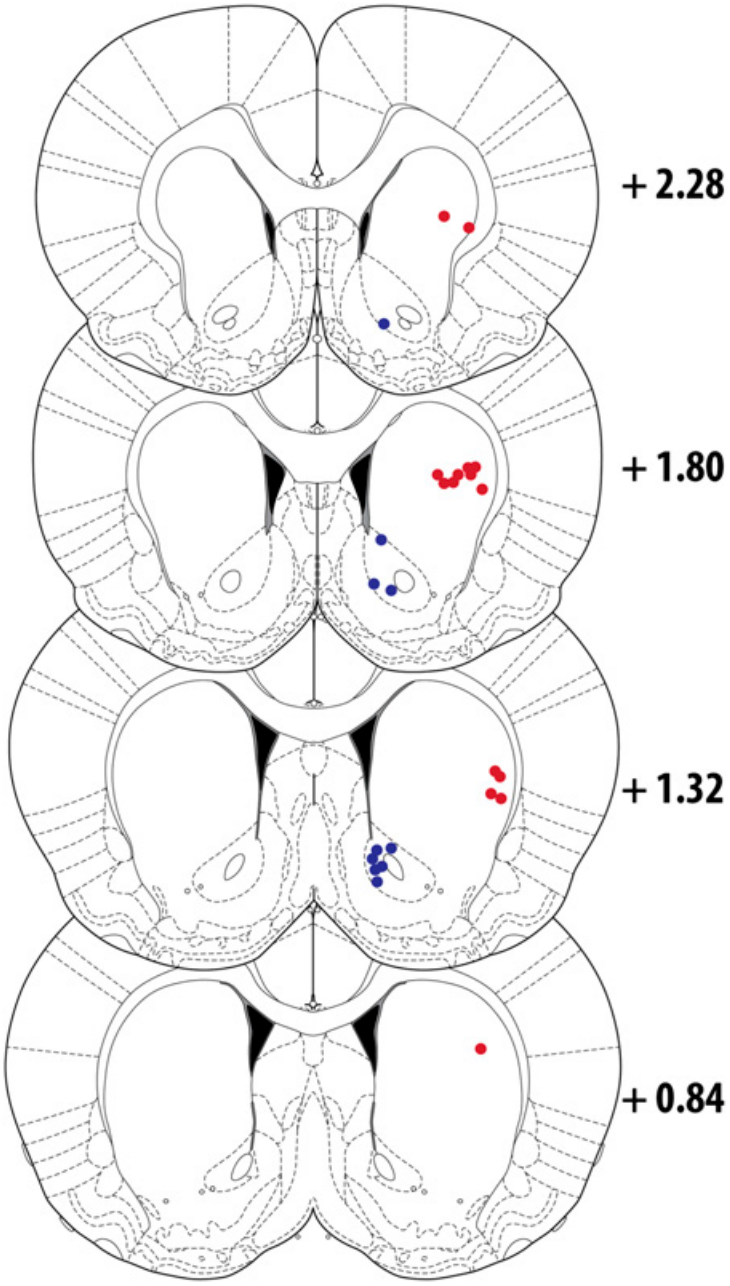
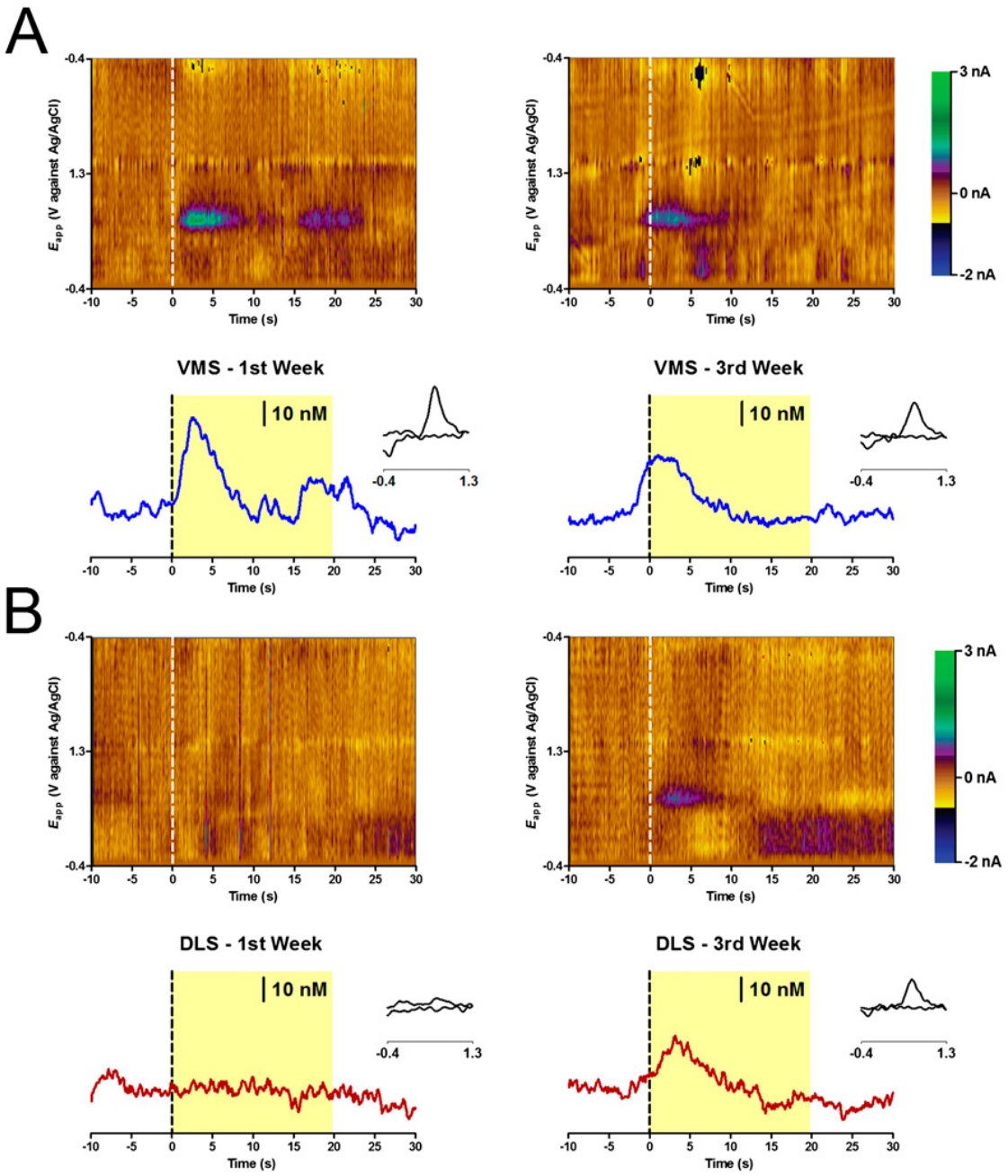
Author contributions

I.W., B.J.E. and P.E.M.P. designed the experiments, I.W. and L.M.B. conducted the experiments, I.W. analyzed data, and I.W. and P.E.M.P. wrote the paper.

Supplementary Figures

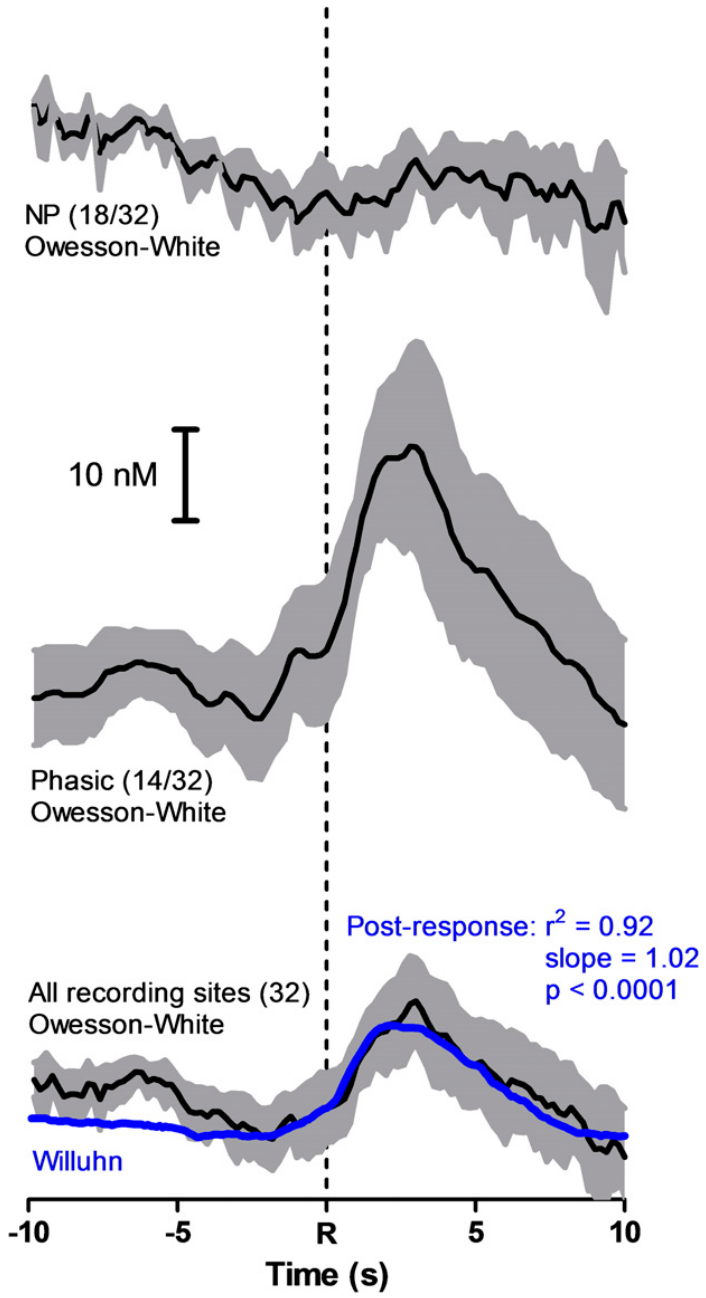


Supplementary Figure 1. Verification of recording sites in the VMS and DLS (first experiment)
Histological verification of recording sites in the VMS and DLS (first experiment). VMS recording sites (blue circles) were confirmed to be within the nucleus accumbens core, and DLS recording sites (dark red circles) were in the lateral half of the dorsal striatum. The numbers on each plate indicate the distance in millimeters anterior from bregma[109].

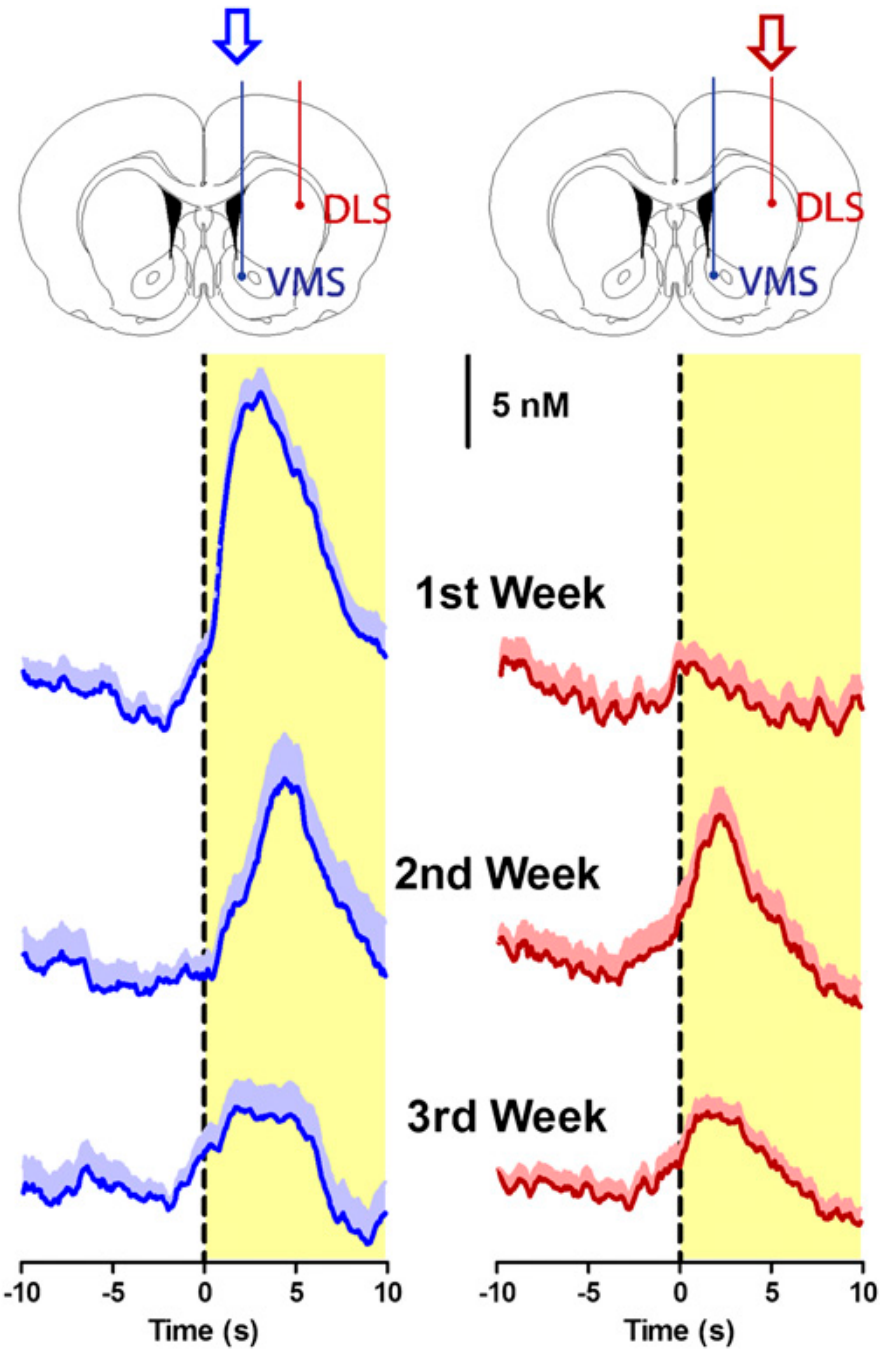


Supplementary Figure 2. Examples of phasic dopamine release in the VMS and DLS associated with an individual nose poke (single trial) into the active port.

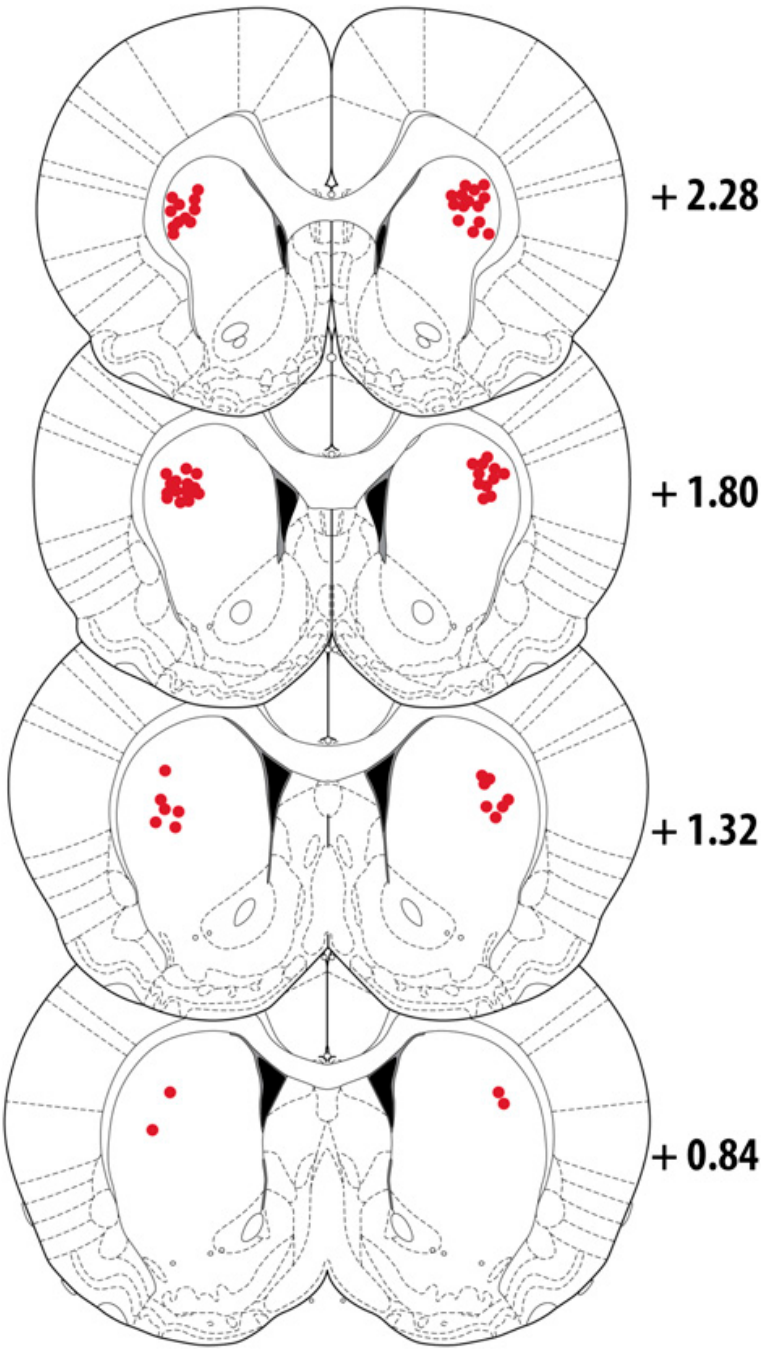
Examples of phasic dopamine release in the VMS and DLS associated with an individual nose poke (single trial) into the active port. **a)** Pseudocolor plots (Upper), dopamine traces (Lower), and cyclic voltammograms (Insets in lower panel) for representative current fluctuations recorded in the VMS for the period 10 s before an operant response (dashed line), during the subsequent 20-s presentation of the CS (yellow box), and 10 s after the offset of the CS during the first (Left) and third (Right) weeks of cocaine self-administration. **b)** Pseudocolor plots (Upper), dopamine traces (Lower), and cyclic voltammograms (Insets in lower panel) for representative current fluctuations recorded in the DLS during the first (Left) and third (Right) weeks of self-administration. The color plots show current changes across the applied voltages (E_{app}; y axis) over time (x axis).



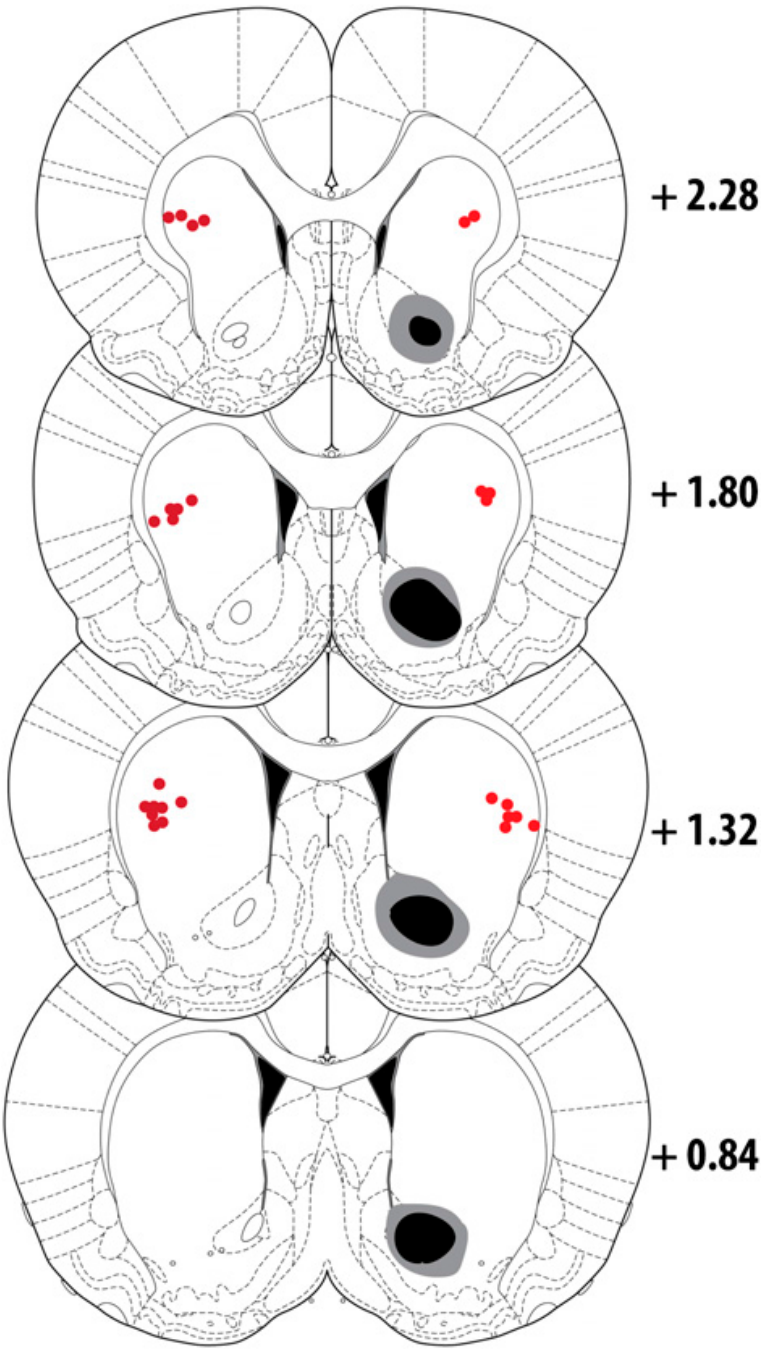
Supplementary Figure 3. Comparison of data from Owesson-White *et al*. 2009[102] and the current findings. Phasic dopamine release (digitized using GetData Graph Digitizer, http://getdata-graph-digitizer.com) from recording sites adjacent to neurons whose firing rates were responsive (Middle) or unresponsive (Top) to cocaine self- administration were combined to produce a weighted average (Bottom). This average response was similar to the average response in the current study obtained from the nucleus accumbens core in the first week of cocaine self-administration (Bottom, blue trace). The phasic responses that follow an operant response (R) for cocaine from the two studies have concentration profiles that match closely in shape ($r^2 = 0.92$) and amplitude (slope = 1.02).



Supplementary Figure 4. Phasic dopamine signaling in the VMS and DLS for an individual animal.
The pattern of average phasic dopamine release in the VMS (Left) and DLS (Right) is depicted 10 s before and 10 s after responses into the active nose-poke port across 3 wk of cocaine self-administration in an individual animal. Data are expressed as mean + s.e.m. per week.

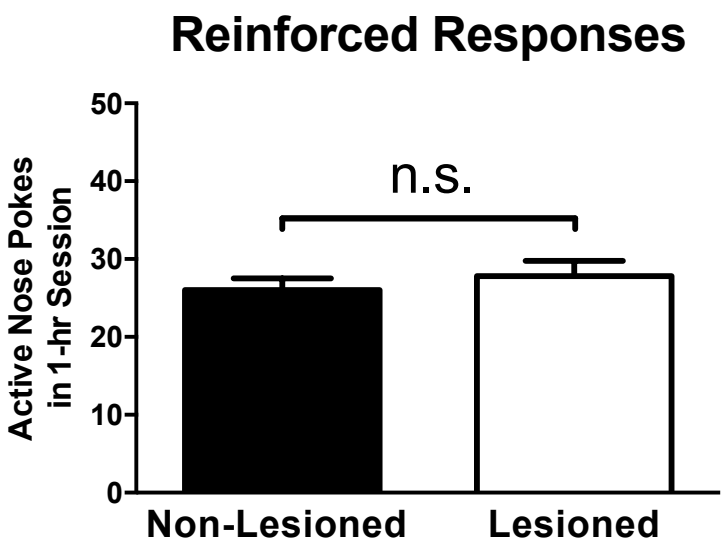


Supplementary Figure 5. Histological verification of infusion sites in the DLS (pharmacological experiment). DLS infusion sites (dark red circles) were verified to be in the lateral half of the dorsal striatum. The numbers on each plate indicate the distance in millimeters anterior from bregma[109].



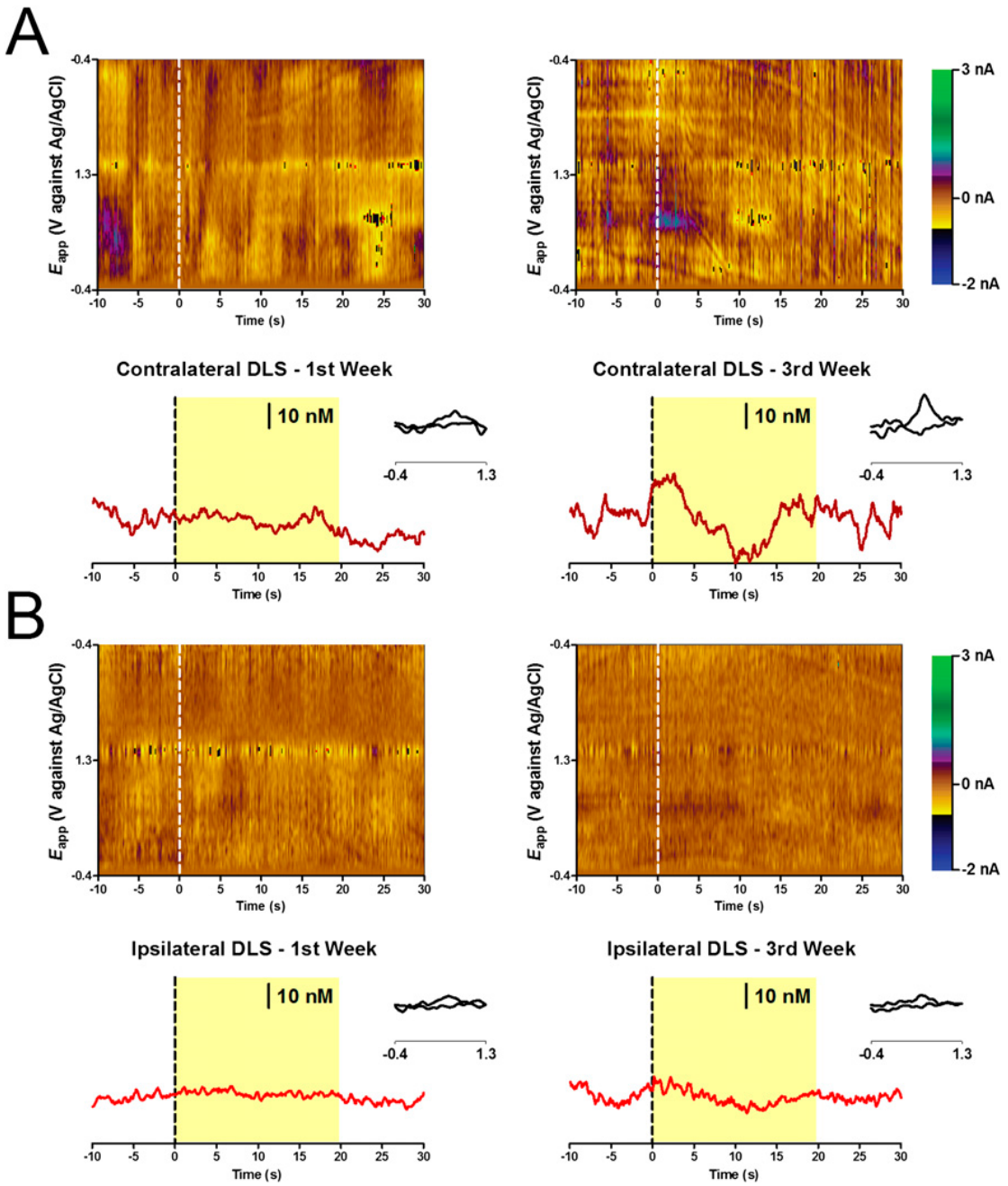
Supplementary Figure 6. Histological verification of lesion sites in VMS and recording sites in the DLS (lesion experiment).

DLS recording sites were verified to be in the lateral half of the dorsal striatum, and infusions of quinolinic acid produced lesions in the nucleus accumbens core (VMS). Recording sites in the DLS ipsilateral to the lesion are shown in light red (Right). DLS recording sites in the contralateral hemisphere are shown in dark red (Left). Areas shaded in gray and black represent the largest and smallest extent of neuronal damage, respectively. The numbers on each plate indicate the distance in millimeters anterior from bregma[109] .



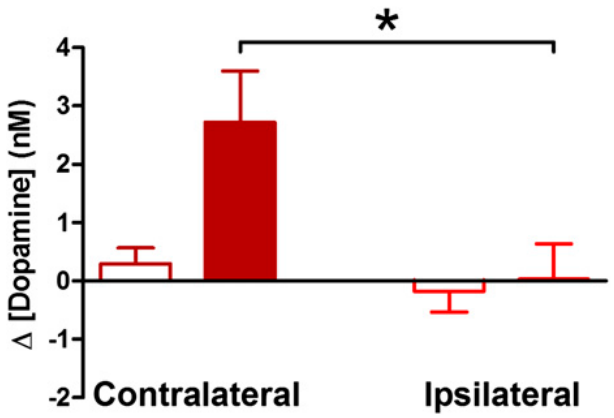
Supplementary Figure 7. Responses to obtain cocaine infusions by animals with intact VMS (first experiment) and by animals with unilateral lesion of the VMS (lesion experiment).

The average total number of reinforced nose pokes (mean + s.e.m.) did not differ significantly ($p > 0.05$) in nonlesioned rats (filled bar) and rats with unilateral lesion of the VMS (open bar). n.s., not significant.

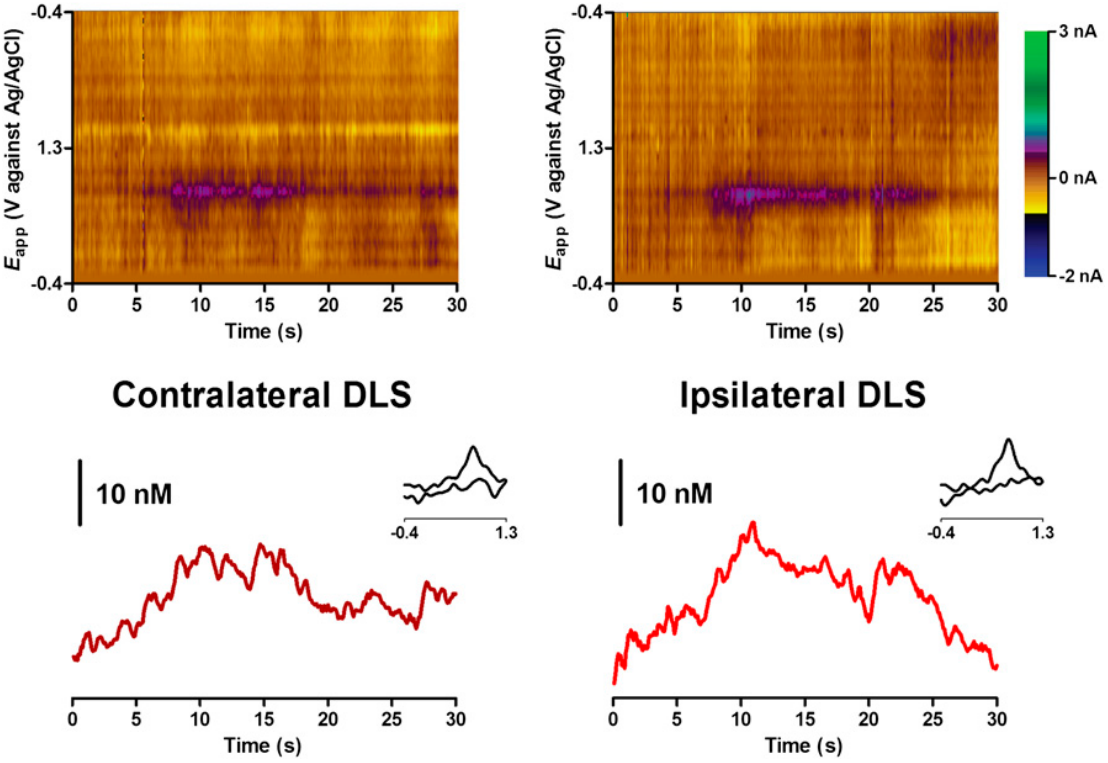


Supplementary Figure 8. Examples of phasic dopamine release in the DLS contralateral and ipsilateral to lesioned VMS associated with an individual nose poke (single trial) in the active port.

a) Pseudocolor plots (Upper), dopamine traces (Lower), and cyclic voltammograms (Insets in lower panel) for representative current fluctuations recorded in the DLS of the hemisphere contralateral to the VMS lesion for the period 10 s before an operant response (dashed line), during the subsequent 20-s presentation of the CS (yellow box), and 10 s after the offset of the CS during the first (Left) and third (Right) weeks of cocaine self-administration. **b)** Pseudocolor plots (Upper), dopamine traces (Lower), and cyclic voltammograms (Insets in lower panel) for representative current fluctuations recorded in the DLS of the hemisphere ipsilateral to the VMS lesion during the first (Left) and third (Right) weeks of self-administration. The color plots show current changes across the applied voltages (Eapp; y axis) over time (x axis).



Supplementary Figure 9.
CS-induced dopamine signaling in the DLS contralateral and ipsilateral to lesioned VMS.
The average phasic release of dopamine in response to the noncontingent presentation of the drug CS is greater in the DLS of the hemisphere contralateral to the VMS lesion than in the ipsilateral DLS (PHolm $p < 0.05$). Data are expressed as mean + s.e.m. (n = 17 and 11 electrodes, respectively, in 17 rats)



Supplementary Figure 10. Spontaneous release of dopamine in the DLS contralateral and ipsilateral to the lesioned VMS.
Averaged pseudocolor plot (Upper), dopamine traces (Lower), and voltammograms (Insets in lower panel) for spontaneous current fluctuations (n = 7 electrodes in 4 rats) recorded in the DLS of the hemisphere contralateral (Left) or ipsilateral (Right) to the VMS lesion for a 30-s period. Dopamine release in the and ipsilateral DLS did not differ significantly [$t(11) = -0.913$, $p = 0.381$], indicating that spontaneous dopamine release (i.e.,during time periods free of operant responding and CS presentation) in the DLS is not affected by the VMS lesion.

Excessive cocaine use results from decreased phasic dopamine signaling in the striatum.

Ingo Willuhn[1,2,3*], Lauren M. Burgeno[1,2], Peter A. Groblewski[2], and Paul E. M. Phillips[1,2,] (2014). *Nature Neuroscience*, 17(5), 704-9.

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Abstract

Drug addiction is a neuropsychiatric disorder marked by escalating drug use. Dopamine neurotransmission in the ventromedial striatum (VMS) mediates acute reinforcing effects of abused drugs, but with protracted use the dorsolateral striatum (DLS) is thought to assume control over drug seeking. We measured striatal dopamine release during a cocaine self-administration regimen that produced escalation of drug taking in rats. Surprisingly, we found that phasic dopamine decreased in both regions as the rate of cocaine intake increased; with the decrement in dopamine in the VMS significantly correlated with the rate of escalation. Administration of the dopamine precursor L-DOPA at a dose that replenished dopamine signaling in the VMS reversed escalation, thereby demonstrating the causal relationship between diminished dopamine transmission and excessive drug use. Thus, together these data provide mechanistic and therapeutic insight into the excessive drug intake that emerges following protracted use.

Introduction

Drug abuse is closely linked to the release of dopamine in the striatum[21,140]. However, drug use-related changes in dopamine neurotransmission vary in duration and subregion[141-143]. Slow increases in the extracellular concentration of dopamine in the ventromedial striatum (VMS), stimulated by many drugs of abuse including cocaine[59], are assumed to reflect the reinforcing properties of drugs[61], as animals regulate their rate of cocaine self-administration in order to maintain an elevated level of ambient dopamine concentration[67]. Within the VMS, overlapping putative roles of dopamine signaling in the core and shell subregions of the nucleus accumbens have been reported, but with an emphasis on the shell for mediating primary drug reward and the core for acting as a substrate for conditioned reinforcement[21]. Indeed, phasic dopamine release in the nucleus accumbens core, lasting for a few seconds, is conditioned to presentation of environmental stimuli that have been repeatedly paired with the drug[69,100-102] and is capable of controlling drug seeking and taking[69]. The encoding of such conditioned stimuli by dopamine release is also found in sensorimotor aspects of the striatum (dorsolateral striatum, DLS)[91], a striatal subregion that has been linked to the development of habitual and compulsive drug seeking[7,22,105]. Thus, the progression of drug taking beyond recreational use is considered to reflect the engagement of dopamine signaling in different striatal subregions[21,92], with an emphasis of shift from the limbic (VMS) to the sensorimotor (DLS) striatum during the development of established drug-seeking behavior[21,93]. However, it is not known whether encoding of drug-related actions or stimuli by phasic dopamine changes as moderate drug-taking behavior escalates.

Rodent paradigms that are deemed to best model the transition from moderate drug use to addiction employ protracted access to the drug[124,144], such as extending access from one (short access, ShA) to six hours (long access, LgA) per day for a period of weeks[33]. Such a drug self-administration regimen is capable of producing escalated[33] and compulsive drug seeking[145], among other cardinal symptoms that characterize substance dependence in humans[5]. Here, we tested how LgA to cocaine affects the regional dynamics of phasic dopamine signaling in the

striatum previously characterized during stable ShA drug use[91] to gain a better comprehension of the neurobiological mechanisms underlying escalation of drug use.

Methods

Subjects

Adult male Wistar rats from Charles River (Hollister, CA, USA) weighing between 300g and 400g were housed individually and kept on a 12-h light/12-h dark cycle (lights on at 0700) with controlled temperature and humidity with food and water available ad libitum. All animal use was approved by the University of Washington Institutional Animal Care and Use Committee, and surgical procedures were performed under aseptic conditions. For the voltammetry experiments 50 animals underwent surgery, of which 29 maintained catheter patency throughout the experiments, had at least one functional and histologically verified electrode, and passed behavioral criteria (see below). For the pharmacological experiment, 28 of 32 rats that underwent catheter implantation, maintained intravenous catheter patency and were used in the study. Animals were counterbalanced into experimental groups based upon their self-administration rate during ShA pre-experimental training. Sample sizes are similar to those reported in previous publications[91].

Stereotaxic Surgery

Rats were anesthetized with isoflurane, placed in a stereotaxic frame, administered with the nonsteroidal anti-inflammatory carprofen (5 mg/kg, subcutaneously), and placed on an isothermal pad to maintain body temperature. The scalp was swabbed with alcohol and betadine, bathed with a mixture of lidocaine (0.5 mg/kg) and bupivacaine (0.5 mg/kg), and incised to expose the cranium. Holes were drilled in the cranium and dura mater was cleared for targeting of the DLS (1.2-mm anterior, 3.1-mm lateral and 4.8-mm ventral to Bregma[109]) and the nucleus accumbens core of the VMS (1.3-mm anterior, 1.3-mm lateral and 7.2-mm ventral to Bregma). One carbon-fiber microelectrode made in-house[89] was positioned in the VMS and another in the DLS, and a Ag/AgCl reference electrode was implanted in a separate part of the

forebrain. In a different set of animals, guide cannulas (26 gauge; Plastics One, Roanoke, VA, USA), occluded by "dummy" cannulas of equal length, were bilaterally implanted to target the VMS. Electrodes and guide cannula were secured with cranioplastic cement anchored to the skull by screws. Following surgery, rats were administered with the long-acting, nonsteroidal anti-inflammatory carprofen (5 mg/kg, subcutaneously) and placed on an isothermal pad to maintain body temperature until ambulatory. All animals were implanted with intravenous catheters during a separate surgery one week later.

Implantation of Intravenous Catheters

Rats were anesthetized with isoflurane, administered with the nonsteroidal anti-inflammatory carprofen (5 mg/kg, subcutaneously), and placed on an isothermal pad to maintain body temperature. Catheters were made of silastic tubing with an outer diameter of 0.6 mm and attached to a "hub" at one end (distal to vein insertion; Plastics One, VA, USA) for connection to an infusion pump. Catheters were pushed subcutaneously through an incision on the back between the shoulders to the front of the body, and anchored into the right jugular vein aided by a silicon rubber bead near the proximal end of the catheter. Optimal positioning of the catheter was verified by drawing blood into it with negative pressure. The hub was then secured by a piece of Teflon mesh sutured to surrounding tissue and incisions were closed, leaving the hub protruding from the rat's back. The catheter was then flushed with a heparin solution (80 U/ml in saline) and filled with a viscous solution of polyvinylpyrolidone (PVP) and heparin (1000 U/ml). The catheter hub was capped with a short, crimped piece of polyethylene tubing and the PVP solution remained in the catheter to ensure patency. Following surgery, rats were allowed to recover for at least five days.

Cocaine Self-Administration

Self-administration sessions were conducted between 0900 and 1700 hr. Rats learned to self-administer cocaine (Sigma, St. Louis, MO, USA) in a modular operant chamber (Med Associates,

VT, USA) equipped with two nose-poke response devices (port with integrated cue lights) located on adjacent panels of the same wall, a house light and speakers to provide pure-tone and white-noise stimuli. The operant chamber was housed within a sound-attenuated outer chamber. Rats (3-4 months old) were trained to obtain cocaine following an operant response on FI20 reinforcement schedule. Nose-poking in the active port (side counterbalanced between animals) resulted in an immediate intravenous infusion of cocaine (0.5 mg/kg over about ten seconds) paired with a 20-second presentation of an audiovisual stimulus (illumination of the light inside the nose poke port and tone; conditioned stimulus, CS). During CS presentation, a 20-second time out was imposed during which nose poking did not result in further drug infusion or any other programmed consequences. Drug availability during the session was signified by white noise and illumination of the house light. To control for response specificity, nose-poking of the second (inactive) port was monitored, but was never reinforced. Following pre-training sessions with a criterion of five or more active responses per session on two successive sessions for inclusion in the study, rats were given daily access to cocaine for one hour per day (short access; ShA) for one week and then six hours per day (long access; LgA) for three weeks (five days per week). The number of sessions to reach criterion varied between animals from two to five sessions. Behavioral results from a previously reported control group[91] were used to as a baseline to compare behavioral data from rats undergoing LgA cocaine self-administration to rats trained under a ShA regimen of an equal number of days.

Subsequent to the three ShA or LgA weeks of FI20 cocaine self-administration, a subset of rats underwent progressive-ratio testing. These sessions were identical to FI20 sessions except that animals were required to perform an increasing number of operant responses for successive infusions of cocaine during this session. The operant requirement on each trial (T) was the rounded-down integer of $1.4^{(T-1)}$ lever presses, starting at 1 lever press (that is, 1, 1, 1, 2, 3, 5, 7, 10, 14, 20, 28, 40, 56, 79, 111, 155, 217, 304, 426). This work requirement becomes so high that eventually animals stop responding and reach a “break point”. The break point was operationally defined as the total number of infusions earned prior to a thirty-minute period during which no infusions were obtained.

L-DOPA/Benserazide Administration

L-DOPA (L-3,4-dihydroxyphenylalanine) was given in combination with the peripherally acting DOPA decarboxylase inhibitor Benserazide to decrease peripheral breakdown of L-DOPA (both from Sigma, St. Louis, MO, USA). Both drugs were dissolved in saline and infused intravenously at a volume of 1 ml/kg body weight. L-DOPA was administered 30 minutes prior to session start at 0, 10, 30, or 90 mg/kg, whereas Benserazide was given consistently at 2 mg/kg irrespective of the L-DOPA dose administered. In a first set of studies (dose response), rats were treated with L-DOPA on a single day (Figure 4). None of the L-DOPA doses used inhibited general performance or caused dyskinesia. To avoid potentially confounding effects of repeated L-DOPA administration, rats were trained without L-DOPA treatment following "L-DOPA sessions". In a second set of studies, animals were treated with these L-DOPA prior to each self-administration session for a period of up to two weeks (Figure 5). In a third set of studies, rats that exhibited escalated cocaine self-administration during LgA, the effects of bilateral infusion of L-DOPA (25-50 µg dissolved in 0.5 µl ACSF into each hemisphere; 0.25 µl/min; Sigma, St. Louis, MO, USA) and ACSF into VMS on drug-taking behavior were examined. On infusion days, the dummy cannula was replaced with a 33-gauge infusion cannula that protruded 1.0 mm beyond the guide cannula. Infusions were given ten minutes prior to session start. After the infusion, the cannulas were left in place for two minutes before removal to allow for diffusion of the drug.

Voltammetric Measurements and Analysis

For dopamine detection by fast-scan cyclic voltammetry during experimental sessions (recordings performed during two sessions per week), chronically implanted carbon-fiber microsensors were connected to a head-mounted voltammetric amplifier, interfaced with a PC-driven data-acquisition and analysis system (National Instruments, TX, USA) through an electrical swivel (Med Associates, VT, USA) that was mounted above the test chamber. Voltammetric scans were repeated every 100 ms to achieve a sampling rate of 10 Hz. During each voltammetric scan, the potential at the carbon-fiber electrode was linearly ramped from -0.4 V

versus Ag/AgCl to +1.3 V (anodic sweep) and back (cathodic sweep) at 400 V/s (8.5-ms total scan time) and held at -0.4 V between scans. When dopamine is present at the surface of the electrode, it is oxidized during the anodic sweep to form dopamine-o-quinone (peak reaction detected at approximately +0.7 V) which is reduced back to dopamine in the cathodic sweep (peak reaction detected at approximately -0.3 V). The ensuing flux of electrons is measured as current and is directly proportional to the number of molecules that undergo electrolysis. Voltammetric data was band-pass filtered at 0.025 - 2,000 Hz. The background-subtracted, time-resolved current obtained from each scan provided a chemical signature characteristic of the analyte, allowing resolution of dopamine from other substances[146]. Dopamine was isolated from the voltammetric signal by chemometric analysis using a standard training set[89] based upon electrically stimulated dopamine release detected by chronically implanted electrodes. Dopamine concentration was estimated based upon the average post-implantation sensitivity of electrodes[89]. Prior to analysis of average concentration, all data were smoothed with a 5-point within trial running average. The concentration of dopamine was averaged over seven seconds (approximate duration of the observed phasic signal) following the operant response (post-response) or non-contingent presentation of the CS and was compared to the average concentration over the two seconds prior to the operant response (baseline). The CS was presented non-contingently during every recording sessions conducted in the second and third weeks (twice per session for 20 seconds each), but not during the first week to avoid interference with the associative conditioning between drug delivery and the cue during a period where this association was presumably still developing.

Statistical Analysis

Individual electrochemical signals were averaged across self-administration session, and then across animals and weeks, to increase statistical power. Signals were compared using multivariate ANOVAs with response, brain region, cocaine intake, and week as factors. For comparison with electrochemical data, behavioral data were also binned into weeks. For L-DOPA experiments, behavioral data (averaged across days if administered on consecutive days)

of a respective drug treatment (no treatment, L-DOPA dose, or vehicle) were analyzed using multivariate ANOVAs with drug treatment, training regimen, cocaine intake, and week as factors. In case of significant main effects or interactions, post-hoc analyses were conducted and *P* values were adjusted according to the Holm-Bonferroni correction method for multiple testing[111]. Plots were made using Prism (GraphPad Software, La Jolla, CA, USA). Statistical analyses were carried out using SPSS, version 17.0 (Chicago , IL, USA) and Prism. Data are appropriate for parametric statistical analysis. Data collection and analysis were not performed blind to the conditions of the experiments.

Histological Verification of Recording Sites

On completion of experimentation, animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg). In animals with electrode implants, recording sites were marked with an electrolytic lesion (300 V) prior to transcardial perfusion with saline followed by 4%-paraformaldehyde. Brains were removed and post-fixed in paraformaldehyde for twenty-four hours and then rapidly frozen in an isopentane bath, sliced on a cryostat (50-µm coronal sections, -20 °C), and stained with cresyl violet to aid in visualization of anatomical structures and the electrode-induced lesion or infusion sites.

Results

Male Wistar rats with indwelling intravenous catheters were trained to self-administer cocaine during daily ShA sessions and following acquisition were switched to LgA sessions in chambers equipped with two nose-poke ports. A nose poke into the active port elicited an infusion of cocaine (0.5 mg/kg/infusion) and 20-s presentation of a light-tone stimulus on a fixed-interval (FI) 20 schedule of reinforcement. Responses in the second (inactive) nose-poke port, or in the active port during stimulus presentation (20-s time-out), were without programmed consequence. For purposes of reporting, nose poke responses in the active port outside the time-out period (i.e., those that elicited a cocaine infusion) are referred to "active nose pokes" and those in the inactive port outside the time out period as "inactive nose pokes". The number of active nose pokes significantly exceeded inactive nose pokes (main effect of nose-poke port: $F_{(1, 23)} = 383.226$, $p < 0.001$; **Figure 1**) during each week ($p < 0.001$). After the switch from ShA to LgA, cocaine intake significantly increased over time (main effect of week: $F_{(3, 69)} = 25.504$, $p < 0.001$; **Figure 1**), as consistently reported by many others[147].

To assess the long-term dynamics of dopamine transmission, longitudinal neurochemical recordings were carried out simultaneously in the nucleus accumbens core of the VMS and in the DLS at chronically implanted microsensors[89] using fast-scan cyclic voltammetry (see **Supplementary Figure 1** for histological verification of electrode placement). In the first week of LgA, we observed a transient increase in extracellular dopamine concentration in VMS following active responses ($p < 0.001$; **Figure 2a**). This pattern of activation declined during LgA where dopamine release in the third week was significantly smaller than in the first ($p < 0.001$) and second ($p = 0.030$) weeks (main effect of week: $F_{(2,72)} = 10.230$, $p < 0.001$; **Figure 2b**). Phasic dopamine release in the DLS emerged in the second week ($p = 0.006$; **Figure 2c**) but was absent in the third week of LgA (main effect of week: $F_{(2,51)} = 3.474$, $p = 0.039$; active poke x week interaction: $F_{(2,51)} = 4.021$, $p = 0.024$; **Figure 2c,d**). These data show that phasic dopamine signals in VMS and DLS emerge sequentially at different stages of drug taking similar to what we reported for a ShA regimen[91]. However, this signaling diminished in both regions over the

course of LgA, a period over which it is known that the pharmacokinetics of intravenously administered cocaine do not change[148,149].

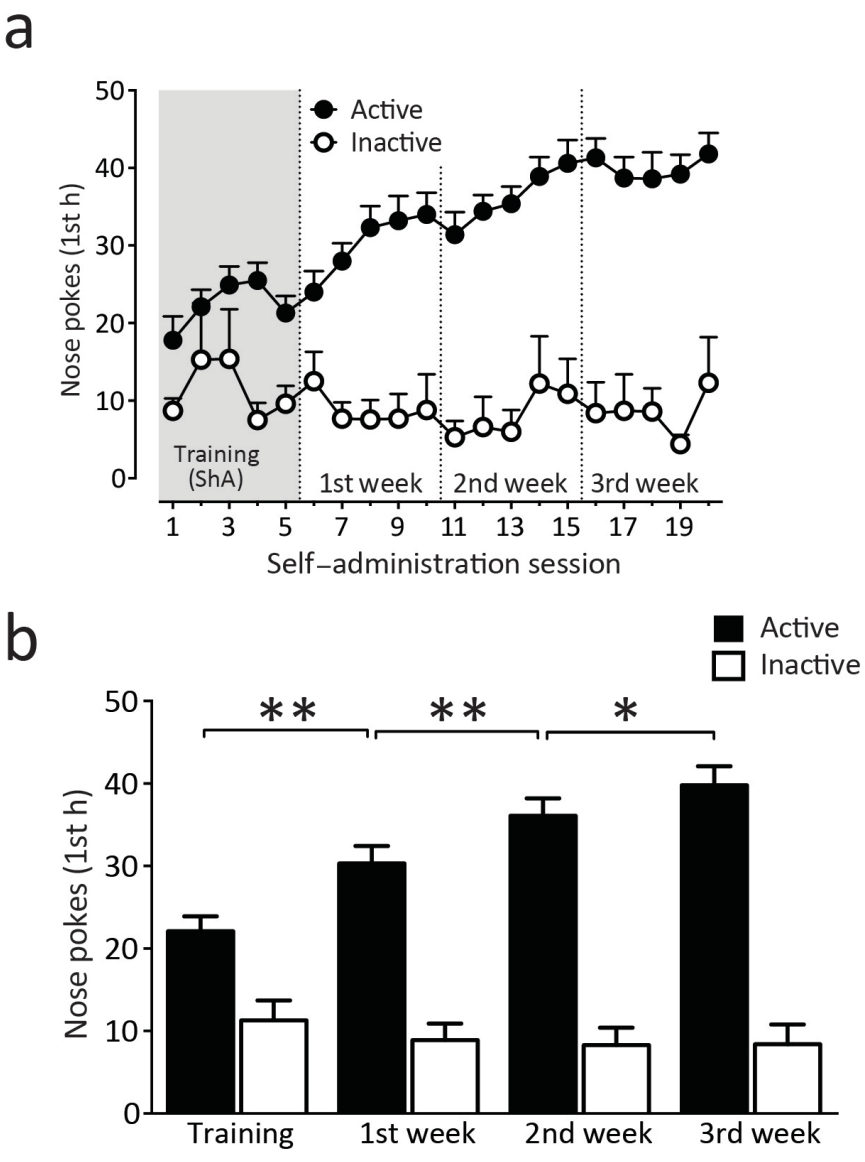


Figure 1. Escalation of drug taking over the course of weeks.

a) Nose pokes into the active (filled circles) and inactive (open circles) ports (excluding pokes inside the time-out period) over 5 d of ShA training (gray background) and the first hour of 15 d of LgA (white background) cocaine self-administration (n = 24 rats). **b**) The number of active nose pokes (filled bars) increased significantly across weeks (training versus first week, p = 0.006; first versus second week, p = 0.003; second versus third week, p = 0.013), whereas the number of inactive responses (open bars) remained stable. Data are mean + s.e.m. * $p < 0.05$, ** $p < 0.01$, *post hoc* two-sided t-tests following two-way ANOVA.

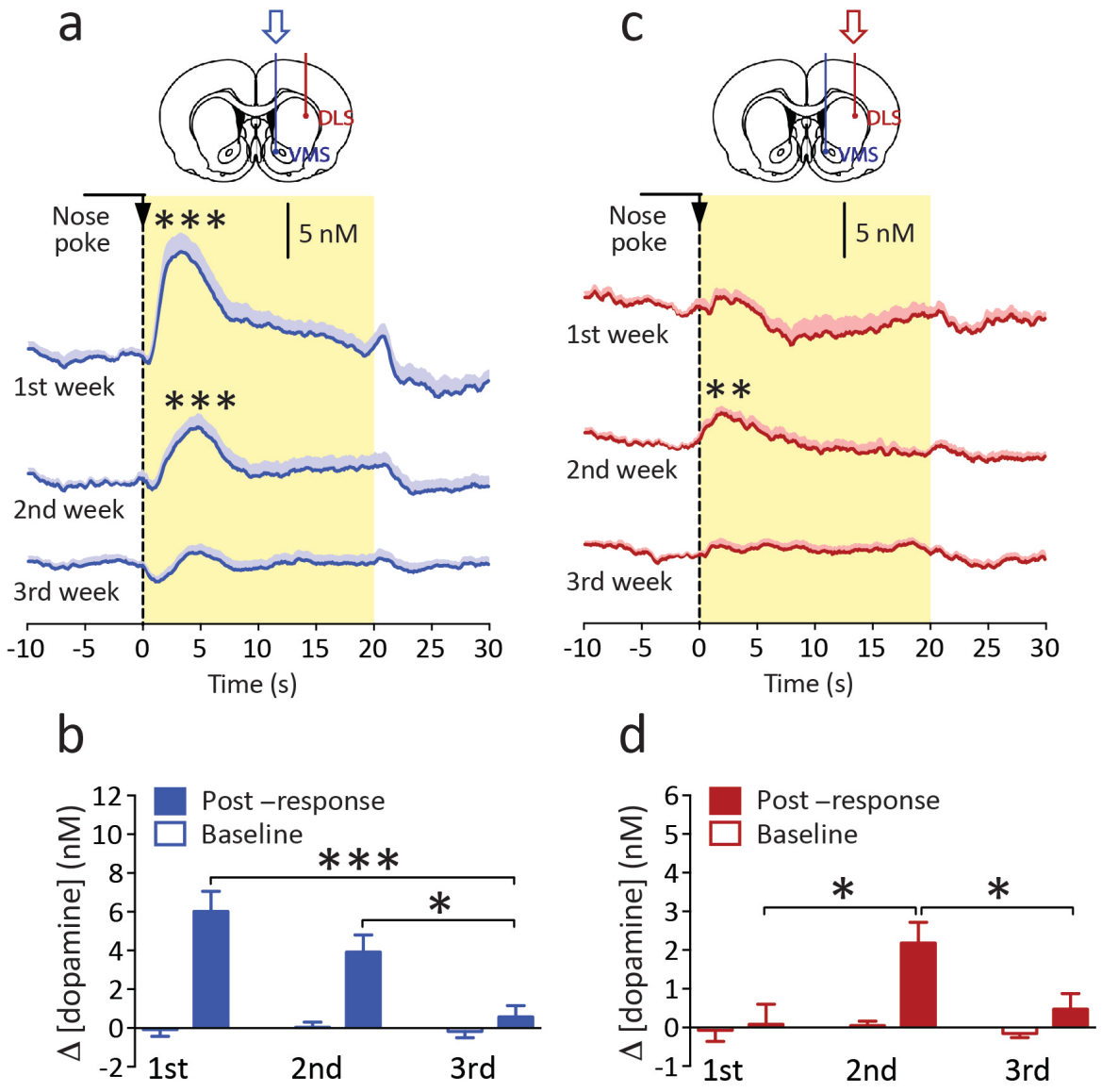


Figure 2. Dopamine signaling in the VMS and DLS over the course of weeks.

A nose poke (dashed line) into the active port elicited an infusion of cocaine (0.5 mg/kg per infusion) paired with the presentation of an audiovisual stimulus (yellow box) during a 20-s time out. **a**) Phasic dopamine release in VMS following active nose-poke responses was observed during the first and second, but not third, weeks of LgA cocaine self-administration (first hour; n = 18 electrodes). **b**) The average amplitude of dopamine release decreased over the course of weeks (first versus third week, $p < 0.001$; second versus third week, $p = 0.030$). **c**) Phasic dopamine release in DLS following active nose-poke responses was observed during the second week of cocaine self-administration only (first hour; n = 18 electrodes). **d**) Dopamine releases in the second week were greater in amplitude than those in the first and third weeks (first versus second week, $p = 0.014$; second versus third week, $p = 0.020$). Data are mean + s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, *post hoc* two-sided *t*-tests following two-way ANOVA.

Decrement in VMS dopamine correlates with drug intake

To test the relationship between the loss of dopamine signaling and escalation of drug consumption, we took advantage of individual differences in susceptibility to escalate drug self-administration during the LgA regimen by separating animals into two groups depending on whether they exhibited significant escalation based upon linear regression of drug consumption over LgA sessions or not (**Figure 3a, b**). Validation of this separation of animals demonstrated that non-escalated animals showed no significant increase in active nose pokes over the course of three weeks of LgA (main effect of week: $F_{(2,18)} = 0.633$, $p = 0.542$; **Figure 3b**, left), whereas escalated rats increased their intake significantly (main effect of week: $F_{(2,26)} = 14.826$, $p < 0.001$; **Figure 3b**, right; intake x week interaction: $F_{(2,44)} = 4.674$, $p = 0.014$) making more active nose pokes than non-escalated animals during the third LgA week ($t_{(22)} = 2.307$, $p = 0.031$; **Figure 3b**). Notably, escalated animals displayed an increased motivation to obtain cocaine, as demonstrated in a progressive-ratio task ($p = 0.028$; **Supplementary Figure 2**). In escalated rats, there was a significant decline in dopamine release in the VMS (main effect of week: $F_{(2,51)} = 15.507$, $p < 0.001$; **Figure 3c**, right, and **Supplementary Figure 3a**). However, VMS dopamine release was stable in non-escalated rats (main effect of week: $F_{(2,18)} = 0.057$, $p = 0.945$; **Figure 3c**, left and Supplementary **Figure 4a**) conferring significantly more phasic dopamine in the third week compared to escalated rats (main effect of intake: $F_{(1,69)} = 6.444$, $p = 0.013$; **Figure 3d**, left; intake x week interaction: $F_{(1,70)} = 4.303$, $p = 0.042$). This difference in dopamine release between escalated and non-escalated rats was evident throughout the entire six hours of self-administration ($t_{(43)} = 2.599$, $p = 0.013$). Importantly, this difference did not result from a general decline in dopamine function in escalated animals, as dopamine release following non-contingent, experimenter-induced infusions of cocaine did not differ between non-escalated and escalated animals ($p = 0.605$; **Supplementary Figure 5a**).

In contrast to the maintained phasic dopamine release in the VMS of non-escalating rats, we previously reported that there was a decrease in dopamine release in animals that had undergone three weeks of limited cocaine access (ShA) of only one hour per daily session[91]. Therefore, we carried out additional analyses on the data obtained from these ShA rats to permit

a detailed characterization of the relationship between dopamine function and drug intake across animals who had undergone ShA or LgA cocaine self-administration. While there was not a significant escalation of the mean drug consumption across animals during ShA, there were individual differences with a subset of animals (6 of 16) exhibiting significant escalation of drug intake over three weeks of ShA cocaine self-administration. Interestingly, VMS phasic dopamine in the third week of ShA cocaine self-administration in the group of animals who maintained stable drug consumption (i.e., did not exhibit significant escalation) was not significantly different from that of non-escalated animals in the third week of LgA ($p = 0.741$; Supplementary Figure 5b). ShA animals that escalated their drug intake, exhibited lower rates of drug consumption (32.7 ± 3.9 versus 43.9 ± 3.1 infusions in the first hour, $p = 0.017$) and less attenuated dopamine release ($p = 0.049$; Supplementary Figure 5b) than animals that escalated their intake under LgA conditions. Nonetheless, there was a non-significant trend for decreased VMS dopamine compared to their non-escalating counterparts ($p = 0.094$) and no significant interaction for dopamine release over time between ShA and LgA escalating rats (no intake x regimen interaction: $F_{(1,57)} = 0.111$ $p = 0.740$; Supplementary Figure 5b). Given these individual differences, we carried out regression analysis across all of the ShA and LgA rats to test for a direct relationship between dopamine levels and the degree of escalation, and found significant negative correlation (ShA and LgA rats pooled together; $r = -0.628$, $p = 0.005$) with greatest escalation in animals that had the lowest dopamine release in week 3 (Figure 3e, left). Therefore, the attenuation of dopamine signaling in the VMS was predictive of escalation of drug self-administration across LgA and ShA drug-access regimens. These data highlight that the germane aspect related to changes in dopamine release is whether animals escalate or not, rather than the self-administration regimen they have been exposed to *per se*. Likewise, we find that across all rats, escalation is a significant predictor of increased motivation for cocaine ($p = 0.037$, Supplementary Figure 6a), but LgA/ShA regimen is not, as assessed in a progressive ratio schedule ($p = 0.340$, Supplementary Figure 6b).

In contrast to the VMS, response-contingent dopamine release in the DLS did not differ between escalated and non-escalated LgA animals (main effect of intake: $F_{(1,48)} = 0.472$, $p = 0.$

496; **Figure 3d**, right and **Supplementary Figures 3b and 4b**), nor was there a significant relationship between the slope of escalation and dopamine release across animals that underwent ShA or LgA cocaine self-administration (r = -0.112, *p* = 0.649; **Figure 3e**, right). Thus, whereas dopamine in the VMS correlated with the escalation of drug taking, a similar correlation was not observed in the DLS, a brain region that has been widely associated with extended drug self-administration[7,21,93,105].

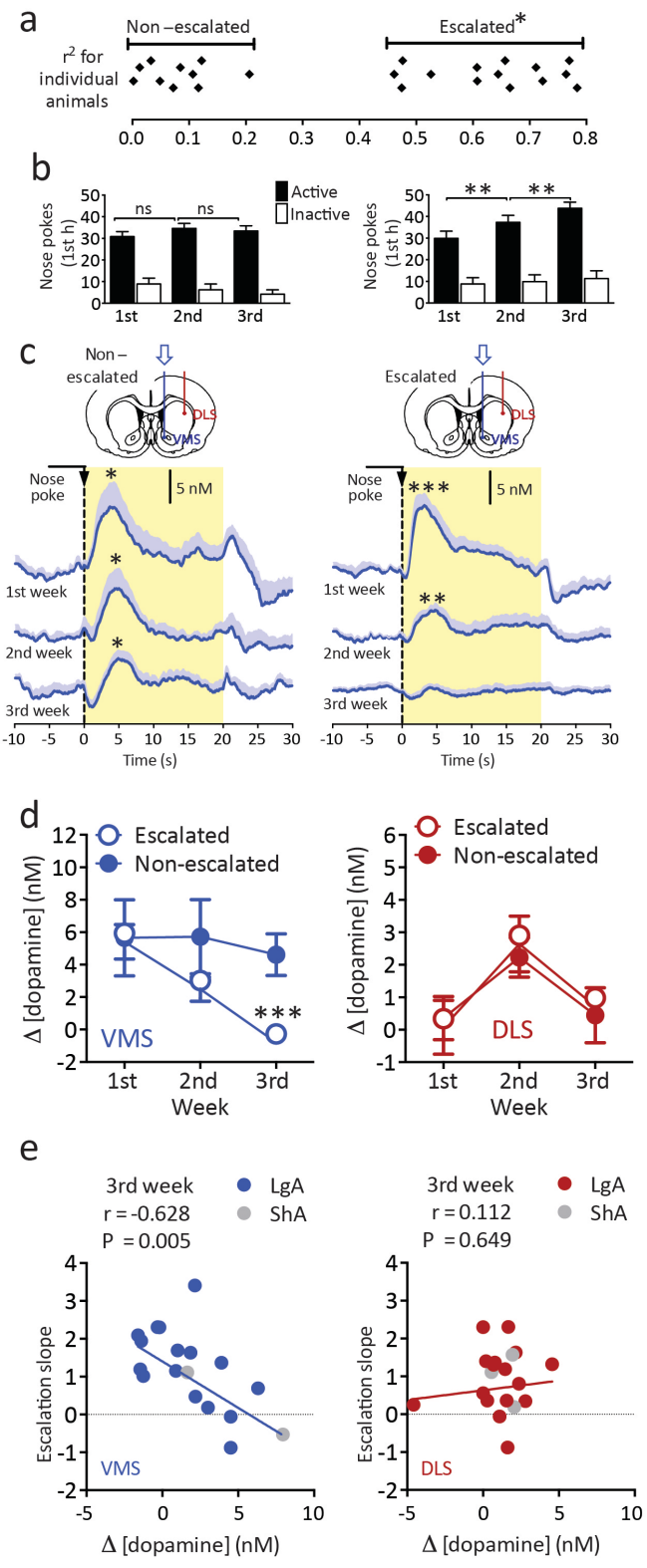


Figure 3. Individual differences in drug-taking behavior and striatal dopamine signaling.
a) A linear regression between the number of active nose pokes per session and the number of days of self-administration revealed a non-escalated ($P > 0.05$; $n = 10$ rats) and an escalated ($P < 0.05$; $n = 14$ rats) population of rats. **b**) Non-escalated animals showed no significant increase (NS) in cocaine intake over the course of LgA (left; first versus second week, $p = 0.956$; second versus third week, $p = 0.338$), whereas escalated rats increased their intake significantly (right; first versus second week, $p = 0.009$; second versus third week, $p = 0.008$). **c**) Phasic dopamine release in VMS of non-escalated animals (left) following active nose-poke responses was observed during all three weeks of cocaine self-administration ($p = 0.039$, 0.034 and 0.048, respectively), whereas release in VMS of escalated rats (right) was observed during the first ($P < 0.001$) and second ($p = 0.006$), but not third ($p = 0.754$), weeks. **d**) Consequently, VMS dopamine release was significantly different between non-escalated and escalated animals during the third week (left). DLS dopamine signaling did not differ between non-escalated and escalated animals at any time point (right). **e**) A significant relationship between the slope of escalation and dopamine release was detected in VMS, but not in DLS (ShA (gray circles) and LgA (colored circles) rats pooled). Data are mean + s.e.m. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, *post hoc* two-sided *t*-tests following ANOVA.

L-DOPA restores VMS dopamine and de-escalates drug-taking

Given this provocative correlation between neurochemistry and behavior, we hypothesized that the decline in phasic dopamine signaling was causal in producing escalation of drug taking, akin to the increase in drug taking produced by dopamine-receptor antagonists[150-152], and so restoring it would produce a reversal in escalation (**Figure 4a**). Therefore, we treated escalated animals (p = 0.024; **Figure 4b**) with L-DOPA prior to session start to increase phasic dopamine release[153]. L-DOPA dose-dependently (0, 10, 30, and 90 mg/kg, intravenous) decreased cocaine intake (main effect of L-DOPA: $F_{(3,53)}$ = 5.053, p = 0.004; **Figure 4b**), with 30 mg/kg returning intake to the pre-escalated level. Importantly, the 30 mg/kg dose of L-DOPA was sufficient to completely restore phasic dopamine signaling in the VMS (see **Supplementary Figure 7** for recording sites) during drug taking ($F_{(2,8)}$ = 6.316, p = 0.023; **Figure 4c**), an effect also observed for the full six hours of self-administration ($F_{(2,8)}$ = 7.610, p = 0.0141). Thus, the amount of phasic dopamine release in the VMS predicted the amount of drug intake during a cocaine self-administration session (r = -0.525, p = 0.046; **Figure 4d**). This behavioral effect of L-DOPA cannot be explained by changes in the pharmacological response to cocaine, as the slow concentration changes in VMS dopamine following contingent drug infusion were not altered by L-DOPA treatment and, in fact, did not differ between pre-escalation, escalation, and escalated L-DOPA-treated states ($F_{(2,8)}$ = 0.020, p = 0.980; **Supplementary Figure 8**). Furthermore, the effect of L-DOPA on drug consumption was also observed when L-DOPA was locally infused into the VMS (see **Supplementary Figure 9** for infusion sites) of escalated rats prior to a session ($t_{(7)}$ = 6.517, p < 0.001; **Figure 4e**). Taken together, this set of studies demonstrates that a single dose of L-DOPA administered prior to drug access is effective in restoring dopamine signaling and normalizing cocaine use to the pre-escalated state.

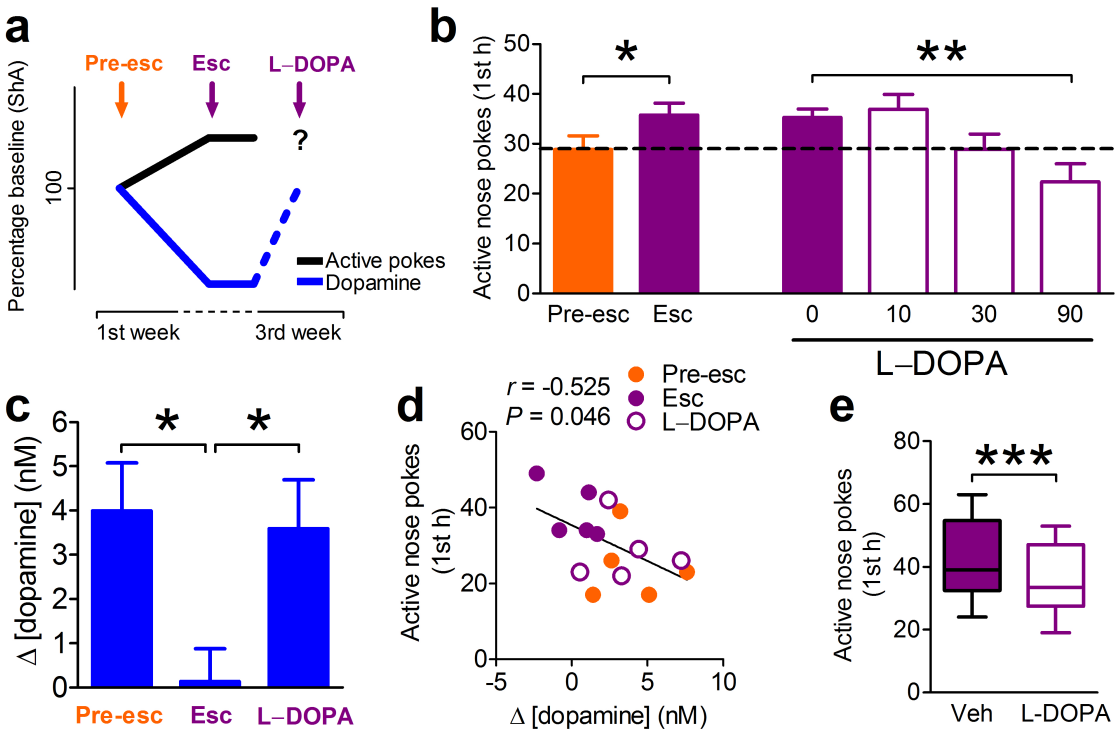


Figure 4. L -DOPA decreases escalated drug intake by replenishing VMS dopamine release.
a) Schematic of findings posing the question of whether L-DOPA will normalize LgA-induced escalation of drug-taking behavior by correcting the observed neurochemical deficit. Esc, escalation. **b**) Escalated rats received an intravenous injection of the dopamine precursor L-DOPA (0, 10, 30, and 90 mg/kg) and DOPA decarboxylase inhibitor benserazide (2 mg/kg) 30 min before a self-administration session during the third week of LgA. **c**,**d**) Administration of L-DOPA (30 mg/kg) and benserazide (2 mg/kg) restored escalation- related decremented phasic dopamine release in the VMS following active nose-poke responses. **e**) L-DOPA infused into the VMS of escalated rats was effective at reducing drug consumption. Veh, vehicle control. Data are mean + s.e.m. $*P < 0.05$, $***P < 0.001$, two-sided *t*-tests or *post hoc* Newman-Keuls. $**P < 0.01$, one-way ANOVA.

We next tested whether the use of L-DOPA would be effective at reducing escalated drug consumption in longer-term dosing regimens, more relevant to clinical applications. First, we conducted experiments introducing repeated infusion of L-DOPA on consecutive days during the induction of escalation. Animals were trained to stably self-administer cocaine and then either switched to LgA or remained on ShA during which time they were injected with L-DOPA (30 mg/kg, intravenous) or saline prior to each session for two weeks (**Figure 5a**). L-DOPA significantly affected drug intake in a regimen-specific manner (main effect of treatment: $F_{(1,53)}$ =

9.297, p = 0.004; main effect of regimen: $F_{(1,53)}$ = 5.968, p = 0.018; **Figure 5a**) with decreased cocaine intake in LgA animals (p = 0.004), but not ShA animals (p = 0.170; **Figure 5a**), and without effect on inactive nose pokes (LgA, p = 0.202; ShA, p = 0.101; data not shown). Therefore, the L-DOPA treatment was effective at preventing escalation of drug consumption during LgA. However, upon treatment cessation, this effect did not endure (p = 0.789; **Figure 5a**). Second, we repeatedly administered L-DOPA on consecutive days in animals with established escalated drug consumption. Animals were trained to stably self-administer cocaine and subsequently were either switched to LgA, or remained on ShA for three weeks. These animals were then treated with L-DOPA or saline prior to self-administration sessions in the third week (**Figure 5b**). LgA-trained animals showed a significant increase in cocaine use during the first two weeks compared to ShA-trained animals (main effect of regimen: $F_{(1,51)}$ = 15.706, p < 0.001; data not shown). L-DOPA treatment produced a regimen-specific effect (main effect of treatment: $F_{(1,51)}$ = 5.303, p = 0.025; main effect of regimen: $F_{(1,51)}$ = 11.884, p = 0.001; **Figure 5b**), decreasing cocaine intake in LgA animals (p = 0.048), but not ShA animals (p = 0.210; **Figure 5b**) without effecting inactive responding (LgA, p = 0.641; ShA, p = 0.664). Importantly, the differential effect of L-DOPA on active nose pokes was more robust when animals were grouped into escalated and non-escalated, instead of ShA and LgA (escalated animals, p = 0.005; non-escalated animals, p = 0.421; **Figure 5c**), indicating that L-DOPA reduced escalated cocaine intake preferentially rather than affecting drug consumption *per se*, an interaction that developed over days (intake x treatment (day 1) interaction: $F_{(1,51)}$ = 0.562, p = 0.457; but intake x treatment (day 5) interaction: $F_{(1,51)}$ = 4.091, p = 0.048). Importantly, these differences between escalated and non-escalated sub-populations as well as the de-escalating effects of acute and chronically administered L-DOPA are also observed across all six hours of self-administration (**Supplementary Figure 10**). Together these findings demonstrate that phasic dopamine release decreases in animals that escalate their cocaine intake and restoring it with repeated administration of the dopamine precursor, L-DOPA, prevents and reverses this escalation, providing evidence that decreased dopamine drives escalation of drug self-administration.

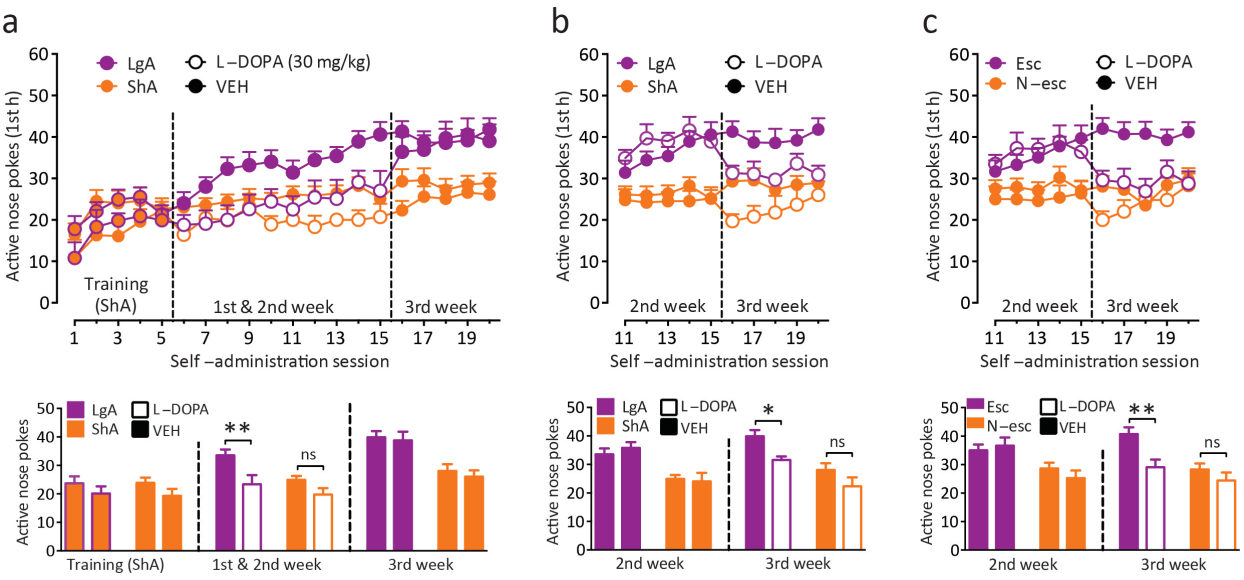


Figure 5. L-DOPA prevents and reverses the escalation of drug-intake.

a) Animals were trained to self-administer cocaine (ShA) and subsequently either switched to LgA or remained on ShA (*n* = 57 rats). Rats that received an intravenous injection of the dopamine precursor L-DOPA (30 mg/kg) before each LgA session during the first and second weeks (open purple circles) did not escalate their drug intake (first hour) compared to animals that received vehicle (filled purple circles). Upon cessation of L-DOPA treatment (third week), we observed no differences between these LgA groups. L-DOPA-induced changes in drug intake of ShA rats (orange circles) were not significant. **b**) In another experiment, animals were trained to self-administer cocaine (ShA) and subsequently were either switched to LgA or remained on ShA (*n* = 55 rats). LgA-trained animals (purple circles) showed a significant increase in cocaine use compared to ShA-trained animals (orange circles) during the second week. During the third week, a subset of rats were treated with L-DOPA. In LgA animals (open purple circles), L-DOPA treatment decreased escalated cocaine intake. In ShA animals (open orange circles) L-DOPA treatment did not yield a significant change. **c**) The differential effect of L-DOPA on active nose pokes (**b**) was more robust when animals were divided into escalated and non-escalated groups, instead of ShA and LgA. Data are mean + s.e.m. NS, $P \geq 0.05$; *$P < 0.05$; **$P < 0.01$; *post hoc* two-sided *t*-tests following two-way ANOVA.

Discussion & Conclusions

In the present study, we investigated phasic dopamine release in the VMS and DLS during escalation of drug intake, a phenomenon that models a key diagnostic criterion for drug dependence[5,33]. Our findings demonstrate that escalation is associated with decreased dopamine signaling in both the VMS and DLS, with the decrement in dopamine in the VMS significantly correlated with the rate of escalation. This effect appears to be selective for phasic dopamine as comparable changes were not observed in tonic dopamine in the current study, in previous work using the same regimen in rats[149] or related self-administration paradigms in non-human primates[154,155]. There have been a number of reports of reduced phasic dopamine function during drug withdrawal (tested between 18 hours and seven days from the last self-administration session) which is associated with reduced sensitivity to cocaine[156-159]. While we observed a similar reduction in the dopamine response to cocaine between ShA and LgA rats (**Supplementary Figure 5a**), this effect did not appear to be pertinent to escalation as the neurochemical response to non-contingent cocaine is not different between rats that escalated and those that did not (no intake x regimen interaction: $F_{(1,34)} = 1.964$ $p = 0.170$; **Supplementary Figure 5a**). Similarly, peak changes in tonic dopamine concentration up to 90 seconds after contingent cocaine, presumably due to the pharmacological actions of cocaine, did not differ between the pre-escalated and escalated state within the same animals (**Supplementary Figure 8**). Thus, the only aspect of dopamine transmission that we observed which predicted escalation of drug intake was the phasic response that occurred immediately following an active nose poke, which is a conditioned response primarily to drug-associated cues[69,91,101]. This neurochemical response diminished in animals that escalated their drug intake, which is reminiscent of a normal learning process wherein dopamine release in the VMS elicited by a reward-related stimulus decreases as that stimulus becomes temporally predicted[73,160]. However, the attenuation of dopamine release during self-administration occurs much later in the learning process than would be expected for contingency learning, long after the acquisition of established drug taking. Moreover, in animals that do not escalate their drug intake, attenuation of phasic dopamine

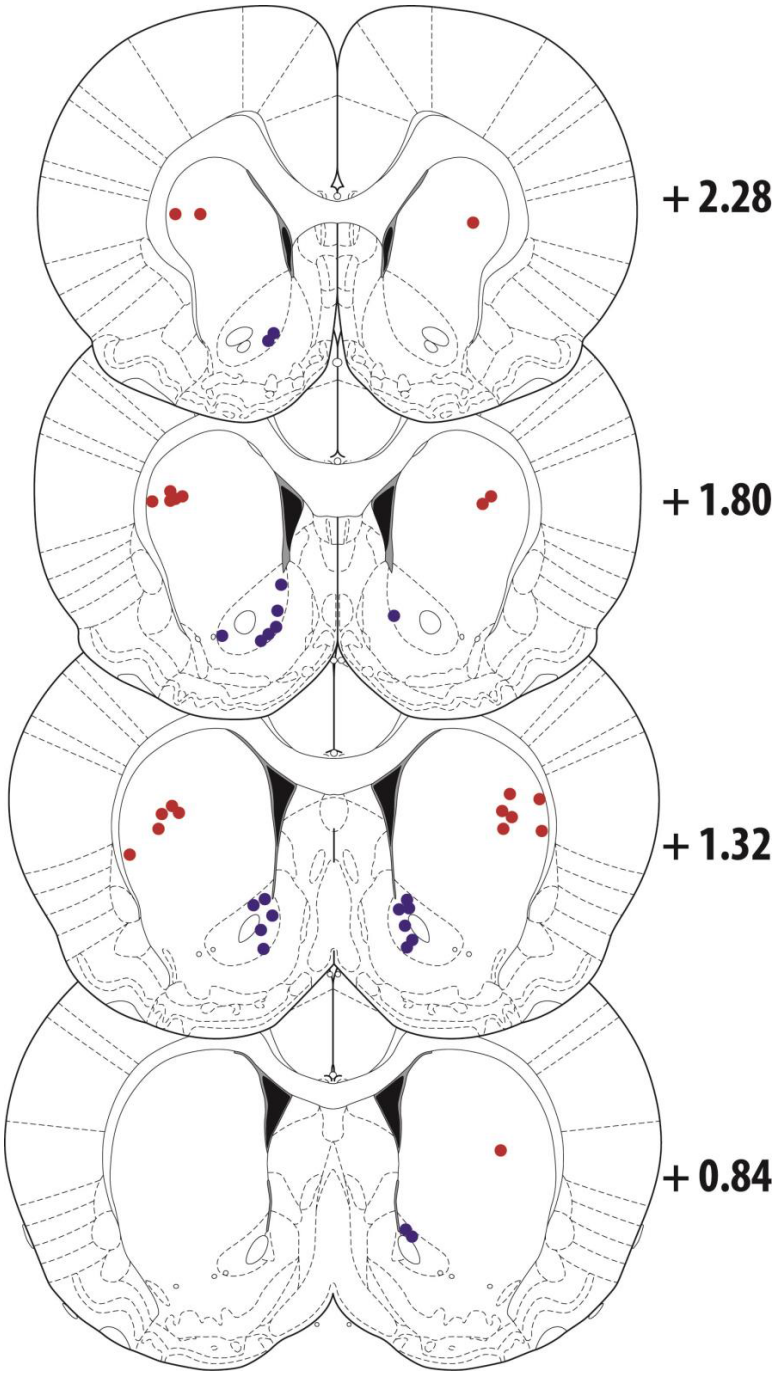
release does not take place even though these animals exhibit asymptotic discriminative instrumental behavior.

At face value, our observations of declining dopamine release as drug use progresses appear to be at odds with several contemporary theories of addiction. Theories focusing on drug-induced incentive sensitization processes postulate increasing reactivity of the VMS dopamine system upon repeated exposure to drugs of abuse that mediates a sensitized response to drug and cue exposure[87], a phenomenon that is specifically robust after LgA[161]. Conceptualizations on the role of aberrant learning and habit formation in drug addiction suggest that emerging dopamine signaling in the DLS increasingly assumes control over drug seeking[21,22,105]. Moreover, prominent computational models of addiction specifically implicate increased dopamine signaling to drug-associated cues as a driving force towards addiction[162,163]. Conversely, our findings appear to be more consistent with the dopamine depletion hypothesis of addiction, proposed by Dackis and Gold[164], and related opponent-process theories[33] that emphasize drug-abuse-induced suppression of reward-related processes. Such suppression has been hypothesized to cause compensatory self-regulation of drug use to maintain a preferred level of drug intoxication[33]. Specifically, humans and animals compensate for lowered unit doses of cocaine with increased responding[28,165]. This process is regulated by dopamine transmission in the VMS[67] and, consequently, lowering dopamine transmission (e.g., by dopamine receptor antagonism) elicits an increase in the rate of drug consumption[150,151]. Therefore, the reduction in dopamine signaling that we observed during LgA may stimulate compensatory upregulation of drug intake to achieve the preferred level of intoxication. In support of this hypothesis, the reduction of dopamine in the VMS was most pronounced in animals that exhibited greater escalation of drug taking.

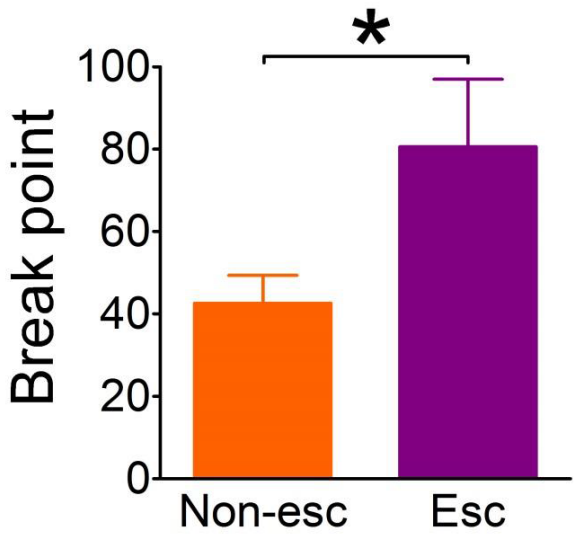
Thus, we reasoned that restoring dopamine transmission would attenuate escalation. Indeed, L-DOPA administration was effective at both preventing and reversing the escalation of drug intake. Notably, the effects of L-DOPA on drug use did not endure following termination of treatment, suggesting that it did not prevent the underlying neuroadaptation. Therefore, our data indicate that escalation is mediated by a process that is manifested through a decrease in

phasic dopamine during drug taking. These findings provide mechanistic information for the use of L-DOPA in the clinical treatment of psychostimulant abuse, a strategy that has had some promising, but overall mixed, outcomes in a small number of recent clinical trials[166]. Specifically, since L-DOPA reduced escalated drug use without producing abstinence, we suggest it is better suited for harm-reduction approaches and, in particular, allowing addicts to regain a degree of control of their drug use while entering behavioral therapy programs. Overall, our findings reveal a decrement in phasic dopamine release that takes place during protracted drug access which mediates the shift from recreational to uncontrolled drug use.

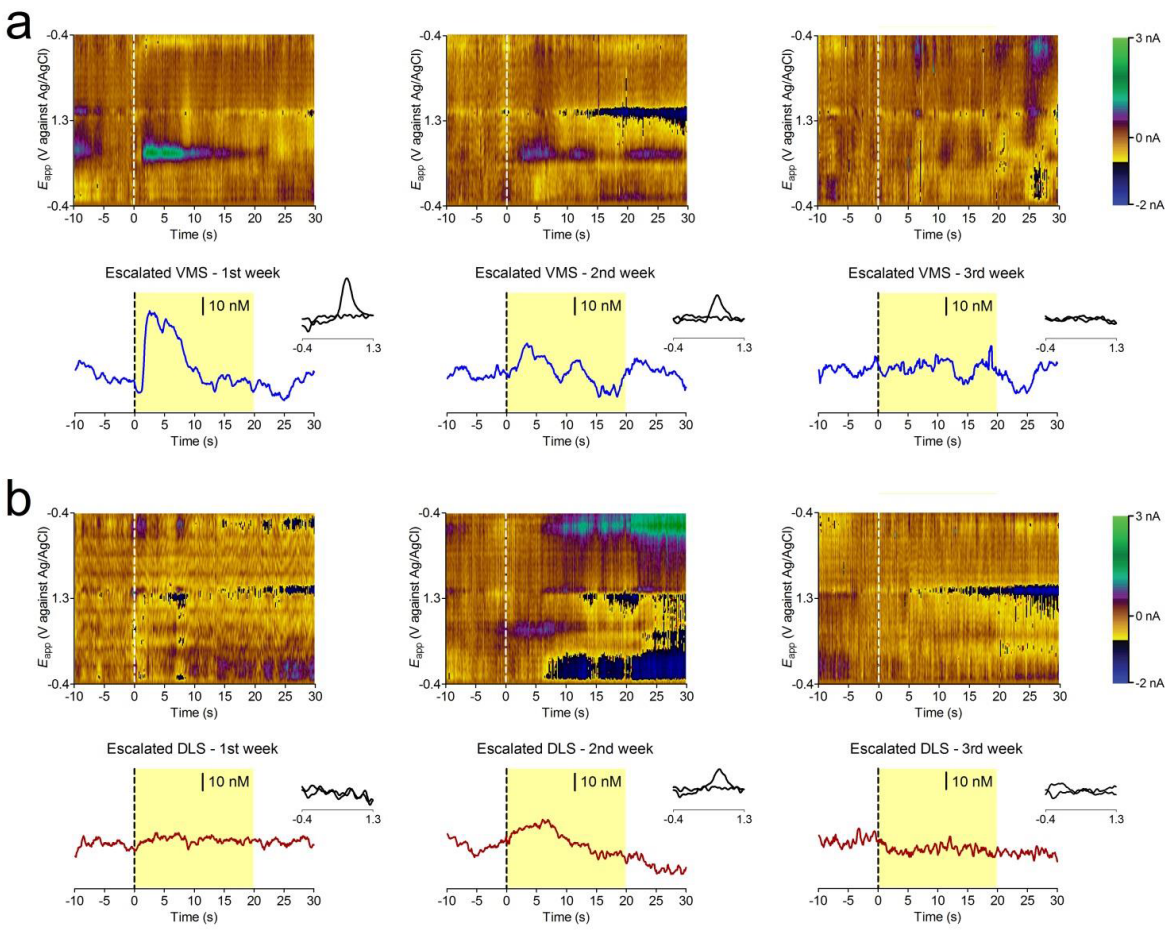
Supplementary Figures



Supplementary Figure 1. Histological verification of recording sites in VMS and DLS (1st Experiment)
VMS recording sites (blue circles) were confirmed to be within the nucleus accumbens core, and DLS recording sites (red circles) were in the lateral half of the dorsal striatum. The numbers on each plate indicate distance in millimeters anterior from bregma[109]

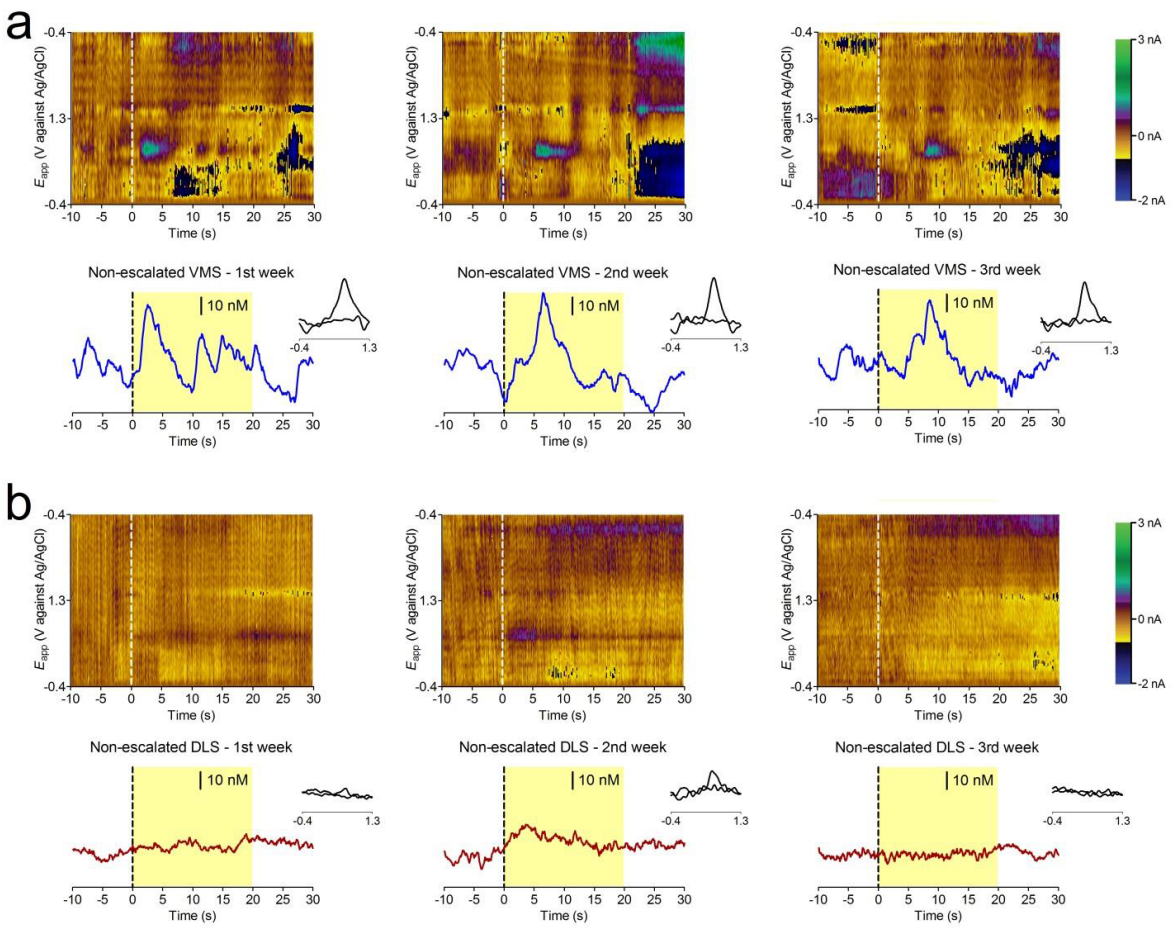


Supplementary Figure 2. Escalated animals display increased motivation to obtain cocaine.
Subsequent to FI20 cocaine self-administration, a subset of LgA rats (n = 19) underwent progressive-ratio testing. Progressive-ratio sessions were identical to FI20 sessions except that animals were required to perform an increasing number of operant responses for successive infusions of cocaine. The break point was operationally defined as the maximum number of responses. Average break point values are depicted (mean + s.e.m.). Escalated rats (purple bar) displayed significantly more responses (and earned more infusions) than non-escalated animals
(orange bar). *P<0.05.



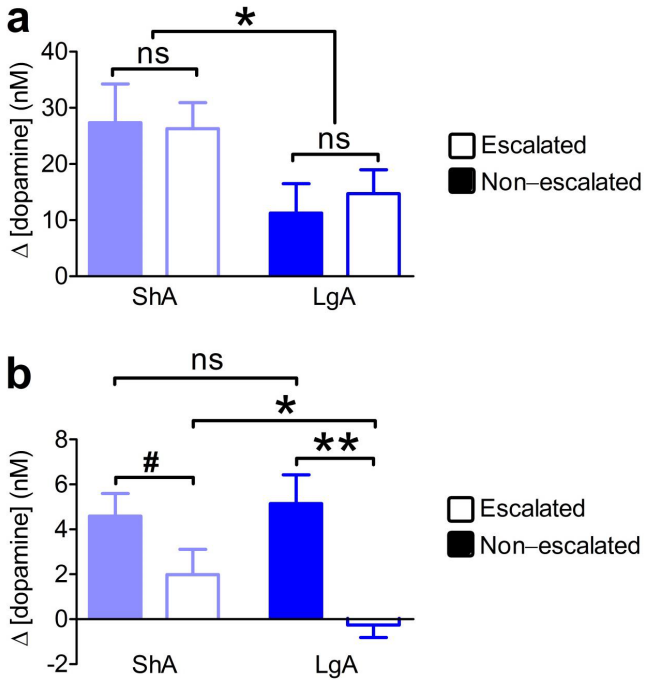
Supplementary Figure 3. Examples of phasic dopamine release in VMS and DLS associated with an individual nose poke (single trial) into the active port for animals with escalated cocaine intake.

a) Pseudocolor plots (top panel), dopamine traces (bottom panel), and cyclic voltammograms (inset in bottom panel) for representative current fluctuations recorded in VMS for the period 10 seconds before an operant response (dashed line), during the subsequent 20-second presentation of the CS (yellow box; includes cocaine infusion), and 10 seconds after the offset of the CS during the first (Left), second (Middle), and third (Right) weeks of LgA cocaine self-administration (first hour). **b)** Pseudocolor plots (top panel), dopamine traces (bottom panel), and cyclic voltammograms (inset in bottom panel) for representative current fluctuations recorded in DLS during the first (Left), second (Middle), and third (Right) weeks of LgA cocaine self-administration. The color plots show current changes across the applied voltages (Eapp; yaxis) over time (x-axis).

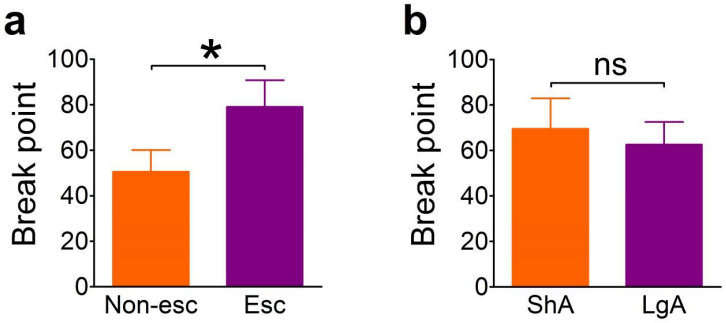


Supplementary Figure 4. Examples of phasic dopamine release in VMS and DLS associated with an individual nose poke (single trial) into the active port for animals with non-escalated, stable cocaine intake

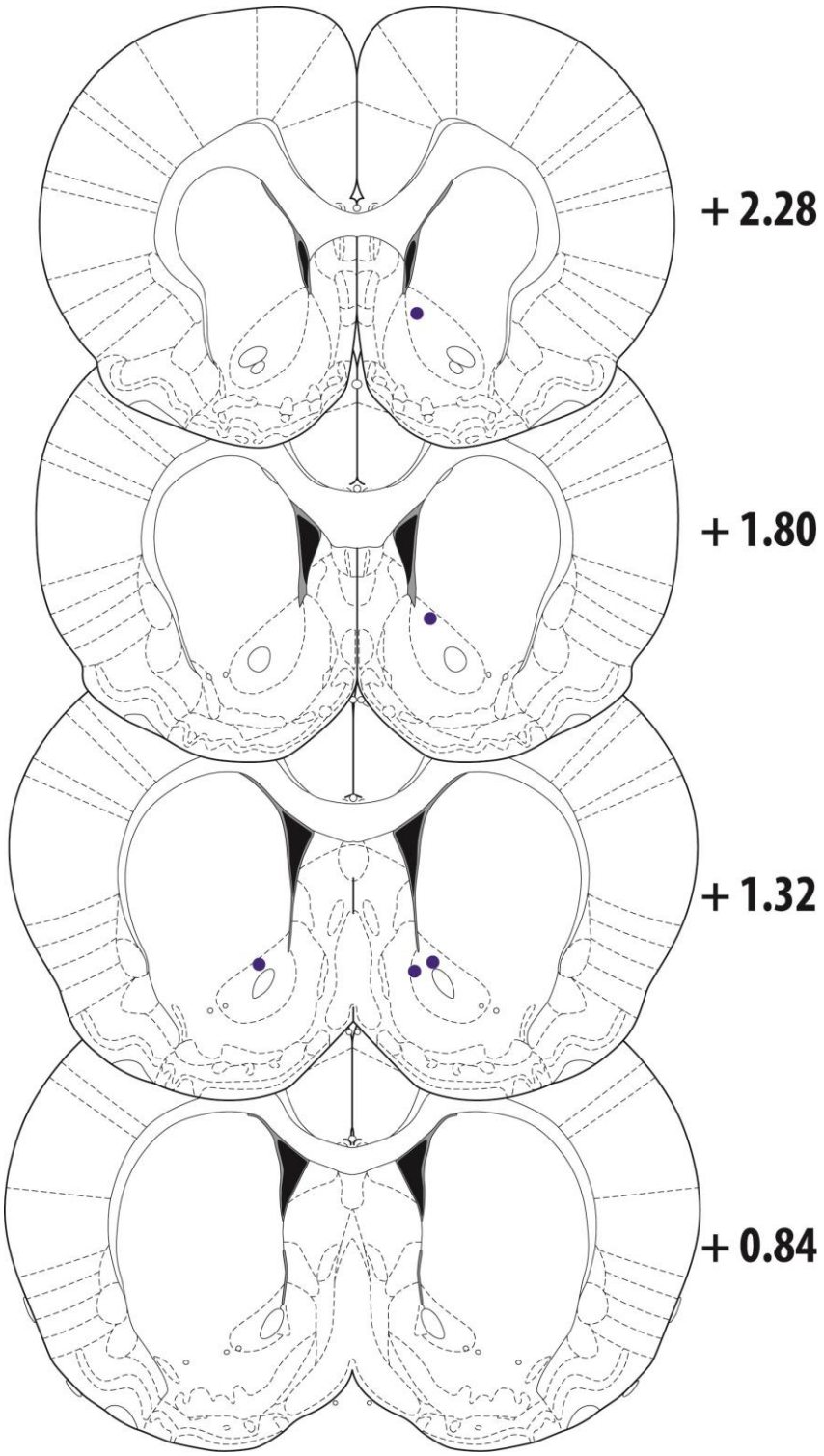
a) Pseudocolor plots (top panel), dopamine traces (bottom panel), and cyclic voltammograms (inset in bottom panel) for representative current fluctuations recorded in VMS for the period 10 seconds before an operant response (dashed line), during the subsequent 20-second presentation of the CS (yellow box; includes cocaine infusion), and 10 seconds after the offset of the CS during the first (Left), second (Middle), and third (Right) weeks of LgA cocaine self-administration (first hour). **b**) Pseudocolor plots (top panel), dopamine traces (bottom panel), and cyclic voltammograms (inset in bottom panel) for representative current fluctuations recorded in DLS during the first (Left), second (Middle), and third (Right) weeks of LgA cocaine self-administration. The color plots show current changes across the applied voltages (Eapp; y-axis) over time (x-axis).



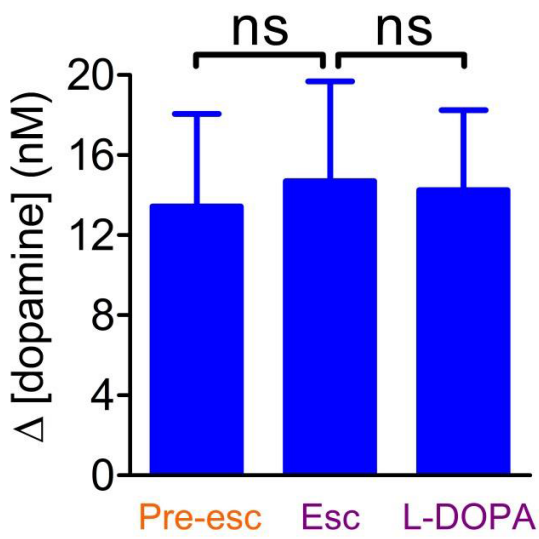
Supplementary Figure 5. Effects of cocaine (pharmacological) and responding for cocaine delivery (behavioral) vary with access regimen (ShA/LgA) and intake pattern (Esc/ Non-esc). **a**) Average increases in extracellular concentration of dopamine in the VMS over a thirty second period following a non-contingent (response-independent; no CS) intravenous infusion of cocaine (0.5 mg/kg) are depicted for non-escalated (closed bars) and escalated (open bars) animals (mean + s.e.m.) given ShA (left) or LgA (right). Cocaine-induced dopamine release in the VMS was significantly decreased in rats given LgA compared to ShA, but release did not differ significantly between non-escalated and escalated rats ($P>0.05$). **b**) Phasic dopamine in the VMS of non-escalated animals (n = 6/16) in the third week of LgA was not different to that of non-escalated ShA rats (n = 10/16). Escalated ShA animals (n = 6/16) displayed a nonsignificant trend for decreased VMS dopamine compared to non-escalating ShA rats (n = 10/16); #p = 0.094). Escalated LgA animals exhibited less dopamine release than escalated ShA animals. Data are mean + s.e.m., *$P<0.05$, **$P<0.01$.



Supplementary Figure 6. Intake pattern, but not access regimen, affects motivation to obtain cocaine. Subsequent to FI20 cocaine self-administration, a subset of ShA and LgA rats (n = 32) underwent progressive-ratio testing. Progressive-ratio sessions were identical to FI20 sessions except that animals were required to perform an increasing number of operant responses for successive infusions of cocaine. The break point was operationally defined as the maximum number of responses. Average break point values are depicted (mean + s.e.m.). a, Escalated rats (purple bar; ShA and LgA pooled) displayed significantly more responses (and earned more infusions) than non-escalated animals (orange bar; ShA and LgA). b, Access regimen (ShA in orange and LgA in purple) had no significant effect on the number of responses rats performed to receive an infusion of cocaine ($P>0.05$). *$P<0.05$.

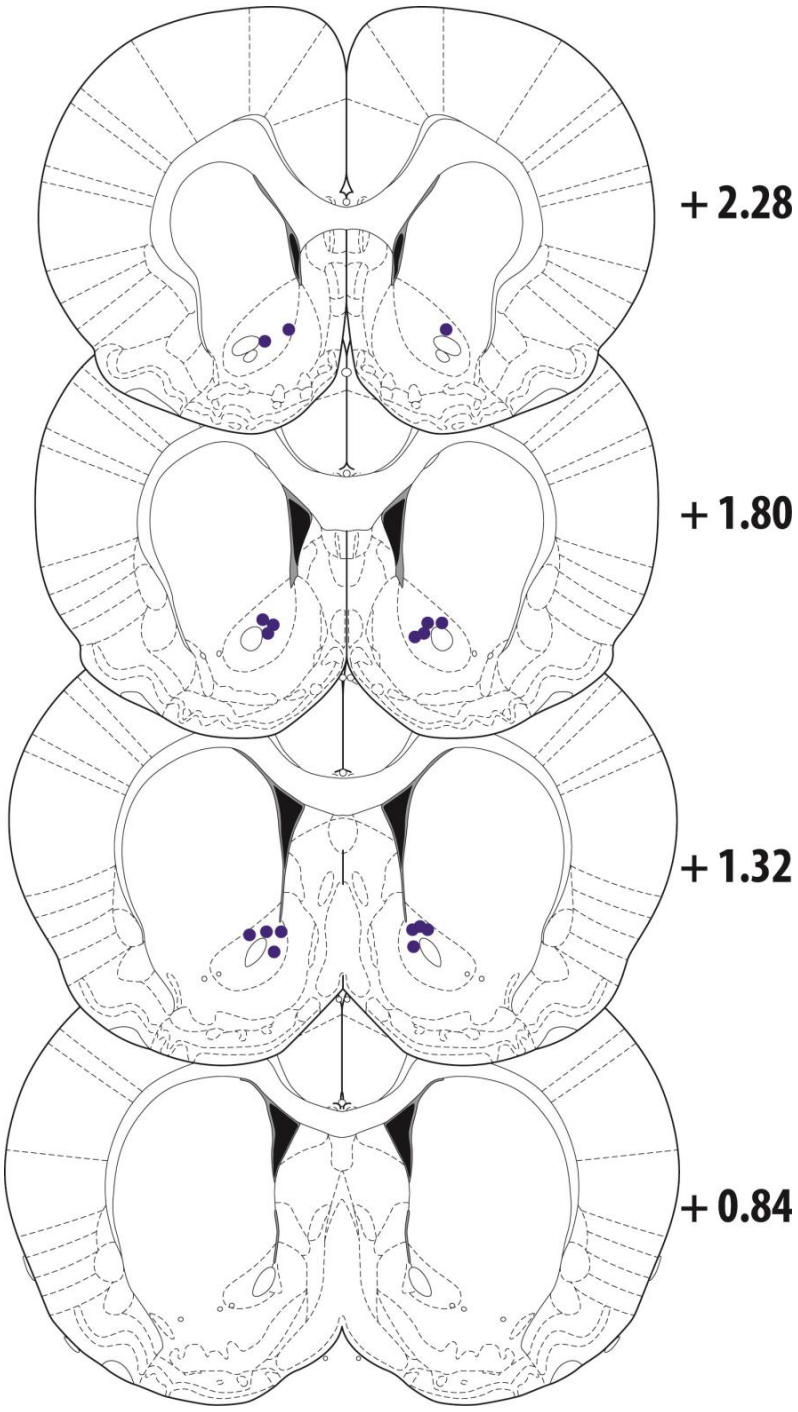


Supplementary Figure 7. Histological verification of recording sites in VMS (2nd experiment).
VMS recording sites (blue circles) were confirmed to be within the nucleus accumbens core. The numbers on each plate indicate distance in millimeters anterior from bregma[109].

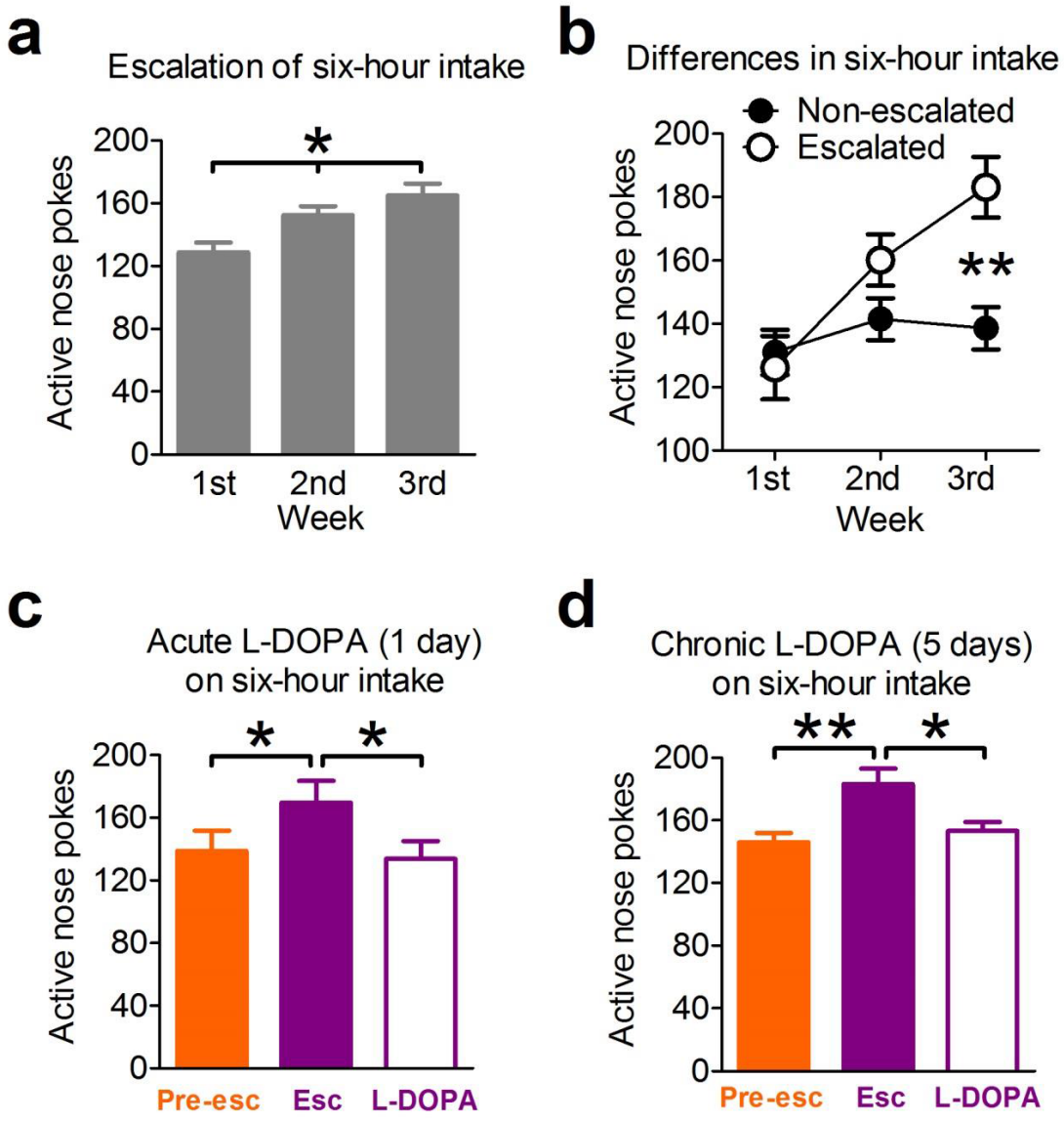


Supplementary 8. Slow changes in dopamine release in the VMS following a cocaine infusion induced by an active nose-poke response.

Increases in peak concentration of extracellular dopamine in the VMS measured during LgA cocaine self-administration sessions. Measurements were conducted over 90 seconds following an infusion of cocaine induced by a nose poke into the active hole (that occurred without additional operant responses within 90 seconds following this infusion) prior to escalation (pre-esc), after escalation (esc), and after escalation with L-DOPA treatment (L-DOPA). Average changes in such "tonic" dopamine concentration did not differ significantly from each other (ns, not significant; P >0.05). Data are mean + s.e.m.



Supplementary Figure 9. Histological verification of infusion sites in VMS (3rd experiment).
VMS infusion sites (blue circles) were confirmed to be within the nucleus accumbens core. The numbers on each plate indicate distance in millimeters anterior from bregma[109].



Supplementary Figure 10. Escalation of cocaine intake and effects of L-DOPA during LgA over six hours of cocaine self-administration.

a) LgA animals showed a significantly increasing number of active nose pokes during six hours of cocaine self-administration across weeks (n = 24). **b**) Non-escalated animals (n = 10) showed no significant increase in cocaine intake during six hours of self-administration over the course of LgA (closed circles), whereas escalated rats (n = 14) increased their intake significantly (open circles). **c**) A single i.v. injection of L-DOPA (30 mg/kg) and Benserazide (2 mg/kg) prior to session start decreased the escalated number of active nose poke responses (purple bar) to a number (open purple bar) comparable to the pre-escalation stage (orange bar; n = 5). d, Repeated i.v. administration of L-DOPA (30 mg/kg) and Benserazide (2 mg/kg) on five consecutive days reliably reduced the escalated number of active nose poke responses (purple bar) and maintain the responses at a rate (open purple bar) comparable to the pre-escalation stage (orange bar; n = 5). Data are mean + s.e.m. *P<0.05, **P<0.01.

CHAPTER 3

*Diametric Changes in Dopamine Release During Drug-Taking and Drug-Seeking**

*This chapter (along with work presented in Chapter 4) is currently in preparation for submission as a manuscript by Lauren M Burgeno, Nicole L Murray, Ryan Farero, Ingo Willuhn, and Paul EM Phillips. I carried out all experiments with help from NLM and RF, designed experiments with help from IW and PEMP. I am writing the manuscript with help from PEMP.

Introduction

Though altered dopamine transmission is implicated in most contemporary theories of drug abuse, the timing, context, and directionality of these changes remain a matter of debate. In addition to mediating the reinforcing effects of drugs of abuse themselves[48-51], the mesolimbic dopamine pathway, projecting from the ventral tegmental area (VTA) to the nucleus accumbens core (NAcc), also signals learned associations between drugs (and natural reinforcers) and the environmental stimuli that predict them[69-71]. Following repeated pairing with drug, drug-associated conditioned stimuli (CS) gain the ability to evoke NAcc dopamine release when presented alone[69,72]. This enables CS to exert powerful control over behavior in both drug-taking and drug-seeking contexts[69,72]. Even when experienced in the absence of drug or following long periods of abstinence, CS alone can precipitate drug craving in humans[40,75-77], and promote drug seeking in rodents[23,24,44,78,79], both dopamine mediated phenomena[80-85]. Although it is clear that CS-elicited dopamine release is a key regulator of drug-taking and drug-seeking behaviors, it has yet to be determined whether these dopamine signals change over the course of drug taking once acquired, and if the duration of daily drug access impacts the dynamics of these signals over the course of drug taking. Allowing animals protracted access (long-access, 6hr/day) to drug each day is considered a more robust model of addiction as it yields changes in many of the behavioral phenomena that mirror human addiction criteria[5,33,124,144]. Many long-access (LgA) rats escalate their drug intake over time, as is seen in drug abuse patients, while rats only allowed short-access (ShA, 1hr/day)do not[33]. Those animals that escalate their intake have also been shown to exhibit enhanced motivation to obtain drug and an increased reinstatement of drug-seeking compared to ShA animals[161,167,168].

The development of the chronically implantable microelectrode within our lab has allowed for the measurement of CS-elicited phasic dopamine release in the NAcc of the same animal, at the same site over periods of weeks to months[89]. Our initial studies characterizing the dynamics of CS-elicited NAcc dopamine responses during an extended period of drug taking,

demonstrated a causal link between the progressive decreases in CS-elicited NAcc dopamine observed and the escalation of cocaine intake[72] (Chapter 2). These findings were consistent with the long standing theory that dopamine in the NAcc plays an important role in producing drug satiety and regulating drug intake[31,33,48,50,169]. However, at face value these data are at odds with another, equally well supported body of work that implicates CS-elicited NAcc dopamine in mediating drug-craving and promoting drug-seeking[80,87,168,170,171].

How might cue-elicited dopamine transmission both serve as a satiety signal and produce craving in the same brain region?

Drug associated CS serve different purposes in different situations. During active drug taking, CS confirm the success of drug seeking actions and indicate imminent drug delivery, signaling that drug seeking can be terminated. In contrast, the same CS, experienced non-contingently (unexpectedly) during a period of abstinence, signals the possibility of drug availability nearby and promotes initiation of drug seeking. **For CS-elicited NAcc dopamine to be able to both suppress drug-taking and promote drug-seeking, we hypothesized that there must be a divergence in CS-elicited dopamine release in these two differing contexts: a decrease in CS-elicited NAcc dopamine release over drug history when the CS is presented in a behavior contingent manner during drug-taking (as we previously characterized in Willuhn *et al*., 2014[72]), and an increase in CS-elicited NAcc dopamine release over drug history when the CS is presented non-contingently during a drug free period, as is used in cue-initiated drug-seeking paradigms.** Furthermore, as rats given protracted drug access exhibit robust drug-seeking behavior, we predicted that non-contingent CS-elicited dopamine responses would increase following protracted drug access.

We employed fast-scan cyclic voltammetry (FSCV), an electrochemical detection method that allows for the measurement of subsecond changes in dopamine release, in the NAcc of awake behaving rats to measure non-contingent and behavior-contingent CS-elicited dopamine responses throughout the course of weeks of cocaine self-administration. We found that following protracted daily access to drug, the directionality of the changes in cue-elicited signals

obtained in drug-taking and drug-seeking contexts oppose one another in animals that escalate their drug intake. Thus, NAcc dopamine signals elicited by the same cues in drug-taking and drug-seeking contexts may contribute to different, but equally important, core symptoms of substance use disorders

Methods

Subjects

A total of 175 adult male Wistar rats (Charles River), weighing 300-400g upon arrival, were housed individually and kept on a 12-h light/12-h dark cycle (lights on at 0700) with controlled temperature and humidity, and food and water available *ad libitum*. All animal use was approved by the University of Washington Institutional Animal Care and Use Committee, and surgical procedures were performed under aseptic conditions. Thirty rats completed the study in the short-access cohort, and 55 completed the study in the long-access cohort. Approximately 20% of the attrition was a result of headcap loss or catheter failure during the surgery recovery period. The remainder of subjects dropped out due to headcap loss, catheter failure, or failure to acquire self-administration after training began.

Electrode & Catheter Implantation

Chronically implantable carbon fiber microelectrodes were constructed as described in Clark et al., 2009[89] and implanted bilaterally via standard stereotaxic procedure into the ventral striatum (nucleus accumbens core, AP: +1.3mm, ML: +1.3mm, DV: -7.2mm). Following two weeks of recovery, animals were outfitted with indwelling intravenous catheters and allowed to recover for an additional two weeks prior beginning behavioral training and testing. Catheters were flushed with saline daily, and heparin as needed, to maintain catheter patency before and throughout experimentation.

Self-Administration Training

Rats were trained to self-administer cocaine during daily one hour (short-access, ShA) sessions in an operant chamber outfitted with a liquid swivel and containing two nose-poke ports **(Figure 1a)**. During self-administration sessions, the illumination of a house light paired with white noise signaled the availability of drug. A nose poke into the active port elicited a 0.5 mg/kg cocaine infusion (fixed-ratio one schedule), accompanied by a 20-second presentation of an audiovisual

stimulus (nose-poke light + tone= conditioned stimulus (CS, see schematic **Figure 1b**)), during which additional nose pokes were without consequence (time out). Nose pokes into the inactive port at any time were without consequence.

Following acquisition of the self-administration task (acquisition criterion= three sequential sessions earning 10 or more infusions) animals received daily one hour sessions for five more days to establish baseline intake and were then divided into two drug access groups, each receiving the same number of sessions but with sessions differing with respect to the number of hours the animals had access to self-administer cocaine. The short-access (ShA) cohort continued with one hour sessions daily for 10 more days, while the long access (LgA) group received daily six hour sessions for 10 days. A subset of LgA animals continued six hour access for and additional five sessions (mirroring the duration of LgA used in previous studies[72]). Using our previously validated method for separating escalators and non-escalators (further detailed in Willuhn *et al*., 2014, figure 3 & methods[72]), a linear regression analysis of each animal's first hour drug intake over sessions (beginning the day after acquisition) was used to determine whether the relationship between session and drug intake had a significant, non-zero, positive slope. Rats having a significant, positive non-zero slope were considered escalators.

*In Vivo Recordings: Fast-Scan Cyclic Voltammetry (FSCV)**

* A brief description of FSCV follows, however I also contributed to a book chapter which includes a more detailed review of theory and implementation of FSCV (Arnold, Burgeno, & Phillips, 2015[90]).

Behaviorally relevant stimuli elicit rapid changes in dopaminergic neuron firing, resulting in transient changes in dopamine release over the course of seconds, and requiring the use of a detection method with high temporal resolution. In the studies presented throughout this thesis , phasic dopamine release events were measured at carbon fiber microelectrodes in the ventral striatum using fast-scan cyclic voltammetry (FSCV)[89,90]. Briefly, chronically implanted carbon-fiber microelectrodes[89] were connected to a head-mounted voltammetric amplifier, interfaced with a PC- driven data-acquisition and analysis system (National Instruments, TX, USA) through a

commutator (Med Associates, VT, USA) that was mounted above the test chamber. A potential was applied to the electrode as a triangular waveform such that it was linearly ramped from the initial holding potential (-0.4 V vs Ag/AgCl) to a maximum voltage (1.3 V vs Ag/AgCl, anodic sweep), then returned to the holding potential (cathodic sweep). Each voltage scan lasted 8.5 ms, yielding a scan rate of 400 V/s. The holding potential was maintained between voltage scans. Scans were applied every 100 ms (10 Hz sampling). When dopamine was present at the surface of the electrode, it was oxidized during the anodic sweep to form dopamine-*o*-quinone (peak reaction detected at approximately +0.7 V), which was reduced back to dopamine in the cathodic sweep (peak reaction detected at approximately −0.3 V). The ensuing flux of electrons was measured as current and was directly proportional to the number of molecules that underwent electrolysis. Voltammetric data was band-pass filtered at 0.025–2,000 Hz. The background-subtracted, time-resolved current obtained from each scan provided a chemical signature characteristic of the analyte, allowing resolution of dopamine from other substances[146]. Dopamine was isolated from the voltammetric signal by chemometric analysis using a standard training set[89] based on electrically stimulated dopamine release detected by chronically implanted electrodes. Dopamine concentration was estimated on the basis of the average post-implantation sensitivity of electrodes[89]. Before analysis of average concentration, all data were smoothed with a five-point within-trial running average. The concentration of dopamine was averaged over seven seconds (approximate duration of the observed phasic signal) following the non-contingent presentation of the CS or operant response to obtain drug. Data collected on recording days was included if there was a detectable dopamine response at any point within the session. In cases where electrical noise exceeded 0.2 nA, animals became disconnected from the drug delivery tubing, or when tethering for recordings altered the animal's regular behavior patterns (some animals won't move when tethered), data from that session was excluded. In cases where more than one electrode was functioning in an animal during a given session, the average of the signals obtained from both electrodes was used for analysis.

Non-Contingent CS Probe Sessions

Both ShA and LgA animals received time-matched recorded non-contingent probe sessions during their first (ShA Week 1 for both) and third (ShA Week 3 and LgA Week 3 respectively) weeks of training received (see schematics in **Figures 1, 2**). On test days' rats underwent continuous FSCV recording during a non-contingent CS probe session and the self-administration session which immediately followed. Rather than beginning self-administration immediately, they received two unexpected non-contingent presentations of the CS that was usually paired with drug during self-administration, three minutes apart (see **Figure 2** for session schematic). For each animal, the two CS-elicited dopamine responses were averaged for each session. During probe sessions, clear tape was placed over the nose poke holes to allow animals to experience the cues, but prevent extinction of responding for drug. The tape was removed just prior to the self-administration session beginning. FSCV recordings of the self-administration sessions following CS probe sessions allowed us to compare dopamine responses elicited by behavior-contingent versus non-contingent CS.

Non-Contingent CS probe sessions were first carried out on Day 0 (in rats that were drug and CS naïve) to assess whether any response to the intrinsic properties of the CS and/or novelty existed. The subset of rats that continued with an extra week of LgA self-administration also received an additional recording after LgA Week 4.

Histological Verification of Recording Sites

On completion of experimentation, animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg). Electrode recording sites were marked with an electrolytic lesion (300 V) before transcardial perfusion with saline followed by 4% paraformaldehyde. Brains were removed and postfixed in paraformaldehyde for 24 h and then rapidly frozen in an isopentane bath, sliced on a cryostat (50µm coronal sections, −25°C), and stained with cresyl violet to aid visualization of anatomical structures and the electrode-induced lesion. See Histology appendix for all histological placements.

Results & Discussion

To verify that CS-elicited NAcc dopamine responses observed during our studies were due to the learned association with drug and not intrinsic features of the CS itself we first measured non-contingent CS evoked dopamine release in rats prior to training, when they were both drug and CS naïve, and after one week of short-access (ShA) self-administration training (**Figure 1c**). As was previously shown[69], CS-elicited dopamine responses significantly increased following training **(Figure 1d**, $p < 0.01$**),** indicating that CS-elicit NAcc dopamine responses only after learned association with drug.

Non-Contingent CS-elicited NAcc dopamine release increases following protracted drug access

To test the prediction that NAcc dopamine signals elicited by non-contingent CS presentations become larger over the course of self-administration history, both in terms of the number of days spent in self-administration and daily duration of access, we measured non-contingent CS-elicited phasic dopamine release at multiple time points during drug taking in two groups, a ShA cohort that received one hour sessions daily for three weeks, and a long-access (LgA) cohort that received one week of ShA and was then switched to daily six hour sessions for two additional weeks **(Figure 2a)**. At the end of weeks one and three of self-administration, we recorded phasic dopamine release throughout a non-contingent CS probe session, in which CS were presented unexpectedly (two trials), and the self-administration session that immediately followed in both ShA and LgA Cohorts **(Figure 2a)**. During non-contingent CS probe sessions animals were in a drug free state, as these sessions occurred well beyond the point that the previous day's cocaine had been eliminated from the animal's system. It should be noted that at the week one time point ShA and LgA cohorts had equivalent drug history, as the LgA cohort had yet to switch to LgA sessions. There was a three-fold increase in non-contingent CS-elicited phasic dopamine release in the LgA cohort **(Figures 2c and 2d,** week three versus week one: $p < 0.01$**),** but no change in the ShA cohort **(Figure 2b and 2d,** $p > 0.05$**)**.

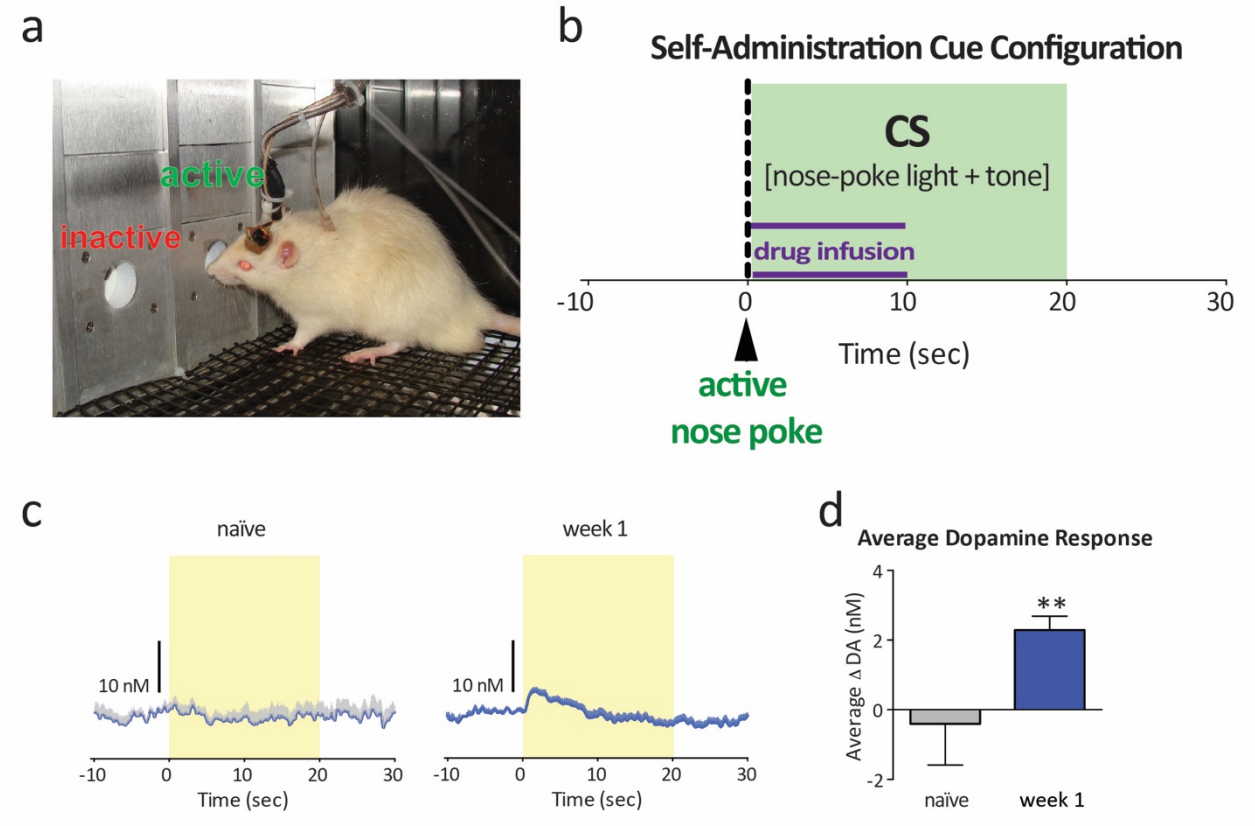


Figure 1. Non-contingent CS evoke NAcc dopamine release following self-administration training

a) Rats were trained to self-administer cocaine during daily short-access (ShA, one hr/day) sessions. **b)** During self-administration sessions, a nose poke into the active port (marked by triangle and dotted line) elicited a cocaine infusion (FR1 schedule, 0.5 mg/kg/infusion) accompanied by a 20-second presentation of an audiovisual stimulus (nose-poke light + tone = conditioned stimulus (CS)/ delivery cue, duration marked by green box), during which additional nose pokes were without consequence (time out). **c)** We used fast-scan cyclic voltammetry (FSCV) to measure NAcc phasic dopamine responses to non-contingent (unexpected) presentations of this same CS in rats prior to training, when they were drug/CS naïve (left), and after one week ShA training (right). FSCV traces are mean + s.e.m. of the background subtracted response (background taken two seconds before CS onset). Non-contingent (unexpected) CS presentation duration is marked by the yellow shaded area. **d)** There was a significant increase in phasic dopamine release elicited by CS following training. Average change in dopamine was calculated as mean + s.e.m. of the seven second window following CS-onset. Statistics: t-test: naïve versus week one, $p < 0.01$.

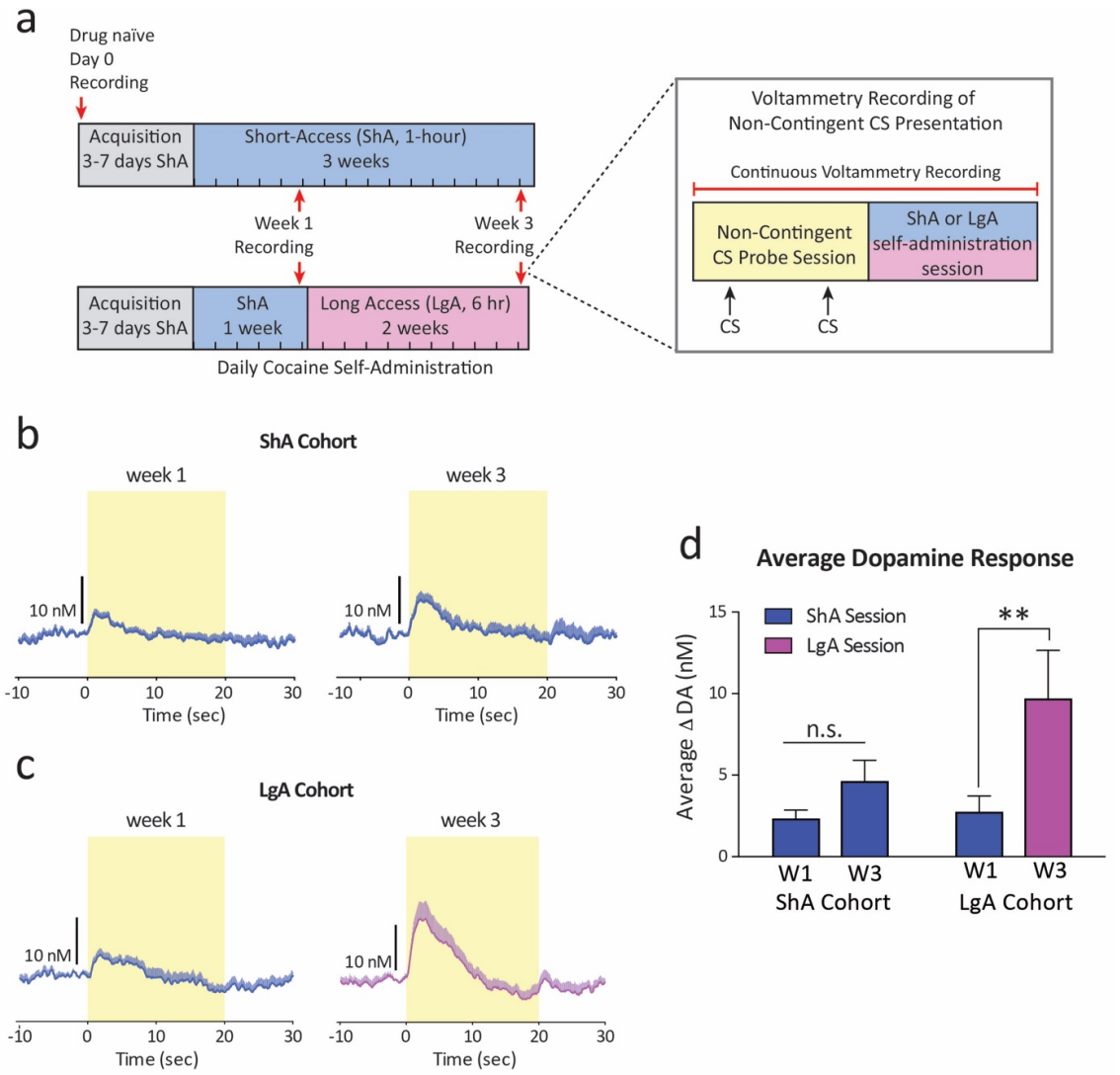


Figure 2. Non-contingent CS evoked NAcc dopamine release increases following LgA

a) Experimental design schematic: Rats were trained to self-administer cocaine in daily short-access (ShA, one hr/day) cocaine self-administration sessions. Following acquisition (three subsequent days earning >10 infusions) they were given another week of ShA to ascertain baseline intake then either remained in ShA for two additional weeks (ShA Cohort) or switched to long-access (LgA, six hr/day) for two additional weeks (LgA Cohort). Red arrows denote days of FSCV recorded non-contingent CS probe sessions. On recording days, the self-administration session was preceded by a non-contingent CS probe session in which rats received 2X unexpected CS presentations (illustrated in box). Average CS evoked FSCV dopamine responses obtained after weeks one and three from the **b)** ShA cohort and **c)** LgA cohort. **Note:** In ShA and LgA cohorts, week one is equivalent as each have only had one week of ShA. Traces and bars illustrating data from ShA sessions are blue and LgA sessions magenta regardless of which cohort the data came from. Area shaded in yellow marks the duration of the non-contingent CS presentation. FSCV traces are mean + s.e.m. of background subtracted response. **d)** There was a three-fold increase in non-contingent CS-elicited dopamine release following LgA but not ShA. Graph illustrates the comparison of average CS-elicited dopamine responses (mean +s.e.m. of seven second window following CS-onset) from time-matched sessions in ShA and LgA cohorts (week one and week three). Statistics: There was a significant main effect of week on CS-elicited phasic dopamine release (Two-way ANOVA, $F_{(1,47)}=6.591$, $p < 0.05$). Holm-Sidak post-hoc analysis reveals a significant difference between weeks one and three in the LgA cohort ($p < 0.01$), but not the ShA cohort ($p > 0.05$).

Non-contingent CS evoked dopamine responses increase in rats that escalate their drug intake

Though as a population LgA rats exhibited a significant escalation of drug intake over time (**Figure 3a**, One-Way ANOVA, $F_{(3,211)}=3.422$, $p < 0.05$), there were individual differences in the extent of escalation between rats. As we have previously validated[72], we used linear regression analysis of each animal's first-hour drug intake over self-administration sessions to separate rats who escalate their drug-intake from those that do not (**Figures 3b and 3c)**.
In this previous work[72] the most robust changes in dopamine release were observed after three weeks of LgA, therefore, we allowed a subset of rats in the current studies to undergo a fourth week of self-administration (one week ShA followed by three weeks LgA).

Following separation of escalators and non-escalators, rats assigned to the non-escalator group are those that maintained stable drug-intake, whereas escalators significantly increased their first-hour intake over the course of weeks of LgA (**Figure 3d** , Two-Way ANOVA, main effect of week: $F_{(3,207)}=5.76$, $p < 0.001$; escalation group x week interaction: $F_{(3,207)}=7.92$, $p < 0.0001$). Post-hoc analysis reveals significantly greater first hour drug-intake at weeks two, three and four relative to week one in escalators (Holm-Sidak: $p < 0.05$, $p < 0.001$, $p < 0.001$, respectively), but not non-escalators ($p > 0.05$ for all).

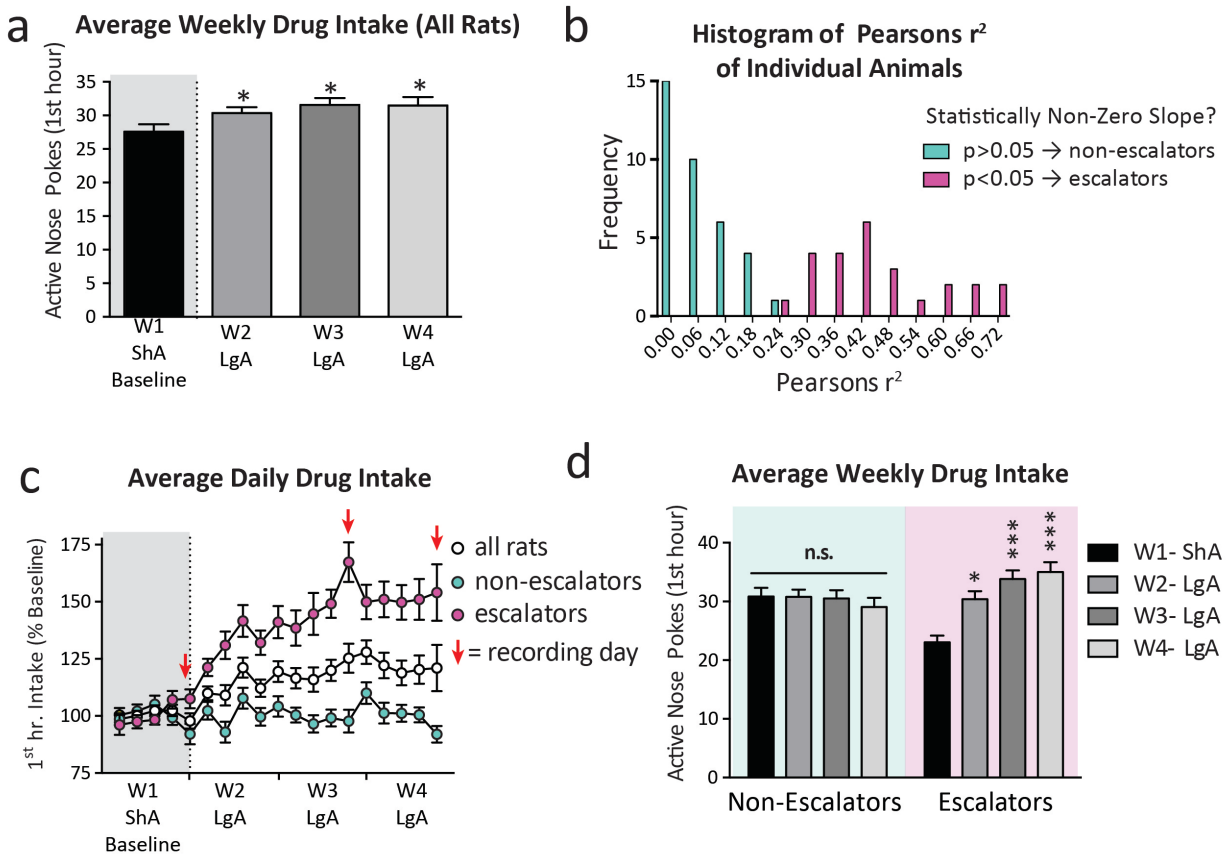


Figure 3. Long-Access Self-Administration Behavior: Separation of Escalators and Non-Escalators

a) Average weekly drug intake (mean + s.e.m. active nose pokes earn 0.5mg/kg cocaine injection) of all LgA subjects. Statistics: There was a significant effect of week on drug-intake (One-Way ANOVA, $F_{(3,211)}=3.422$, $p < 0.05$), and drug intake was significantly increased from ShA W1 baseline following LgA W2, LgA W3, and LgA W4 (Holm-Sidak post-hoc, $p < 0.05$ for all comparisons). A linear regression of active nose pokes over sessions was performed for each rat and those with significant positive slope (escalators) were separated from those with non-significant slope (non-escalators, as in Willuhn et al., 2014[72]). **b)** Histogram of the r^2 values of individual animals obtained from linear regression of drug intake over time in escalators (magenta) and non-escalators (cyan). **c)** Plot of average daily drug intake over time for all rats before separation into escalation groups (white circles), non-escalators (cyan circles), and escalators (magenta circles). Data are expressed as mean ± s.e.m. percent change from baseline (average intake in ShA W1 baseline). Red arrows mark the days during which voltammetry recordings of non-contingent and behavior-contingent CS occurred. **d)** Average weekly drug intake (mean + s.e.m.) in non-escalators and escalators. Statistics: There was a significant main effect of time (Two-Way ANOVA, $F_{(3,207)}=5.76$, $p < 0.001$) and a significant time x escalation group interaction ($F_{(3,207)}=7.92$, $p < 0.0001$). Post-hoc analysis revealed significant increases in drug intake (relative to ShA W1 baseline) in escalators at LgAW2, LgA W3, and LgAW4 (Holm-Sidak, $p < 0.05$, $p < 0.0001$, $p < 0.001$ respectively, but no difference in drug intake in the non-escalator group. *Asterisks denote significant difference relative to ShAW1 (black bar) within the same escalation group (* $p < 0.05$, ***$p < 0.001$).

Reanalysis of the non-contingent CS-elicited dopamine responses from LgA animals, taking individual differences in escalation into account (**Figures 4a and 4c**), revealed a five-fold increase in non-contingent CS-elicited phasic dopamine release in escalators but not non-escalators (**Figure 4c**, Two-way ANOVA, main effect of week: $F_{(1,25)}=7.566$, $p < 0.05$, post-hoc Holm-Sidak week 1 versus 3 in escalators $p < 0.05$, non-escalators $p > 0.05$) . In contrast, analysis of the phasic dopamine responses evoked by the behavior-contingent CS from the drug-taking period **(Figure 4b)** reflected a significant decrease dopamine release over weeks in escalators but not non-escalators **(Figure 4d**, Two Way ANOVA: main effect of week: $F_{(1,28)}=7.098$, $p < 0.05$, post-hoc Holm-Sidak week one versus three in escalators $p < 0.05$, non-escalators $p > 0.05$). Though the recording windows are slightly shifted between this study and our previous work (This Study: week one= ShA, weeks two, three, and four = LgA, Willuhn, *et al*. 2014: weeks one, two, and three= LgA) we replicate the same basic finding, observing decreases in behavior-contingent CS-elicited dopamine in the NAcc following LgA in escalators but not non-escalators.

Despite being underpowered to detect changes in non-contingent CS-elicited dopamine release at week four in the subset of animals given the extra week of LgA, we maintained the same basic findings when we analyzed responses across all four weeks of LgA **(Supplementary Figure 1)**, observing significant increases in non-contingent CS-elicited NAcc dopamine at weeks three and four compared to week one in escalators ($p < 0.01$, $p < 0.05$ respectively) but not non-escalators ($p > 0.05$ for all comparisons, see figure legend for detailed stats). Furthermore, analysis of behavior-contingent CS-elicited dopamine responses across all four weeks also produced results consistent with data from both this study and that which we previously published[72] **(Supplementary Figure 2)**. Behavior-contingent CS-elicited dopamine release decreased over weeks selectively in rats that escalate their drug intake (Two-Way ANOVA: significant main effect of week $F_{(2,41)}=9.23$, $p < 0.001$; significant escalation group x week interaction $F_{(2,41)}=4.36$, $p < 0.05$. Post-hoc Holm-Sidak: Escalators-week one versus week three, $p < 0.01$; Escalators-week one versus week four, $p < 0.0001$).

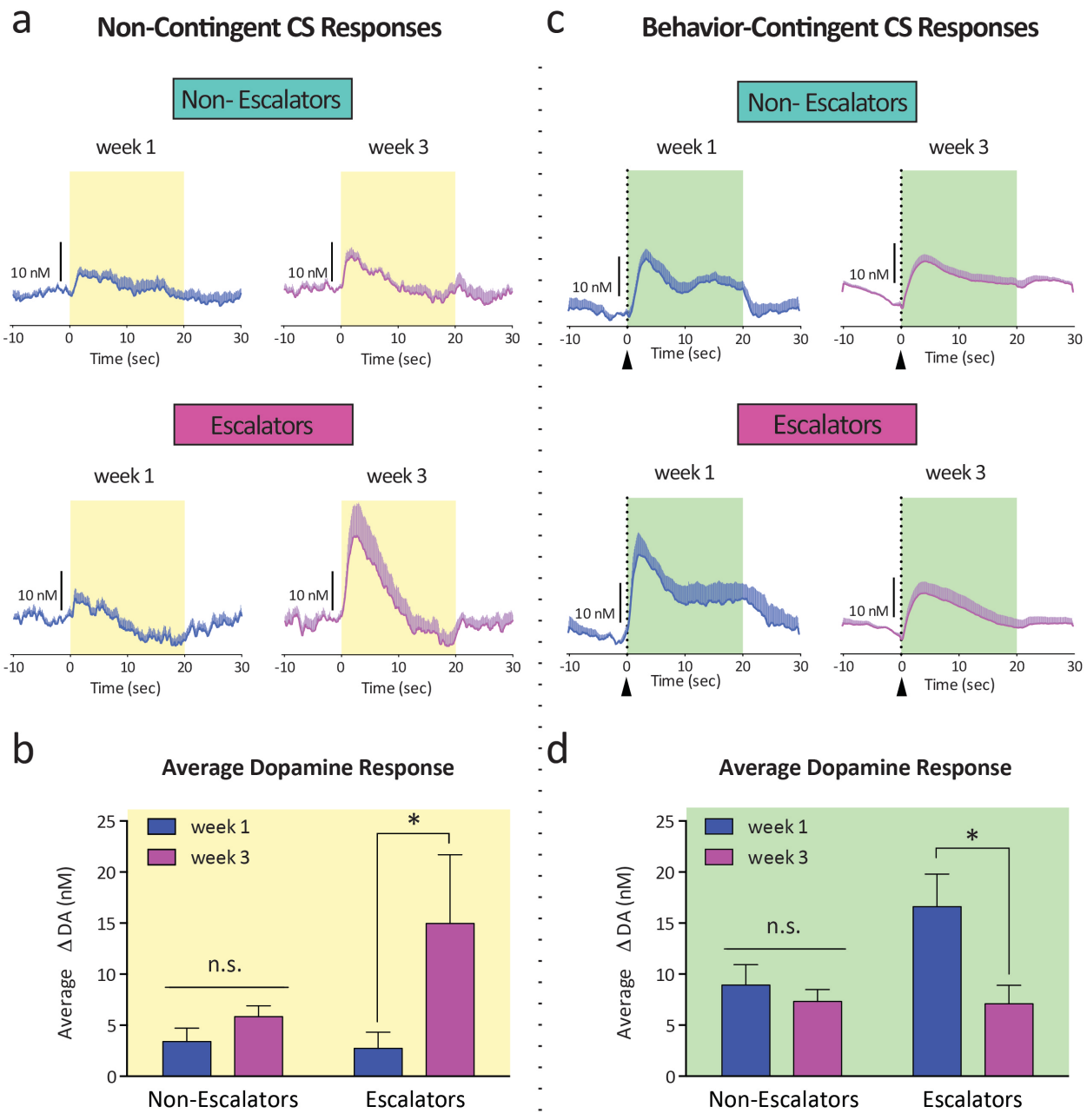


Figure 4. CS-elicited dopamine responses are dynamic in escalators for both CS presentation contingencies
Rats were separated into groups based on whether their drug intake escalated over time or not, as previously validated in Willuhn *et al.* 2014[72]. LgA data was then reanalyzed to assess differences in both non-contingent and behavior-contingent CS evoked dopamine release between the two escalation groups. **a)** Average CS evoked FSCV dopamine responses obtained at weeks one and three in non-escalators (top) and escalators (bottom). Area shaded in yellow marks the duration of the non-contingent CS presentation. FSCV traces are mean + s.e.m. **b)** Average CS evoked FSCV dopamine responses elicited by behavior-contingent CS during self-administration. Weeks one and three in non-escalators (top) and escalators (bottom). Area shaded in green marks the duration of the non-contingent CS presentation. FSCV traces are mean + s.e.m. of background (continued on next page)

subtracted response. **c)** Average non-contingent CS-elicited dopamine responses increase five-fold following LgA in animals that escalate their drug intake (mean + s.e.m., seven second window following CS-onset). Statistics: Two-Way ANOVA, main effect of week: $F_{(1,25)}=7.57$, $p < 0.05$.; non-significant trend towards escalation group x week interaction $F_{(1,25)}=3.38$, $p = 0.08$; Post-hoc Holm-Sidak reveals a significant increase in between weeks one and three in escalators ($p < 0.05$). **d)** As in Willuhn *et al.*,2014[72], average behavior-contingent CS-elicited dopamine responses decrease following LgA in animals that escalate their drug intake (mean + s.e.m., seven second window following CS-onset). Statistics: Two-Way ANOVA, main effect of week: $F_{(1,28)}=7.10$, $p < 0.05$; non-significant trend towards an escalation group x week interaction, $F_{(1,28)}=3.60$, $p = 0.07$; Post-hoc Holm-Sidak reveals a significant decrease between weeks one and three in escalators ($p < 0.05$).

Diametric changes in CS-elicited NAcc dopamine in drug-taking and drug-seeking contexts

Results from our studies of NAcc phasic dopamine release elicited by both non-contingent and behavior-contingent CS over the course of LgA together reveal robust changes in dopamine release following LgA in animals that escalate their drug intake. Direct comparison between the trajectories of phasic dopamine responses elicited by non-contingent and behavior-contingent CS in escalators support our hypothesis that diametric changes in dopamine release are elicited by CS when they are experienced in drug-taking and drug-seeking contexts **(Figure** 5, Two Way ANOVA: significant CS-contingency x week interaction $F_{(1,24)}=11.35$, $p < 0.01$). Analysis across all four weeks of LgA produced the same result **(Supplementary Figure 3)**. Furthermore, this result also holds up when non-escalators are included (data not shown, Two Way ANOVA: significant CS-contingency x week interaction $F_{(1,57)}=11.24$, $p < 0.01$).

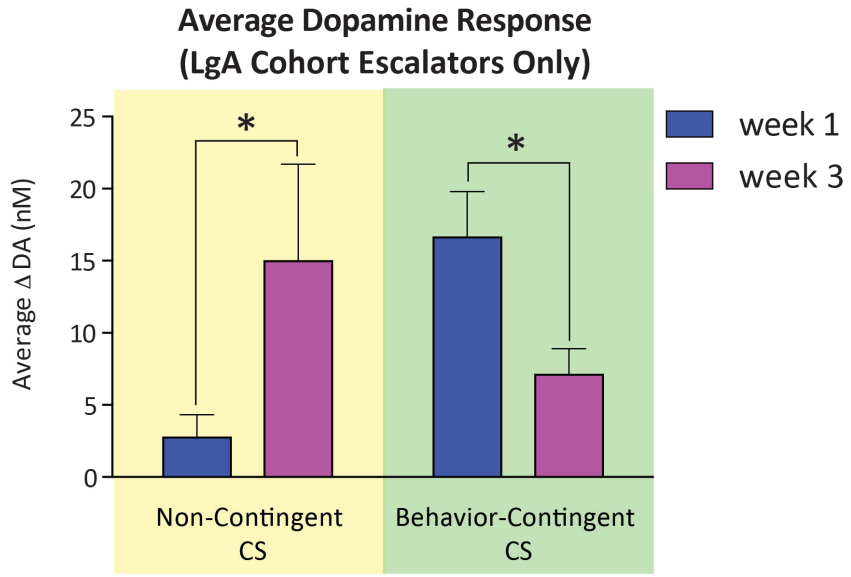


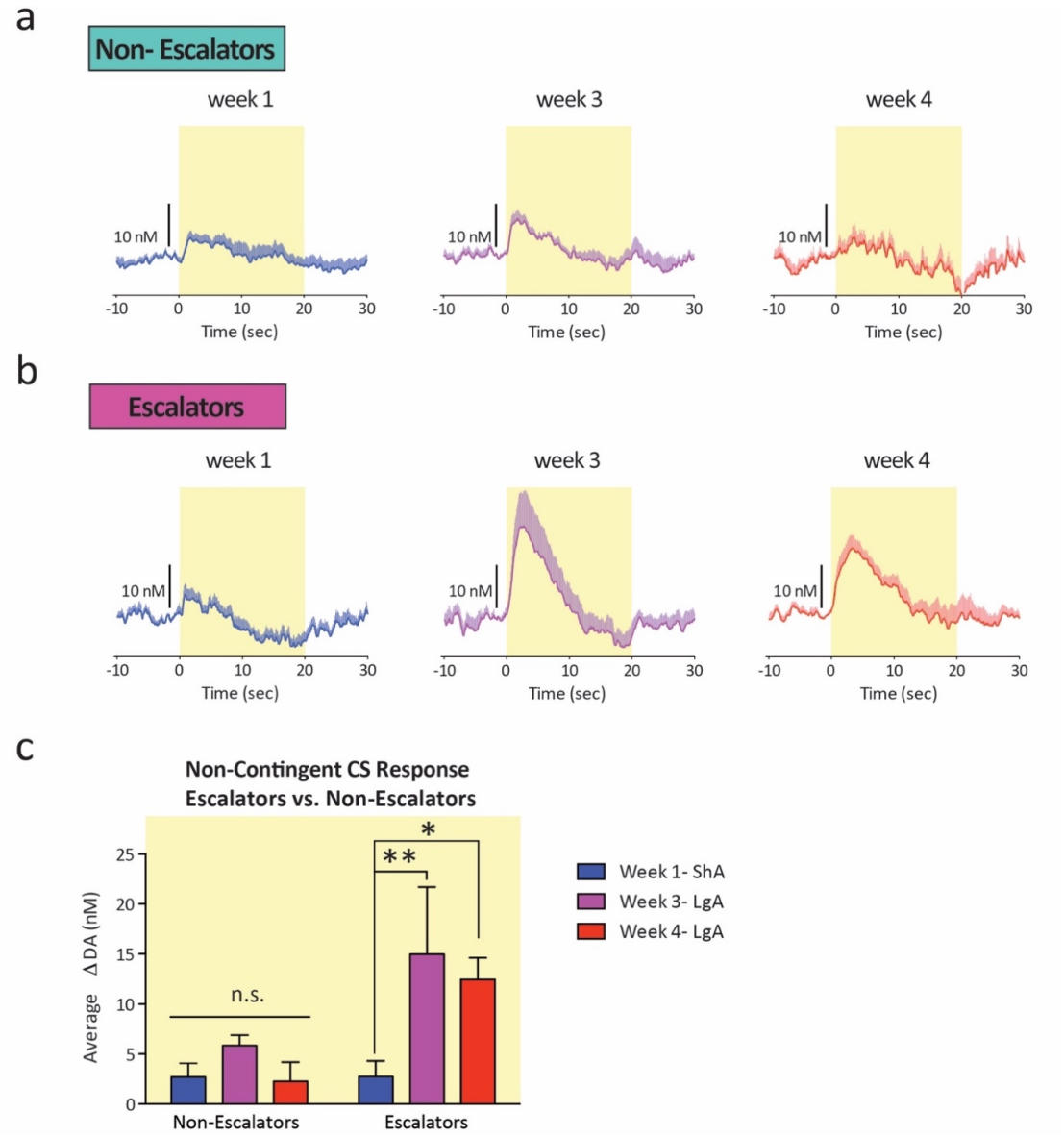
Figure 5. Divergence of NAcc CS-elicited phasic dopamine release over weeks is mediated by CS presentation contingency
Non-contingent and behavior-contingent presentations of the same drug-cue (CS) produce opposing changes in NAcc dopamine following LgA in escalators. Statistics: Two-Way ANOVA reveals a significant CS-contingency x week interaction, $F_{(1,24)}=11.35$, $p < 0.01$. Post-hoc Holm Sidak reveals, as was shown in Figure 3, a significant increase in non-contingent CS-elicited dopamine ($p < 0.05$) and a significant decrease in behavior-contingent CS-elicited dopamine following LgA ($p < 0.05$).

Conclusions

These studies investigated the dynamics of CS-elicited phasic dopamine release in the NAcc in both drug-taking and drug-seeking contexts. We found that when CS are experienced non-contingently, in the absence of drug, they elicit larger signals following protracted drug access. This effect was only observed in rats that escalate their drug intake. In contrast, the trajectory of the dopamine responses elicited by behavior-contingent CS in escalators decreased over the same time period. Whereas CS-elicited dopamine responses in both contexts were dynamic over long-access in escalators, these responses did not change in non-escalators in either case.

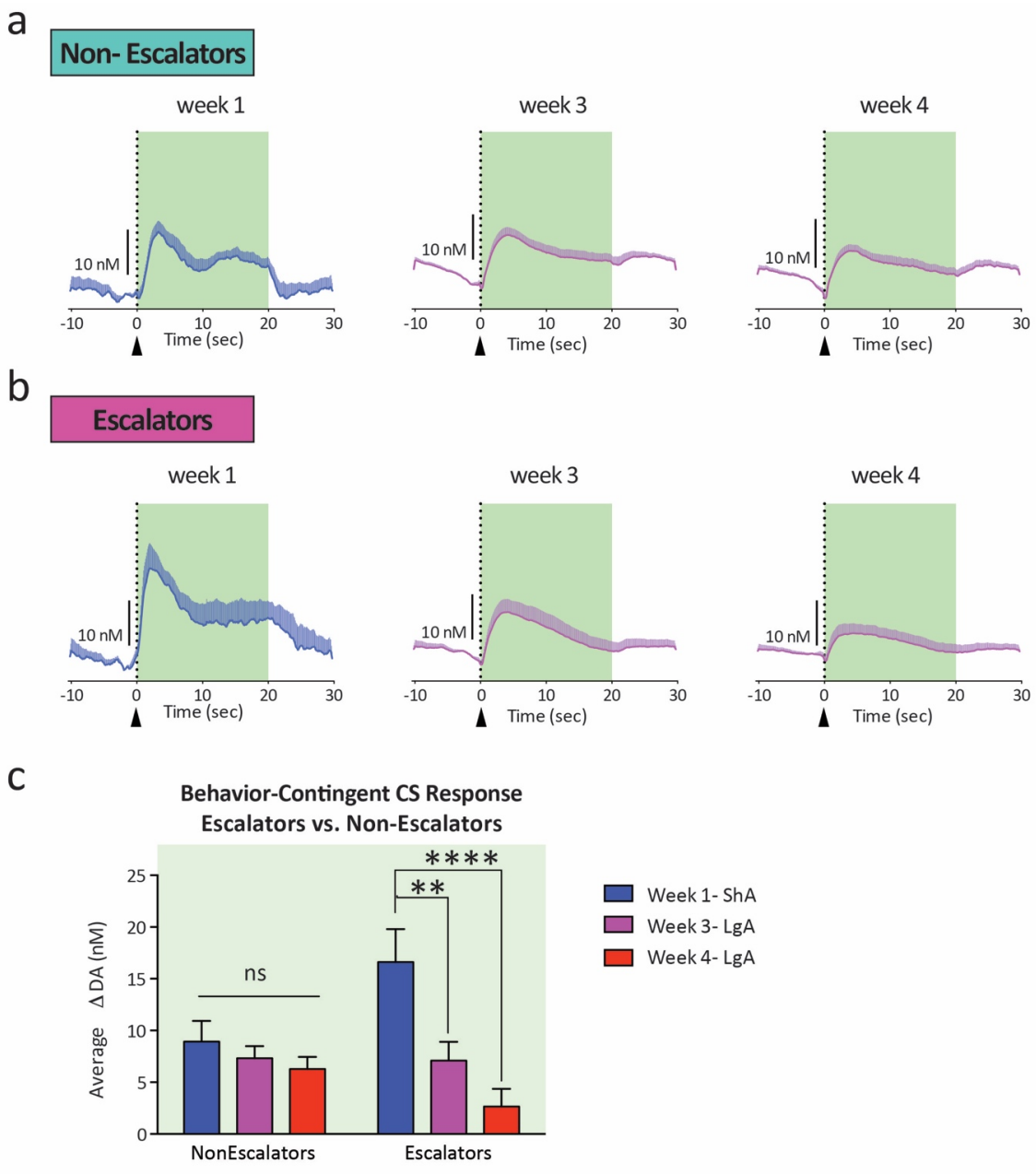
Overall, these studies have helped reconcile competing theories of dopamine's role in addiction by demonstrating that NAcc dopamine is bidirectionally regulated by the same CS during different phases of the drug abuse cycle. Furthermore, these data support the hypothesis that, in drug-taking and drug-seeking contexts, diametric changes in CS-elicited phasic dopamine release contribute to different, but equally important core symptoms of substance use disorders[5]. During drug taking, when CS presentation is predicted, CS-elicited dopamine responses signal the success of drug seeking actions, and suppress further responding for drug. These signals decrease over time in a subset of animals, and this lack of a confirmatory response, signaling the success of drug seeking action, leads to an escalation of drug intake[72]. In this same subset of animals, unexpected CS presentation elicits larger phasic dopamine responses at the same time points, promoting drug seeking during drug free periods[80,87,171].

Supplementary Figures



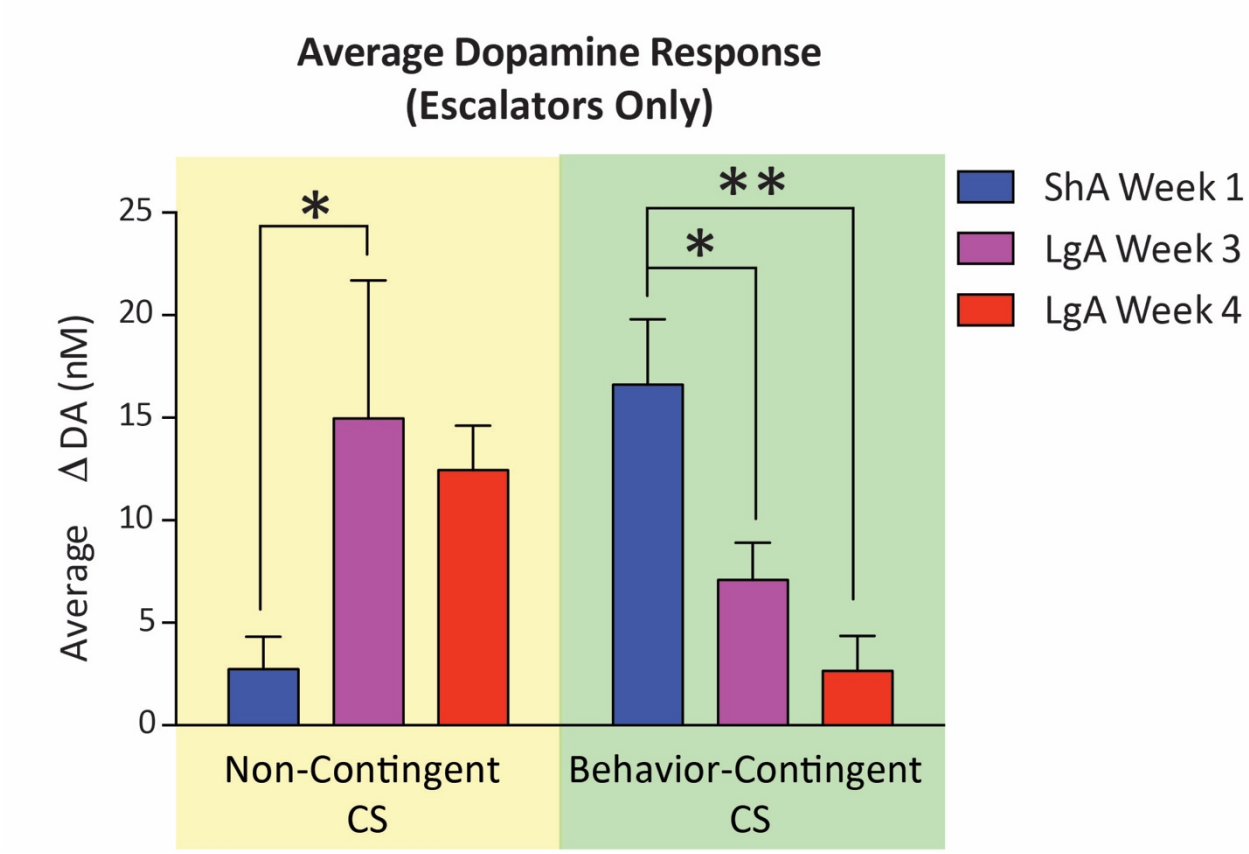
Supplementary Figure 1. Non-contingent CS-elicited dopamine increases in escalators but not non-escalators

Average CS evoked FSCV dopamine responses obtained at weeks one, three and four in **a)** non-escalators and **b)** escalators. Area shaded in yellow marks the duration of the non-contingent CS presentation. FSCV traces are mean + s.e.m. of background subtracted response. **c)** Average dopamine responses obtained at weeks one, three and four in non-escalators and escalators (mean + s.e.m., seven second window following CS-onset). Statistics: Two-way ANOVA: There was a significant main effect of escalation group ($F_{(1,33)}$=8.3, $p < 0.01$), and time ($F_{(2,33)}$=4.52, $p < 0.05$) on CS-elicited dopamine release, and a non-significant trend towards an escalation group x week interaction ($F_{(2,33)}$=2.94, $p = 0.07$). Post-hoc Holm Sidak revealed significant increases in dopamine at weeks three and four compared to week one in escalators ($p < 0.01$, $p < 0.05$ respectively) but not non-escalators ($p > 0.05$ for all comparisons).



Supplementary Figure 2. Behavior-contingent CS-elicited dopamine selectively decreases in escalators

a,b) Behavior-contingent CS-elicited dopamine responses in escalators and non-escalators during the first hour of self-administration. During active drug taking, a nose poke into the active port (indicated by triangle and dotted line), results in a single cocaine infusion along with a 20 second presentation of the CS (behavior-contingent CS-duration marked by green box). **c)** Average behavior-contingent CS-elicited dopamine responses decrease following LgA selectively in animals that escalate their drug intake (mean + s.e.m., seven second window following CS-onset). Statistics: Two-Way ANOVA: significant main effect of week $F_{(2,41)}=9.23$, $p < 0.001$; significant escalation group x week interaction $F_{(2,41)}=4.36$, $p < 0.05$. Post-hoc Holm-Sidak: Escalators-week one versus week three, $p < 0.01$; Escalators-week one versus week four, $p < 0.0001$.



Supplementary Figure 3. Divergence of NAcc CS-elicited phasic dopamine release over time is mediated by CS presentation contingency.

Non-contingent and behavior-contingent presentation of the same CS produce opposite changes in NAcc dopamine following LgA in escalators. Statistics: Two-Way ANOVA reveals a significant CS-contingency x week interaction, $F_{(2,34)}=10.10$, $p < 0.001$. Post-hoc Holm Sidak reveals, as was shown in Supplementary Figures 1-2, a significant increase in non-contingent CS-elicited dopamine at week three versus week one($p < 0.05$) and significant decreases in behavior-contingent CS-elicited dopamine following LgA weeks three and four, versus week one ($p < 0.05$ and $p < 0.01$, respectively).

CHAPTER 4

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*Incubation of Cue-elicited Dopamine Release Parallels Enhanced Drug Seeking and Craving During Abstinence**

*This chapter (along with work presented in Chapter 3) is currently in preparation for submission as a manuscript by Lauren M Burgeno, Nicole L Murray, Ryan Farero, Ingo Willuhn, and Paul EM Phillips. I carried out all experiments with help from NLM and RF, designed experiments with help from IW and PEMP. I am writing the manuscript with help from PEMP.

Introduction

Treatment for addiction has proven difficult, as those afflicted with substance use disorders exhibit a high risk of relapse following both periods of self-imposed and forced abstinence, even at time points far beyond those at which drugs maintain their pharmacological effects[5,172]. One of the main goals of addiction research is to understand the neurobiological mechanisms that underlie drug craving so that we may develop more efficacious therapeutics for relapse prevention[38]. During abstinence, exposure to cues that were previously associated with drug taking can exert powerful influence over behavior and precipitate relapse[38,40,47,75-77]. Drug-associated cues, or conditioned stimuli (CS), also promote drug seeking in rodent models of addiction[23,24,44,78,79,115]. It is thought that CS precipitate craving, which drives subsequent drug seeking and ultimately lead to relapse[47]. This effect appears to be dynamic over prolonged periods of abstinence, with CS exerting stronger behavioral control over time, and precipitating more robust feelings of craving[47]. Numerous studies in rodents, many from the laboratory of Dr. Yavin Shaham, also support the theory that responsivity to CS increases over time during prolonged periods of abstinence[1,173]. More specifically, animals exhibit progressive increases in resistance to extinction, and enhanced cue-induced reinstatement of drug seeking over the course of weeks to months of abstinence, an effect which has been termed the 'incubation of craving'[1].

Molecular neuroadaptations in the mesolimbic dopamine pathway occur over the course of abstinence and are thought to contribute to the incubation of craving (reviewed in Lu *et al*., 2004[173], and Pickens *et al*., 2011[174]). This includes changes in protein-kinase A and adenylate cyclase enzyme activity[175], and changes in protein expression of cyclin-dependent kinase 5[176], tyrosine hydroxylase[177], dopamine active transporter[178], multiple glutamate receptor subunits (GluR1, GluR2, NMDAR1)[179,180], and brain-derived neurotrophic factor[181,182]. These neuroadaptations might alter the mesolimbic dopamine neurotransmission elicited by drug-paired cues during abstinence, however, it has yet to be determined whether CS-elicited dopamine release changes over the course of abstinence. We hypothesize that over the course

of prolonged abstinence, CS-elicited dopamine release in the nucleus accumbens core (NAcc) increases, and that this enhancement in dopamine release mediates the incubation of craving in rats, and the CS driven invigoration drug seeking following long periods of abstinence.

We employed fast-scan cyclic voltammetry in the NAcc to determine whether CS-elicited dopamine release changes over the course of one month of abstinence. We found that CS-elicited dopamine release significantly increases over the course of one month of abstinence, a period in which animals exhibit an incubation of craving, and work harder for receipt of CS alone. In addition, preliminary results from studies of the drug seeking behavior induced by unexpected presentation of CS show a (nearly significant) trend towards an increase in drug seeking elicited by CS over abstinence.

Methods

Subjects

The following experiments were carried out in animals that maintained catheter patency throughout long-access self-administration studies described in Chapter 3.

Non-Contingent CS Probe Sessions

At one day and one month (28-32 days) following the last long-access self-administration session phasic dopamine responses elicited by non-contingent cues were recorded using fast-scan cyclic voltammetry (FSCV, described in Chapter 3 methods and Arnold *et al*., 2015[90]). On test days, rats underwent continuous FSCV recording during a non-contingent CS probe session identical to that used in Chapter 3 experiments, except for that no self-administration session followed. Rats received two unexpected non-contingent presentations of the CS that was usually paired with drug during self-administration, three minutes apart (see schematic, Figure 1a). For each animal, the two CS-elicited dopamine responses were averaged for each session. During probe sessions, clear tape was placed over the nose poke holes to allow animals to experience the cues, but prevent extinction of responding for drug. Since there were a limited number of subjects that

completed both self-administration and abstinence phases voltammetry data presented includes only those subjects from which we obtained successful recordings after both one day and one month of abstinence. In cases where more than one electrode was functioning in an animal during a given session, the average of the signals obtained from both electrodes was used for analysis. Sessions were video recorded to allow for analysis of conditioned approach behavior elicited by CS presentation.

Conditioned Approach Behavior Scoring

Changes in behavior during each CS presentation (20 sec, nose poke light + tone) were monitored and a scored (from 0-5) using the following criteria: 0 = no response, 1 = slight startle only, 2 = looks towards nose-poke hole, 3 = orients towards nose-poke hole in place, 4 = orients and approaches nose-poke hole, 5 = orients, approaches, and physically interacts with the nose-poke hole area (licking, biting, etc.). Data presented are conditioned approach responses from the first CS presentation from each animal at each abstinence duration. Analysis was carried out using animals from which we obtained useable videos from probe sessions at both time points. In some cases, our view of the animal during cue presentations was obscured because of the orientation of the camera, or location of the animal within a chamber. In these cases, the data were not included.

'Incubation of Craving' Extinction Sessions

One day after each of the recorded CS probe sessions rats underwent a 30-minute extinction session. Extinction sessions were identical to self-administration sessions (described in Chapter 3 methods), except for that active nose pokes elicited a CS without the delivery of drug (infusion pumps were off and lines were backfilled with saline). Comparison of the resistance to extinguish responding for CS (conditioned reinforcement) after short (1 day) and long (1 month) periods of abstinence allowed us to assess the incubation of craving[1] in our animals.

Initial studies of the incubation of craving used separate animals for testing at each incubation time point because once animals underwent extinction and reinstatement studies

any testing at later time points would have been confounded by this previous experience[1,173]. However, at the point we began our studies it had been shown that assessment of the incubation of craving within subjects (with repeated measures early and late in abstinence) could be done without impacting behavior by using a modified protocol in which resistance to extinguish responding for a CS was measured in a very brief (30 minute) extinction sessions at early and late time points during abstinence[183]. To ensure that extinction testing early in abstinence did not affect measurements made later, we compared extinction responding after one month of abstinence between rats that did or did not receive an extinction test on the first day of abstinence. We found that performing a 30-minute extinction test on the first day of abstinence did not significantly impact extinction responding at during the extinction test after one month of abstinence (Supplementary Figure 1a, t-test $p > 0.05$), thus we carried out repeated tests for these experiments.

Results & Discussion

To determine whether NAcc phasic dopamine responses elicited by CS were dynamic over a prolonged period of abstinence we used FSCV to measure CS-elicited dopamine responses after short (one day) and long (one month) periods of abstinence following long-access cocaine self-administration (**Figure 1a)**. We observed a robust increase in phasic dopamine release elicited by CS over one month of abstinence, with five of the seven subjects increasing by at least two-fold (**Figures 1b and 1c**, paired t-test: one day versus one month abstinence, $p < 0.05$**).** In addition, analysis of extinction responding following one day and one month of abstinence revealed a significant enhancement in responding for the CS under extinction conditions over the course of one month of abstinence (**Figure 1e**, t-test: two- fold increase between one day versus one month of abstinence, $p < 0.0001$), consistent with the findings in studies of the incubation of craving[1,3,173,183]. These data support the hypothesis that phasic dopamine responses elicited by unexpected presentation of CS 'incubate' (increase) over the course of abstinence, and suggest that increases in CS-elicited dopamine in the NAcc might play a role in mediating the incubation of craving.

Extinction testing in incubation of craving studies gave us an indication of the reinforcing properties of the CS at multiple time points during abstinence as animals responded to receive the CS alone. In addicts, however, CS do not typically precede drug seeking actions; they follow them[171]. As such, we wanted to assess whether unexpected CS presentation during probe sessions stimulated subsequent drug seeking actions. To test this, we scored videos recorded during the non-contingent CS probe sessions to assess the propensity for CS to stimulate conditioned approach to the nose-poke port that was previously paired with drug during self-administration. There was a significant increase in CS-elicited conditioned approach behavior over the course of one month of abstinence (**Figure 1d**, Wilcoxon signed rank test: $p < 0.05$), with all but one animal exhibiting stronger conditioned approach responses after one month of abstinence. Together, our data demonstrate that CS can both promote drug seeking actions and sustain conditioned responding behavior during extinction.

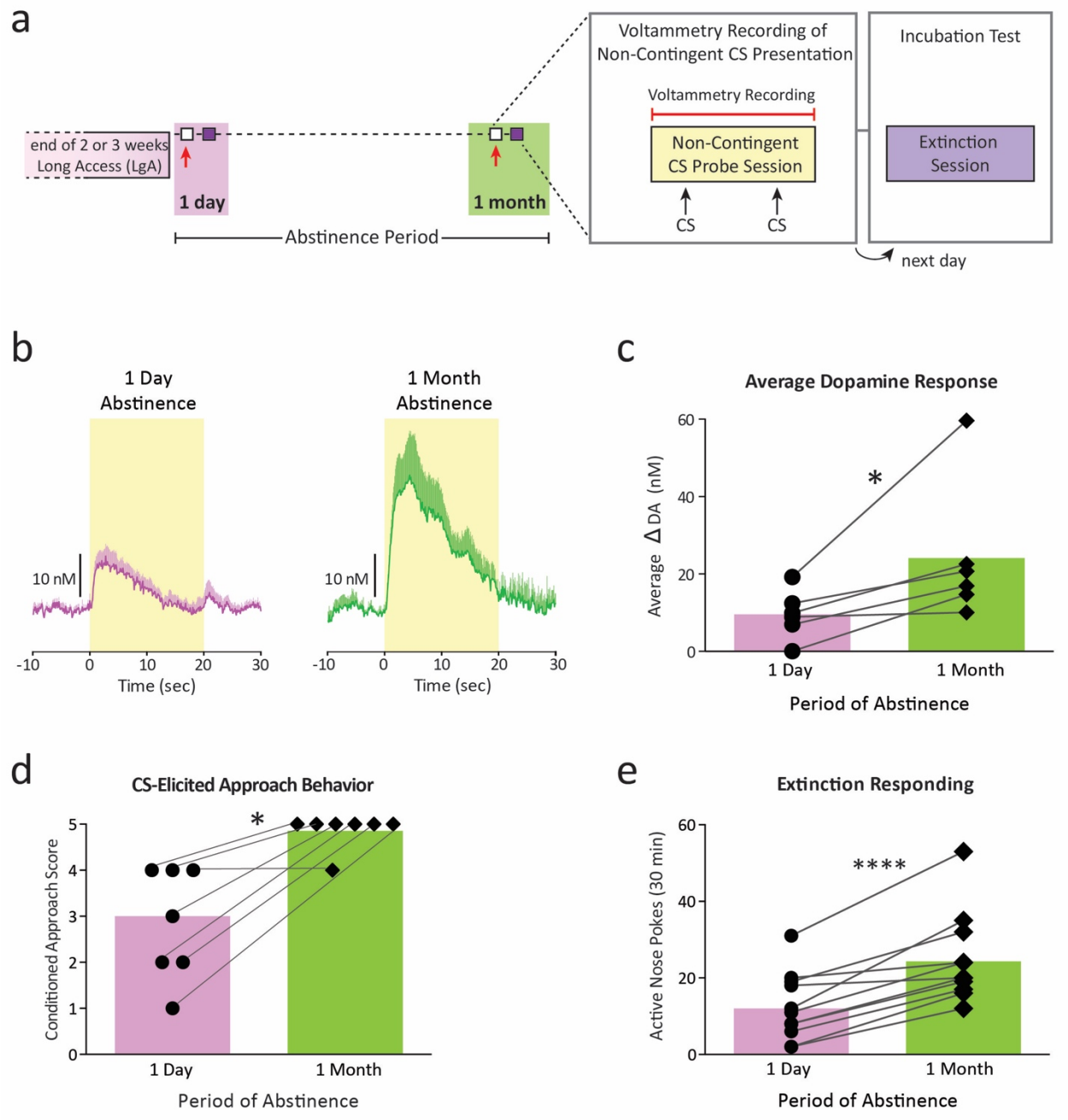


Figure 1. Non-Contingent CS-elicited NAcc dopamine release and CS-elicited drug-seeking increase in parallel with the incubation of craving during prolonged abstinence.

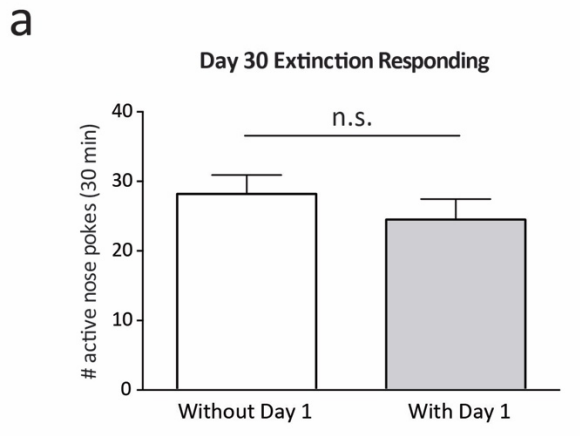
a) Following LgA self-administration, non-contingent CS-elicited NAcc dopamine responses were measured in non-contingent CS probe sessions after both one day and one month of abstinence. One day after each probe session rats were given 30-minute extinction sessions to assess the incubation of craving. Probe session time points are indicated by small white boxes with red arrows, extinction sessions are denoted by small purple boxes, and both probe and extinction sessions are schematized in the large boxes to the right. **b)** Average CS evoked FSCV dopamine responses obtained after one day and one month of abstinence. Area shaded in yellow marks the duration of the non-contingent CS presentation. FSCV traces are (continued on next page)

mean + s.e.m. of background subtracted response. **c)** Average CS-elicited dopamine after one day and one month of abstinence (bars indicate mean during the seven second window following CS-onset, connected dots indicate responses for individual subjects). Statistics: There was a significant, two-fold, increase in CS-elicited dopamine following one month of abstinence (paired t-test, $p < 0.05$). **d)** Non-contingent presentation of CS-elicit significantly stronger conditioned approach responses following one month of abstinence (colored bars indicate the median, and each set of connected points are approach responses to first CS presentation from individual subjects). Statistics: Wilcoxon signed rank test, $p < 0.05$. Conditioned approach scores (defined in methods) range from 0= no response to 5= animal orients, approaches and interacts with nose-poke hole area. **e)** The incubation of craving was assessed by testing the resistance to extinguish responding for the CS in 30-minute extinction tests after one day and one month of abstinence as has been shown previously[1,173] (colored bars indicate the mean, and each set of connected points are responses from individual subjects). Statistics: There was a significant, two-fold, increase in extinction responding following one month of abstinence (paired t-test, $p < 0.0001$).

Conclusions

CS-elicited dopamine release is dynamic over the course of abstinence. Dopamine release elicited by CS increases over the course of one month of abstinence, and conditioned responding for these cues during extinction increases in parallel. The ability of CS to elicit drug seeking also increases over this same time period. Increases in CS-elicited dopamine release during abstinence may serve to invigorate conditioned responding by enhancing the incentive salience ascribed to the CS[87,88,171]. It is thought that by stimulating larger dopamine responses, CS gain the ability to elicit a conditioned motivational state which drives further drug seeking[115].

Supplementary Figure



Supplementary Figure 1. Extinction testing at abstinence day 1 did not impact subsequent extinction responding measured after one month of abstinence.

To ensure that extinction testing early in abstinence did not affect subsequent extinction responding, we compared extinction responding after one month of abstinence between rats that did or did not receive an extinction test on the first day of abstinence. We found that performing a 30-minute extinction test on the first day of abstinence did not significantly impact extinction responding after one month of abstinence (Statistics: t-test $p > 0.05$), thus we carried out repeated tests for subsequent experiments.

CHAPTER 5

Molecular Mechanisms & Therapeutic Strategies

Results from the studies described in Chapters 2-4 demonstrate multiple roles for nucleus accumbens (NAcc) dopamine in mediating different aspects of the addiction process, and indicate that opposite changes in dopamine neurotransmission promote drug-taking and drug-seeking during different phases of the addiction process. Together, these findings suggest that it may be more complicated than previously thought to utilize therapeutic strategies that target dopamine neurotransmission, since drugs that reduce drug taking by altering dopamine release might also enhance drug seeking, or vice versa. This chapter contains preliminary findings from studies of the molecular mechanisms that mediate decreases in dopamine release during drug taking, and therapeutic strategies aimed at reducing both drug-taking and drug-seeking.

Kappa Opioid Receptor Dependent Regulation of Drug-Intake

Introduction

Chronic drug use leads to altered mesolimbic dopamine neurotransmission [140]. We previously demonstrated a causal link between decreases in ventral striatal dopamine release and the escalation of cocaine intake observed when animals are given protracted access to drug[72](Chapter 2). In this section I report preliminary findings from ongoing studies, done in collaboration with the laboratory of Dr. Charles Chavkin, aimed at answering the follow up question: **What molecular mechanisms mediate these observed decreases in dopamine release that drive escalation of drug intake?**

Multiple lines of converging evidence implicate presynaptic kappa opioid receptor activation by the neuropeptide, dynorphin, in mediating decreases in NAcc dopamine during chronic drug taking. First, activation of the kappa opioid receptors (KOR), which are present on dopamine neuron terminals, decreases dopamine release in the NAcc in brain

slices[2,4,184]. Additionally, elevated levels of dynorphin have been observed in the striatum following chronic cocaine exposure[185,186]. Furthermore, long-term KOR antagonism both prevents the escalation of heroin and methamphetamine intake[187,188], and reduces the motivation to work for drug [187,189]. **Thus, the objective of our current work is to test the hypothesis that the decreases in NAcc dopamine that drive the escalation of drug intake are mediated by KOR activation.** Initial studies tested whether blockade of KOR during long-access self-administration prevents the escalation of drug intake. Studies to confirm that the effects of KOR blockade on escalation are dopamine mediated are ongoing.

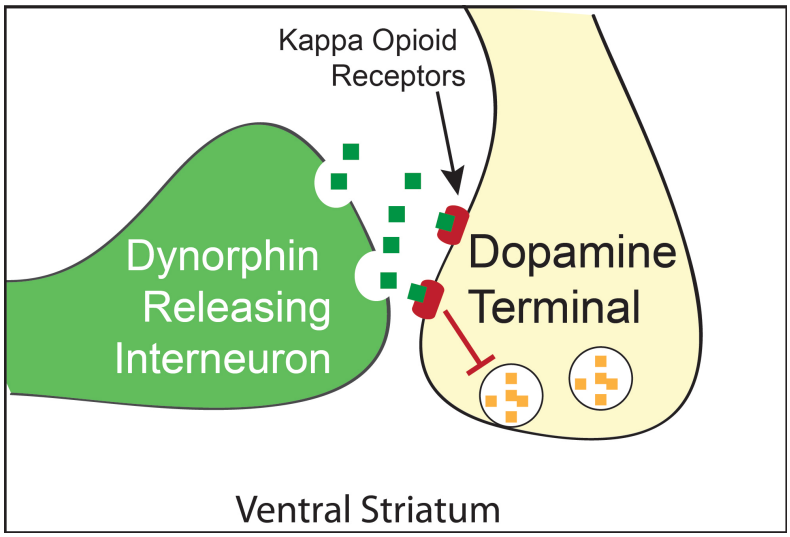


Figure 1. Role of NAcc kappa opioid receptors in regulation of dopamine release
Activation of presynaptic kappa opioid receptors (KOR) on dopamine terminals leads to a reduction in dopamine release[2,4,184].

Methods

Experiment #1 (completed, data presented Below): Male Wistar rats were outfitted with bilateral intra-NAcc cannulas (**Figure 2a,** AP: +1.3 ML: ±1.3, DV: -6.2 for guide cannula, -7.2 internal cannula) and jugular catheters. Following recovery rats were trained to self-administer cocaine (FR1 schedule, 0.5mg/kg/infusion) during short-access (ShA, one-hour) sessions. Once baseline drug intake was established (7-10 sessions total), the long-lasting kappa antagonist, norbinaltorphimine (nor-BNI, bilaterally 5μg in 0.3μL ACSF per side), or vehicle was microinfused within the NAcc and animals were switched to daily long-access (six-hour access) sessions to assess the extent of the escalation of drug intake (**Figure 2b**). Previous studies have demonstrated the duration of action to last between 14-21 days[190], thus only a single injection was required. Escalation was assessed as described previously in Willuhn *et al.*, 2014[72] and Chapter 3 methods. Average baseline intake was no different between animals separated into nor-BNI and vehicle treatment groups (**Supplementary Figure 1**).

Experiment #2 (Ongoing): Injectrodes were designed and constructed by attaching carbon fiber microelectrodes to each side of a bilateral cannula (**Figure 2c**). This allows for microinjection of drug and dopamine recordings to take place within the same region. Male Wistar rats were outfitted with bilateral intra-NAcc injectrodes (AP: +1.3 ML: ±1.3, DV: -6.2 for guide cannula, -7.2 internal cannula/ recording electrode) and jugular catheters. The experimental design was identical to that of Experiment #1, except for that fast-scan cyclic voltammetry (FSCV) recordings were carried out weekly both before and after nor-BNI/ vehicle were administered (**Figure 2d**).

See Chapters 2 and 3 for basic FSCV methods description, or Arnold *et al*., 2015[90] for a more detailed review.

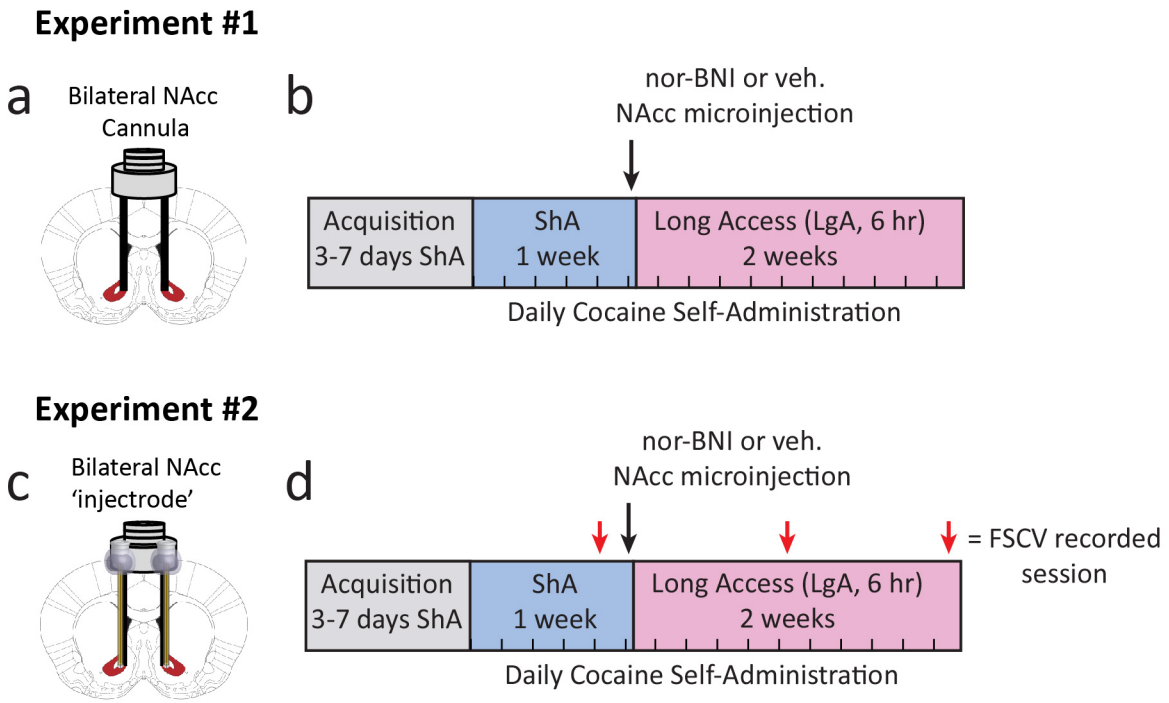


Figure 2. Experimental design schematics

Experiment #1: **a)** Rats were implanted with bilateral NAcc cannulae, and **b)** trained to self-administer cocaine (FR1 schedule, 0.5mg/kg/infusion) during short-access (ShA, one-hour) sessions. Once baseline drug intake was established (7-10 sessions total), the long-lasting kappa antagonist, norbinaltorphimine (nor-BNI, bilaterally 5μg in 0.3μL ACSF per side), or vehicle was microinfused within the NAcc and animals were switched to daily long-access (six-hour access) sessions to assess the extent of the escalation of drug intake. Black arrow indicates time of microinjection.

Experiment #2: **c)** Rats were implanted with bilateral NAcc 'injectrodes' and then **d)** underwent the same paradigm as Experiment #1, except that phasic dopamine release in the NAcc was recorded during one session per week both before and after microinfusions of nor-BNI or vehicle to determine whether behavioral effects of KOR antagonism were due to alterations in dopamine release. FSCV recorded sessions indicated by red arrows. This experiment is ongoing.

Results

In our initial work we sought to test the prediction that blocking KOR during long-access cocaine self-administration would prevent the escalation of drug intake, as has been shown with methamphetamine[188] and heroin[187]. Indeed, we find that bilateral microinjection of the long lasting KOR antagonist, norbinaltorphimine (5μg in 0.3μL ACSF per side, nor-BNI), prior to long-access (LgA) prevents the escalation of drug intake over time (**Figure 3a**, Two-Way ANOVA: significant main effects of treatment group $F_{(13,269)}=4.37$, $p < 0.0001$; and week $F_{(1,269)}=32.33$, $p < 0.0001$; and significant treatment group x week interaction $F_{(13,269)}=2.00$, $p < 0.05$). Comparison of average weekly intake between treatment groups revealed a significant increase in intake between long-access weeks two and three in vehicle but not nor-BNI treated animals (**Figure 3b**, post-hoc Holm-Sidak $p < 0.05$ vehicle, $p > 0.05$ nor-BNI). Analysis of the extent of escalation of animals from both treatment groups revealed that nor-BNI treated animals were less likely to become escalators than their vehicle treated counterparts (**Figures 3c and 3d** $X^2(1, N = 23) = 5.79$, $p <0.05$).

Conclusions

We have determined that KOR activation plays an important role in driving the escalation of cocaine intake. However, we have yet to determine whether these KOR mediated effects on escalation are mediated by changes in dopamine release. This is currently under investigation (see study design in methods Experiment #2). So far, we have built and optimized bilateral injectrodes (cannula / electrode assembly) to allow for NAcc microinjections and FSCV recordings within the same region, and carried out successful recordings in a few animals. Further cohorts will be required to complete this study.

It should be noted that administration of the stress peptide, corticotrophin releasing factor (CRF), leads to an increase in the activation of KOR[191]. This suggests that CRF is stimulating dynorphin release , and may be an upstream regulator of dynorphin release in the drug-taking context. This will be important later in this chapter as we discuss potential therapeutic targets for the regulation of drug intake.

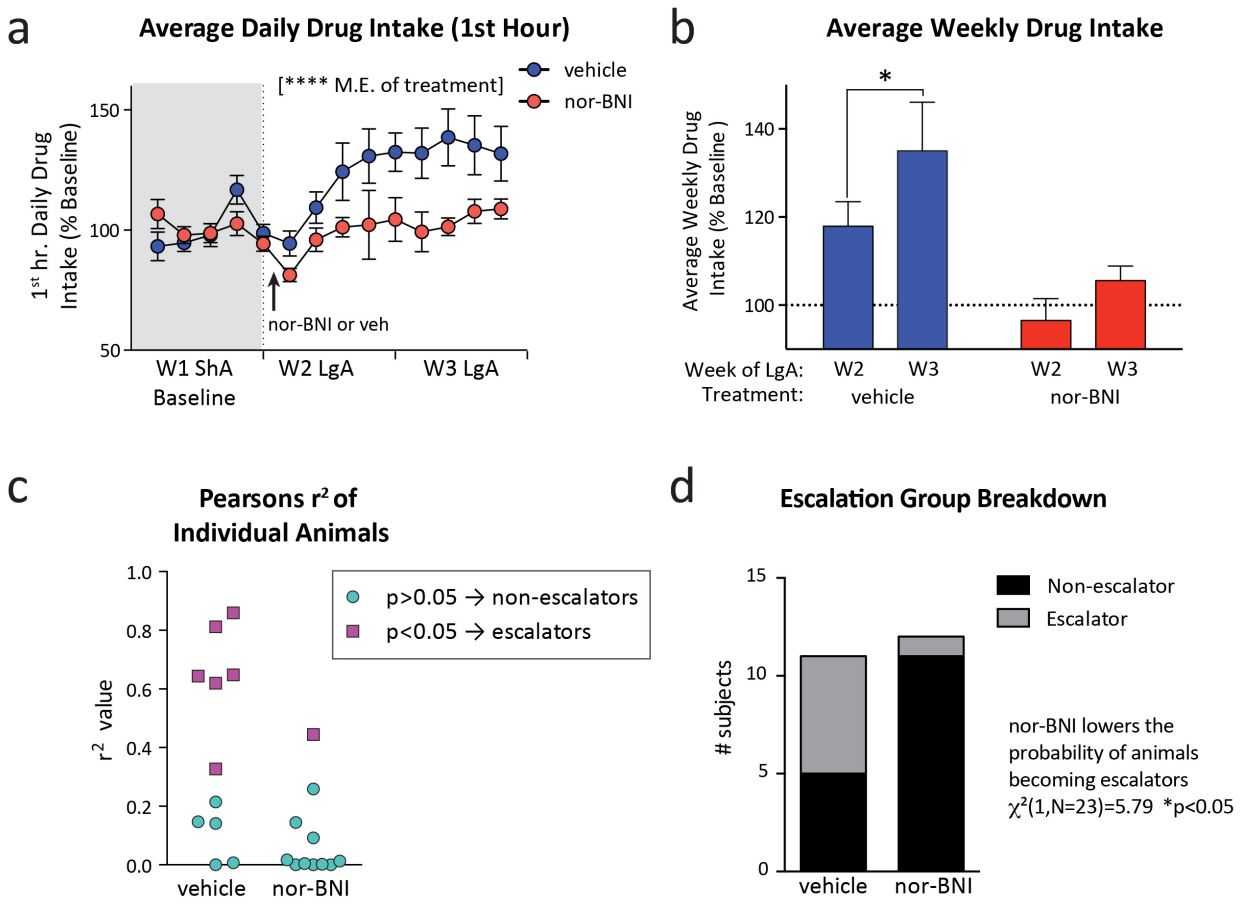
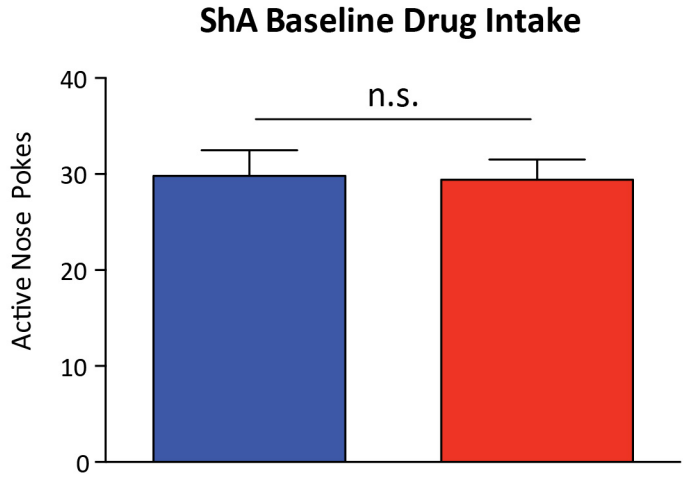


Figure 3. Kappa Opioid Receptor Blockade Prevents the Escalation of Drug Intake

a) Plot of average daily drug intake over time for rats that received either bilateral microinjected of nor-BNI (5μg in 0.3μL ACSF per side, red circles) or vehicle (0.3μL ACSF per side, blue circles) after establishing baseline intake in short-access (ShA, one-hour daily access). Following microinfusion rats were transitioned to long-access (LgA, six-hour daily access). Data are expressed as mean ± s.e.m. percent change from baseline (average intake in ShA W1 baseline). Black arrow marks day of microinjection. Statistics: Two-Way ANOVA revealed significant main effects of both week ($F_{(1,269)}$=32.33, $p < 0.0001$), and treatment group ($F_{(13,269)}$=4.37, $p < 0.0001$), as well as a significant week x treatment group interaction ($F_{(13,269)}$=2.00, $p < 0.05$). Post-hoc Holm Sidak comparison revealed a significant difference between nor-BNI and vehicle groups on days six and seven of LA ($p < 0.05$ for each). **b)** Collapsed data over days into average weekly drug intake (mean + s.e.m.) in nor-BNI and vehicle treated animals. Statistics: Two-Way RM ANOVA revealed significant main effects of both week ($F_{(1,20)}$=11.42, $p < 0.01$) and treatment group ($F_{(1,20)}$=8.22 , $p < 0.01$), and post-hoc Holm Sidak revealed a significant difference between LgA W2 and LgA W3 in the vehicle ($p < 0.05$), but not nor-BNI treated animals ($p > 0.05$). **c)** r^2 values of individual animals obtained from linear regression of drug intake over time from vehicle (left column) and nor-BNI treated (right column) animals. Statistical test of whether each animal's slope was significantly positive, non-zero allowed for the separation of escalators ($p < 0.05$, magenta) from non-escalators ($p > 0.05$, cyan). d) A chi-square test of independence was performed to examine the relation between treatment group and the escalation of drug intake. The relation between these variables was significant, X^2 (1, N = 23) = 5.79, $p < 0.05$), nor-BNI treated animals were less likely to escalate their drug intake than vehicle treated animals.

Supplementary Figure

a



Supplementary Figure 1. There was no difference in baseline intake between nor-BNI and vehicle groups
Statistics: t-test, $p > 0.05$

Therapeutic Potential of Dopamine Stabilizers in Cocaine Addiction

Our previous findings demonstrate that diametric changes in dopamine release mediate drug-taking and drug-seeking during different phases of the addiction cycle. Conceptually, this suggests that therapeutics targeting mesolimbic dopamine neurotransmission should enhance dopamine during drug-taking (when it is low) and reduce dopamine during drug-seeking (when it is high) to effectively reduce both drug-taking and drug-seeking behavior.

(-)-OSU6162 is a compound with unique partial agonist properties and is part of a small group of so-called "dopamine stabilizers"[192-196]. Though the exact mechanisms by which this occurs is still a matter of debate[196-198], these compounds generally have the ability to suppress or enhance dopamine activity depending upon dopaminergic tone[199,200]. Moreover, (-)-OSU6162 administration attenuates ethanol intake in rats[201], and drug seeking in both rats and humans[201,202]. Together these data suggested that (-)-OSU6162 might have promise in reducing both drug-intake and drug-seeking behavior in our cocaine self-administration paradigm. We carried out an experiment like the nor-BNI study previously described, except for we allowed the animals to escalate in two weeks of long-access before administering (-)-OSU6162. We planned to administer (-)-OSU6162 (30mg/kg, i.p. as used in Steensland *et al.* 2012[201]) thirty minutes prior to each self-administration session for the third week of long-access, however on the first day all three of the three rats administered (-)-OSU6162 had seizures within 20-40 minutes of beginning self-administration. We terminated the study at that point.

This outcome challenges the utility of (-)-OSU6162 as a safe therapeutic for use in humans. It is possible that this interaction is specific to cocaine, however further studies are needed to determine whether interactions with (-)-OSU6162 and drugs of abuse will occur in humans before this compound can be used as a therapy.

*Therapeutic Potential of Finasteride for Addiction **

**Much of this work is based upon published and unpublished work (soon to be submitted) done in collaboration with the laboratory of Dr. Marco Bortolato at the University of Utah.*

Introduction

Given that the most obvious approach, a dopamine stabilizer, had such catastrophic consequences, we turned to thinking about whether we could modify the effect of enhanced dopamine on drug seeking through postsynaptic receptors instead. Following a recent collaboration with Dr. Marco Bortolato at the University of Utah, we became particularly interested in the potential therapeutic value of finasteride, an inhibitor of 5-alpha reductase, a key enzyme in the steroid metabolism pathway which converts testosterone to dihydrotestosterone. This drug is currently FDA approved for the treatment of benign prostatic hyperplasia and pattern hair loss in men. Several convergent lines of evidence have directed our attention towards investigating finasteride as a potential therapy for the selective reduction of drug seeking. Recent unpublished work from our collaborator, Dr. Marco Bortolato, demonstrates that finasteride administration leads to a rapid reduction in D3-dopamine receptor expression in the Nac (**Figure 4a**). Previous studies that I carried out in collaboration with the Bortolato lab also demonstrated that D3 receptors are upregulated following dopamine depletion, and that pharmacological activation of postsynaptic D3 receptors selectively impacted risky decision making selectively in animals with dopamine depletion (partially published in Pes *et al*., 2017[203]). Because basal dopamine levels are low during prolonged abstinence[172,204,205], it is possible that D3 receptors are also upregulated under these circumstances. Indeed, it has already been shown that D3 receptor activation is elevated in methamphetamine users[206], and D3 antagonism reduces reinstatement of nicotine and heroin-seeking[207,208]. Therefore, we hypothesized that finasteride's ability to reduce dopamine receptor expression might counteract elevated D3 receptor expression during abstinence and decrease drug-seeking as a result (Schematized in **Figure 4b**).

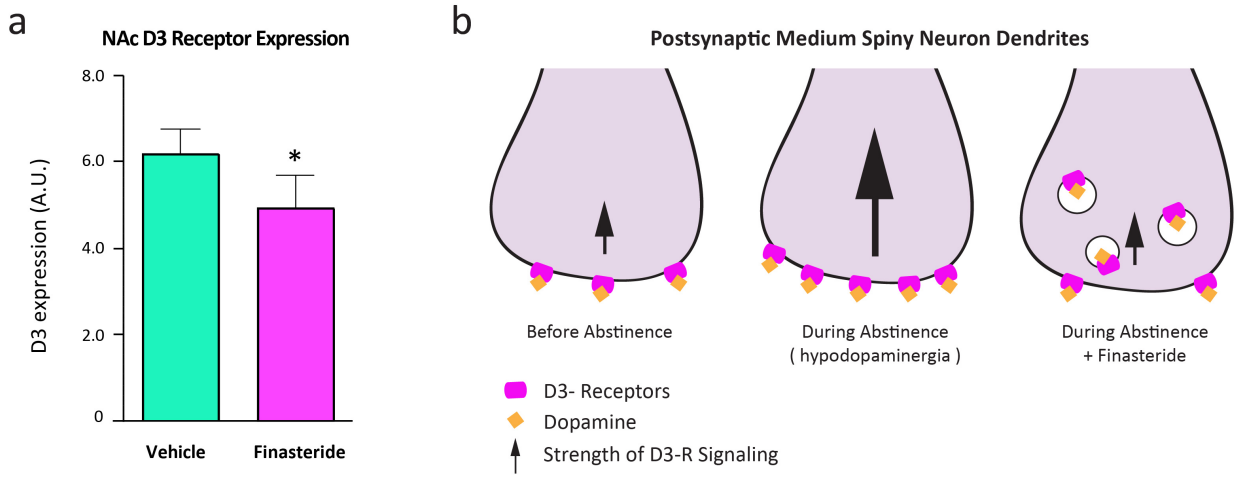


Figure 4. Finasteride is hypothesized to normalize D3 activity following abstinence

a) Finasteride decreases D3 receptor expression in the NAc. Data are mean + s.e.m. levels of D3 receptors (normalized vs. actin) 2-hours after acute treatment of male Sprague-Dawley rats with finasteride (50mg/kg, i.p) n=5/group. These unpublished data were collected by the laboratory of Dr. Marco Bortolato and are used here with permission. **b)** Schematic depicting proposed mechanism by which finasteride might normalize D3 receptor expression during abstinence. The hypodopaminergic state during abstinence may induce an upregulation of D3 receptors, thus treatment with finasteride could reverse this effect.

What was especially interesting about this approach, was that other work from our collaborator, Dr. Marco Bortolato, had also shown that Finasteride has profound effects on corticotrophin releasing factor (CRF). Finasteride treatment completely inhibits stress-induced CRF production in the paraventricular nucleus of the hypothalamus (Figure 5). As mentioned previously, it is possible that CRF is an upstream regulator of dynorphin release during drug taking[191]. Therefore, we hypothesized that finasteride might also reduce drug-taking indirectly by decreasing dynorphin release through a CRF dependent mechanism.

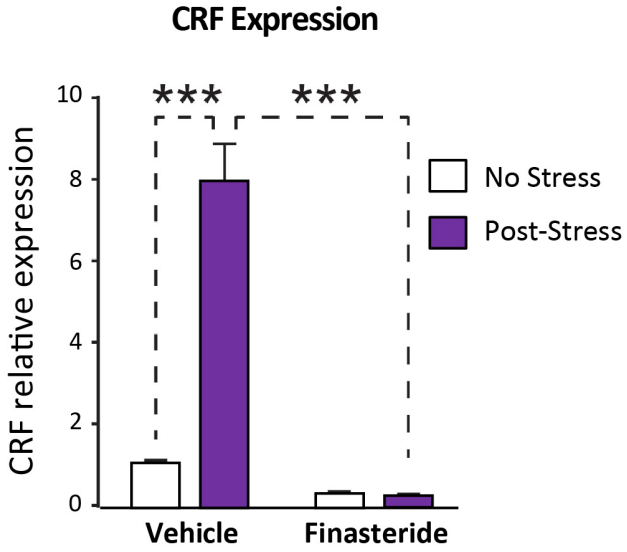


Figure 5. Finasteride blocks stress-induced CRF expression*
Native CRF mRNA expression in the paraventricular nucleus of the rat hypothalamus, as assessed by RT-PCR. These unpublished data were collected by the laboratory of Dr. Marco Bortolato and are used here with permission.

To test the hypothesis that finasteride would both reduce drug-seeking and lower drug-intake, we assessed the affect that finasteride administration had on both drug-seeking, and drug-taking in animals that underwent long-access self-administration. Of course, 5-alpha reductase has widespread downstream targets so there are certainly other actions through which it could influence behavior. However, because this drug is already FDA approved, and there were multiple strong lines of evidence supporting our rationale, we moved forward with testing our hypothesis. Preliminary findings support our hypothesis, with finasteride treated animals exhibiting a suppression of drug seeking, and a prevention of escalation during drug taking.

Methods

Experiment #1: Male Wistar rats were outfitted jugular catheters. Following recovery rats were trained to self-administer cocaine (FR1 schedule, 0.5mg/kg/infusion) during short-access (ShA, one-hour) sessions. Once baseline drug intake was established (7-10 sessions total), animals were switched to daily long-access (six-hour access) sessions for two weeks before beginning daily finasteride or vehicle (25mg/kg, i.p., vehicle 5% tween in saline) injections 30 minutes prior to each self-administration session for the final week of long-access. See experimental design schematic in Figure 6a.

Experiment #2: All vehicle treated rats from Experiment #1 proceed to experiment #2. These rats underwent drug-seeking testing in the incubation of craving paradigm (explained in detail in Chapter 4 methods). All rats underwent a 30-minute extinction responding session after one day of abstinence. Starting at Day 25 of abstinence rats received daily injections of either finasteride or vehicle (25mg/kg, i.p., vehicle 5% tween in saline), until day 30 when they received their final injection and underwent another 30-minute extinction test, 30 minutes after the injection. See experimental design schematic in Figure 6a (within gray box).

Results

In our first experiment (**Figure 6a**, experiment #1) we asked whether daily finasteride administration would alter drug intake in animals that had already undergone escalation. Though we did not expect to see any impact of finasteride on drug intake, finasteride administration blocked further escalation of drug intake, whereas vehicle treated animals continued to escalate (**Figure 6b**, Statistics: Two Way RM ANOVA- significant treatment group x time interaction $F_{(1,8)}=24.29$, $p < 0.01$, t-test LgA W2 vs. LgA W3 in vehicle $p < 0.05$ and finasteride $p > 0.05$ treated). Next (**Figure 5a**, Experiment #2), we sought to determine whether treatment with finasteride prior to extinction testing at the one month abstinence time point would reduce drug seeking / incubation of craving. Indeed, finasteride blocked the incubation of craving effect, which was maintained in vehicle treated animals (**Figure 6c**, Statistics: Two Way RM ANOVA- significant treatment group x time interaction $F_{(1,6)}=9.13$, $p < 0.05$, t-test one day vs. one month abstinence in vehicle $p < 0.05$, and finasteride treated $p > 0.05$).

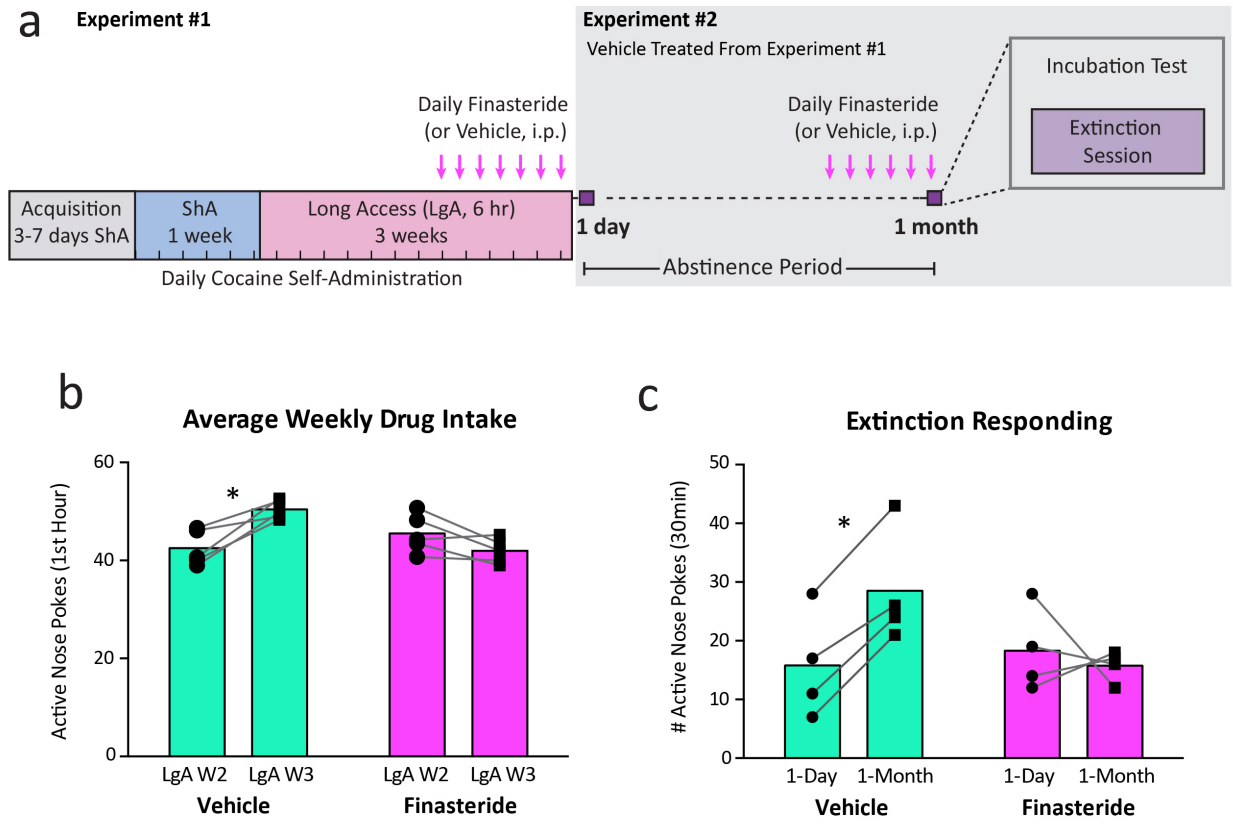


Figure 6. Finasteride prevents escalation of drug intake and blocks the incubation of craving.

a) *Experimental Design: Experiment #1*: Rats were trained to self-administer cocaine (FR1 schedule, 0.5mg/kg/infusion) during short-access (ShA, one-hour) sessions. Once baseline drug intake was established (7-10 sessions total), animals were switched to daily long-access (six-hour access) sessions for two weeks before beginning daily finasteride or vehicle (25mg/kg, i.p., vehicle 5% tween in saline) injections 30 minutes prior to each self-administration session for the final week of long-access.

Experiment #2 (gray box): All vehicle treated rats from Experiment #1 proceed to experiment #2. These rats underwent drug seeking testing using the incubation of craving paradigm (explained in detail in Chapter 4 methods). All rats underwent a 30-minute extinction responding session after one day of abstinence. Starting at Day 25 of abstinence rats received daily injections of either finasteride or vehicle, until day 30 when they received their final injection and underwent another 30-minute extinction test, 30 minutes after the injection.

b) Finasteride administration during drug taking prevented the further escalation of drug intake, vehicle administration did not. Statistics: Two Way RM ANOVA- significant treatment group x time interaction $F_{(1,8)}=24.29$, $p < 0.01$, post-hoc Holm-Sidak LgA W2 vs. LgA W3 in vehicle $p < 0.05$ and finasteride $p > 0.05$ treated. Data from repeated measurements in individual animals are indicated by connected points. **c)** Finasteride treatment prior to the one month abstinence extinction test blocked the incubation of craving (increase in drug seeking) which was preserved in vehicle treated animals. Statistics: Two Way RM ANOVA- significant treatment group x time interaction $F_{(1,6)}=9.13$, $p < 0.05$, post-hoc Holm-Sidak one day vs. one month abstinence in vehicle $p < 0.05$, and finasteride treated $p > 0.05$. Data from repeated measurements in individual animals are indicated by connected points

Conclusions

Despite the limited number of subjects in these preliminary studies, we detected statistically significant effects of finasteride in preventing further escalation of drug intake, and blocking the incubation of craving. This data is very promising and warrants further investigation to determine whether finasteride is producing these effects on drug-taking and drug-seeking through the mechanisms previously described. Because finasteride is FDA approved, it has already undergone extensive tests for safety in humans. This would enable rapid ability to implement clinical trials in human addicts should further studies bolster our current findings.

CHAPTER 6

Discussion and Future Directions

In this dissertation, I have demonstrated that bidirectional changes in drug-cue elicited phasic dopamine release within the NAcc regulates drug-taking and drug-seeking behaviors. This work implicates cue-elicited dopamine release in suppressing drug intake during the intoxication phase of the addiction cycle, and promoting drug-seeking and craving during prolonged periods of abstinence (preoccupation/anticipation phase). Though these studies only involved the study of cocaine self-administration, the consistency of the results of drug intake and relapse studies of most other drugs of abuse suggests that these effects likely extend beyond cocaine addiction. Finally, the results presented in this dissertation unify two contemporary theories of drug abuse which had previously been considered at odds[61,87], and suggest that diametric changes in NAcc dopamine mediate two different, but equally important, core symptoms of substance use disorders. There are, however, some unresolved issues worthy of discussion:

Drug-Seeking in Conditioned Reinforcement and Conditioned Responding Contexts

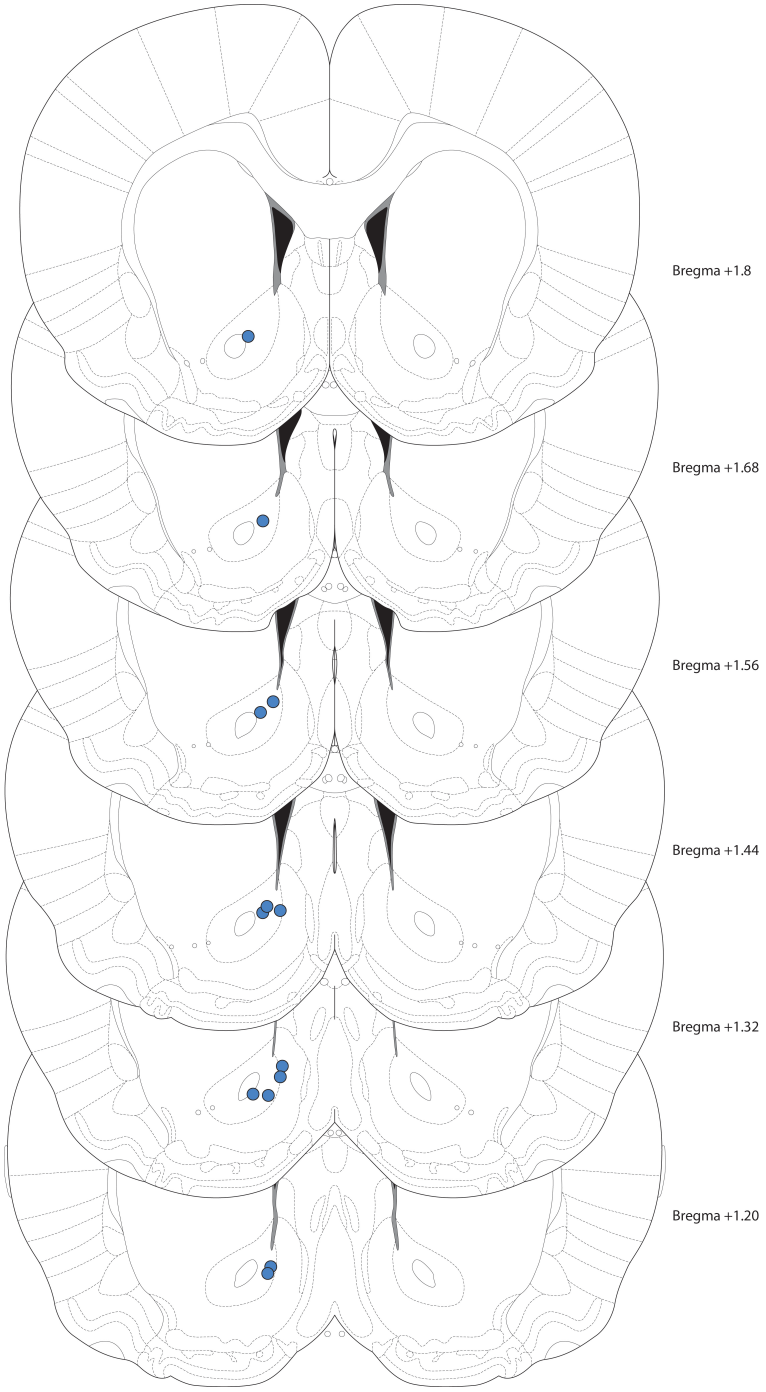
The differences between drug seeking in a conditioned reinforcement context, where animals perform an action to receive subsequent presentation of a drug cue, and the drug-seeking that is stimulated by the presentation of an unexpected cue without further reinforcement need to be addressed. In this dissertation work, measurements of cue-elicited dopamine release during abstinence in a drug-seeking context were made when cues were presented unexpectedly. These animals were not able to respond to receive further cue presentations. Behavioral analysis of video collected during these unexpected drug-cue presentations demonstrated an enhancement of drug seeking, as measured by conditioned approach to the active nose poke hole, following long periods of abstinence. Although the increases in conditioned reinforcement supported drug seeking over prolonged abstinence (incubation of craving extinction responding) paralleled the increases in cue-elicited dopamine release and drug seeking measured during non-contingent cue probe sessions, it should be noted that these drug-seeking situations are not equivalent. In one case drug seeking behavior follows the presentation of the cue, and in the

other the behavior precedes the presentation of the cue. Though the former is most reminiscent of the conditions under which relapse occurs, many cue-induced reinstatement paradigms do not actually measure the behavioral response induced by the cue, rather the cue is used to reinforce behavioral responses already executed. Indeed, this is a major critique of many commonly used cue-induced reinstatement models[23,209,210]. Future studies should assess cue-elicited dopamine responses under conditioned reinforcement to determine whether these signals exhibit similar increases over prolonged abstinence.

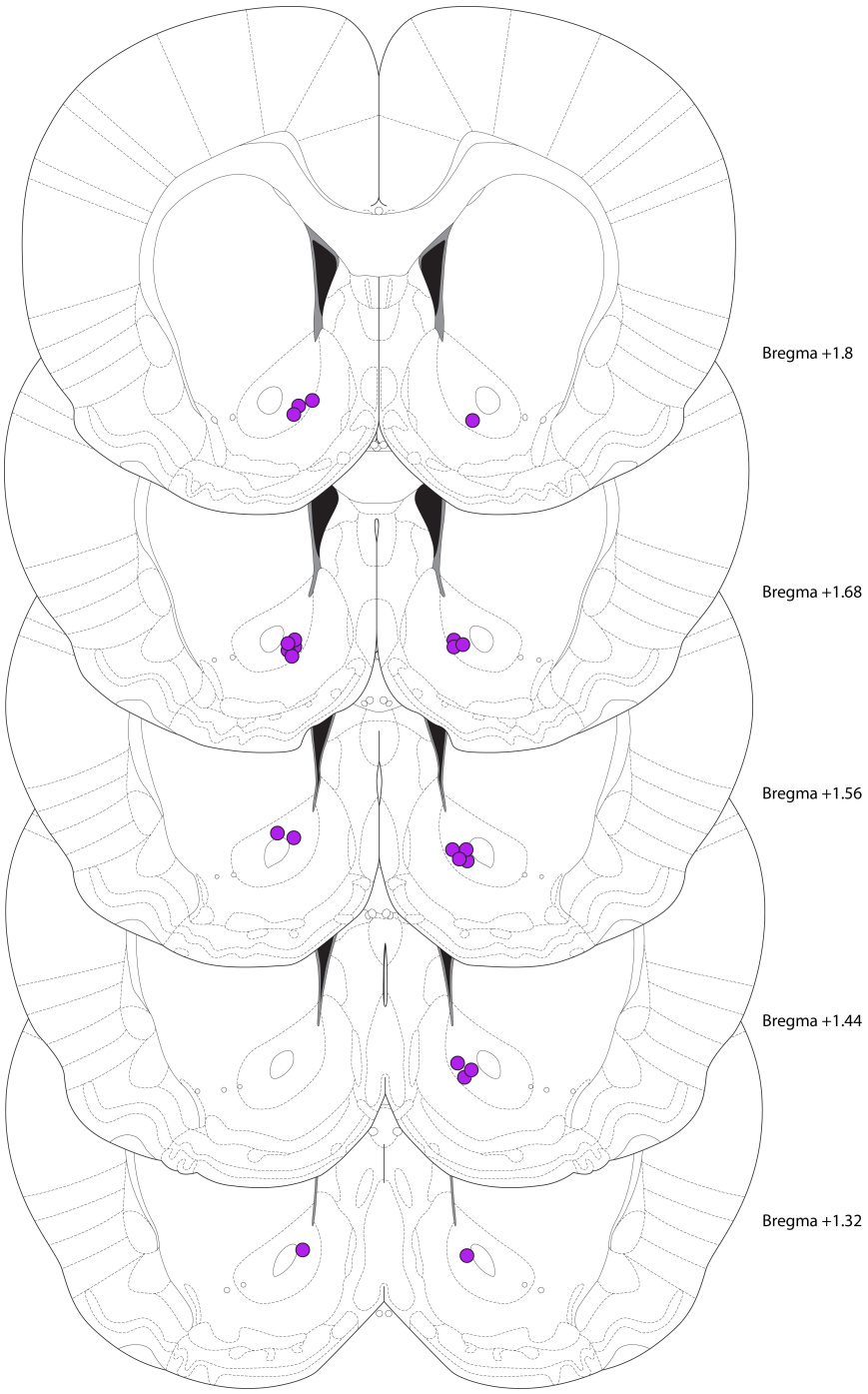
Concerns Regarding the Therapeutic Targeting of the Mesolimbic Dopamine System

If enhanced mesolimbic dopamine neurotransmission both suppresses drug taking and promotes drug seeking, then treatments that reduce drug taking by enhancing dopamine release may promote drug seeking during abstinence. Conversely, relapse treatments that reduce drug seeking by decreasing dopaminergic neurotransmission may increase drug intake in the case of relapse. Further experiments will need to be done to determine whether this is the case. I have described some of our initial studies working towards understanding the molecular mechanisms that underlie these diametric changes in dopamine release and investigate potential therapeutics (Chapter 6). Initial findings suggest that dopamine stabilizers may be problematic, at least in the treatment of cocaine addiction as 100% (3/3) of the subjects treated with a dopamine stabilizer prior to self-administration had seizures soon after beginning drug taking. Therapies which target multiple mechanisms that converge on different parts of the dopamine system (pre- vs. post-synaptic) may be more useful. Initial work has demonstrated that the 5-alpha reductase inhibitor, finasteride, as a potential candidate. Continued studies will be required to replicate this work, and expand it by determining the mechanisms by which finasteride produces effects on drug-taking and drug-seeking behaviors.

Histology



Histology Figure 1. Histological verification of recording sites in ShA Cohort animals (Chapter 3)
In some cases, placements could not be obtained because the lesion could not be found, animals lost headcaps before the end of the study, or brain tissue was damaged making it impossible to resolve lesions. Coronal sections are labeled with anterior-posterior coordinates[109].



Histology Figure 2. Histological verification of recording sites in LgA Cohort animals (Chapters 3 & 4)
In some cases, placements could not be obtained because the lesion could not be found, animals lost headcaps before the end of the study, or brain tissue was damaged making it impossible to resolve lesions. Coronal sections are labeled with anterior-posterior coordinates[109].

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Vita

Lauren Marie Burgeno was born in Torrance, California. She attended high school at Palos Verdes Peninsula High School in Palos Verdes, CA before double majoring in Chemistry and Biology at the University of California Irvine. While at UC Irvine, Lauren spent a great deal of time working in the laboratory of Dr. Daniele Piomelli studying the role of the endocannabinoid system in mediating behavior. Following graduation she worked as a laboratory technician in the laboratory of Dr. Jean C. Shih at the University of Southern California studying the the function of monoamine oxidase enzymes in mediating agression. Lauren began work towards her Ph.D. in 2010 in the Pharmacology Graduate Program at the University of Washington. She joined the laboratory of Dr. Paul E.M. Phillips where she studied the roles that dopamine plays in mediating many aspects of the addictioin process. In summer 2017, Lauren plans to begin her post-doctoral work under the guidance of Drs. Mark Walton and Stephanie Cragg at the University of Oxford, U.K.