Neuropeptidergic Modulation of Canonical Reward Circuitry Underlying Escalation of Cocaine Consumption

Lydia Gordon-Fennell

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Reading Committee:

Paul E.M. Phillips, Chair

Garret Stuber

Larry Zweifel

Program Authorized to Offer Degree:

Neuroscience

Lydia Gordon-Fennell

University of Washington

Abstract

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Lydia Gordon-Fennell

Chair of Supervisory Committee:

Paul E. M. Phillips

Department of Psychiatry and Behavioral Sciences

Substance use disorder (SUD) and its related harms are a major public health concern in the United States and across the globe. Pharmacological treatment options are limited or nonexistent for many types of SUDs. Harm reductionist approaches to SUD, including pharmacological and non-pharmacological, have proven more effective at reducing individual and society impact of SUD than traditional abstinence only based approaches. However, our incomplete understanding of the neurobiology underlying the progression and sustainment of SUD hinders the development of new, more effective treatment. This dissertation focuses specifically on one specific SUD-like phenotype characterized by an increase in drug consumption over time (termed escalation). Preventing escalation and/or reversing escalation decreases harm through decreasing the likelihood of infection, overdose, and potential monetary/social loss of the individual with a SUD. Understanding the neurobiological basis of

escalation will inform future therapeutic strategies. The neurotransmitter dopamine has a long history of being implicated in addictive drugs and there is evidence suggesting its causal relationship to escalation. Here, I will investigate two systems – dynorphin / kappa opioid receptors and corticotropin releasing factor – in their ability to modulate drug consumption, specifically escalation of cocaine self-administration. Both of these systems are capable of modulating dopamine, modulating each other, and have been implicated in the negative affect and withdrawal symptoms of SUD that are theorized to escalate drug consumption. Through pharmacological and gene editing techniques, I demonstrate in Chapter 2 that the kappa opioid receptor system, specifically expressed in ventral tegmental area neurons, is necessary for escalation of cocaine consumption. Using the same techniques, I demonstrate in Chapter 3 that the corticotropin releasing factor system in the nucleus accumbens, specifically CRF-R1, is not necessary for the development nor sustainment of escalation of cocaine consumption. Additionally, in Chapter 4 I provide a survey of cocaine self-administration in female versus male rats and provide evidence that this behavioral paradigm is capable of producing the SUD-like phenotype of escalation equally in females and males. These data provide further evidence for the dynorphin/kappa system to contribute to the β-process of the opponent process theory of SUD, independent of corticotropin releasing factor modulation through CRF-R1. I specifically implicating the dynorphin/kappa system of ventral tegmental area neurons that project to the nucleus accumbens. In the discussion, I theorize about downstream mechanisms and future experiments to investigate the temporal dynamics of dynorphin in the nucleus accumbens and its potential to modulate dopamine release during cocaine self-administration to escalate drug consumption.

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Chapter 1:

Introduction

SUBSTANCE USE DISORDER

Substance use disorder (SUD) and related drug use has negatively impacted the United States and its citizens for decades. The 2023 National Survey on Drug Use and Health reported 59% of Americans over the age of 12 had used an addictive drug (nicotine, alcohol, illicit drugs) within the past month of being surveyed and 17.1% had suffered from a SUD in the past year (Administration, 2024). Additionally, there were 107,942 overdose-related deaths in 2022 (Spencer et al., 2023). Aside from the personal harm to the individual suffering from SUD and their loved ones, substance use also has a broader economic burden on society through healthcare costs, emergency care costs, crime, productivity loss, etc (UN, 2014).

Defining Substance Use Disorder

In the United States, the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-V) is used by medical professionals to diagnose individuals with SUD. The International Classification of Diseases, 10th edition (ICD10) is another manual utilized to diagnose individuals with SUD, more commonly used internationally. The DSM-V details 11 criteria that describe different behavioral and physiological symptoms of SUD (Hasin et al., 2013). Examples of criteria include continued use despite social problems or harm, increase in amount of drug taken, tolerance, unable to lessen or discontinue use (see Table 1 for all criteria). Based on the number of criteria met, an individual can be diagnosed with mild (2-3), moderate (4-5), or severe (6+) SUD. These criteria are broadly used across most addictive drugs, with some criteria exceptions across drugs, such as differences in the definition of withdrawal across drug classes and the exclusion of withdrawal symptoms as a criterion for hallucinogens and inhalants.

In 1997, the director of the National Institute on Drug Abuse (NIDA) at the time, Dr. Alan Leshner, proclaimed SUD to be a brain disease (Leshner, 1997). The purpose was to shape the way that legislators, researchers, clinicians, and society view SUD, the individuals suffering from SUD, and how to combat SUD. In recent years, there has been increased discussion to the validity and usefulness of considering SUD to be a brain disease (Heilig et al., 2021; Pickard, 2021). Regardless, we know that addictive drugs cause neurobiological changes that extend beyond the period of drug consumption, and inherently at least a subset of these changes are the underlying cause of and sustainment of SUD. Therefore, while there are a multitude of angles to approach SUD treatment, such as combatting the societal perspective, understanding the neurobiology behind SUD will likely result in better, more effective behavioral and pharmacological treatments and prevention.

Classes of Drugs of Abuse

There are three main ways in which drugs (including addictive drugs) are classified: behavioral classification, pharmacodynamic classification, and legal classification (Koob et al., 2014a). In behavioral classification, drugs are classified based on their overall behavioral effects and include classes such as: stimulant, depressant, and psychedelic. In pharmacodynamic classification, drugs are classified based on the biological systems with which they interact and include classes such as: indirect dopamine agonist, direct opioid receptor agonist, and serotonin reuptake inhibitor. In legal classification, drugs are classified based on their legal status. Specifically, drugs in the United States are ranked on a schedule system ranging from schedule I (most regulated) to schedule V (least regulated). The schedule system was established by the FDA and is proposed to be constructed based on the abuse liability and medical potential of drugs, with higher scheduled drugs (such as schedule I) being higher abuse liability and lower medical potential than lower class drugs. However, this scheduling system is often criticized for being highly influenced by systemic racism and for scheduling drugs into high schedules without

sufficient research on the abuse and medical potential of those drugs (Nutt et al., 2007; Earp et al., 2021; Marks and Shachar, 2023).

Below, I will briefly detail the behavioral impact, mechanism of action, and current pharmacological treatment options of the main classes of addictive drugs that impact Americans.

Alcohol

The 2023 National Survey on Drug Use and Health reported 47.5% of Americans over the age of 12 had consumed alcohol within the past month of being surveyed and 10.2% of Americans had suffered from an alcohol use disorder in the past year (Administration, 2024). Alcohol is likely the most known addictive drug and includes hard liquor, beer, wine, etc. Alcohol is classified as a sedative (although is also described as a stimulant), acts primarily on GABA-A receptors, and has no legal classification (Koob et al., 2014b). Current pharmacological treatments for alcohol use disorder include Acamprosate, Naltrexone, Disulfiram, Gabapentin, and Topiramate (Volkow, 2020).

Nicotine

The 2023 National Survey on Drug Use and Health reported 17.6% of Americans over the age of 12 had consumed tobacco products within the past month of being surveyed and 9.4% had consumed nicotine vapor (Administration, 2024). Nicotine is classified as a stimulant, acts on nicotinic receptors, and has no legal classification (Koob et al., 2014c). Current pharmacological treatments for nicotine use include nicotine replacement therapy (such as nicotine patches), Varenicline, and Bupropion (Volkow, 2020).

Cannabinoids

The 2023 National Survey on Drug Use and Health reported 15.4% of Americans over the age of 12 had used marijuana within the past month of being surveyed and 6.8% of Americans had suffered from a marijuana use disorder in the past year (Administration, 2024). Cannabinoids include marijuana and THC products, act on cannabinoid receptors, and are currently scheduled

as a schedule I drug (Koob et al., 2014d). However, there are currently ongoing processes to reschedule cannabinoids down to schedule III (Administration and Justice, 2024). Current pharmacological treatments for marijuana use disorder: none.

Opioids

The 2023 National Survey on Drug Use and Health reported 3.1% of Americans over the age of 12 had misused opioids within the past month of being surveyed and 2% of Americans had suffered from an opioid use disorder in the past year (Administration, 2024). The category of opioids can include both prescription and non-prescription drug use. Drugs categorized as opioids include morphine, heroin, fentanyl, etc. All opioids act on opioid receptors but have varying affinity to the different subtypes (kappa, mu, and delta opioid receptors) (Koob et al., 2014e). Different opioids are currently under different schedules, for example, heroin is schedule I while morphine is schedule II. Current pharmacological treatments for opioid use disorder include Methadone, Buprenorphine, extended-release Naltrexone, Lofexidine, and Naloxone (Volkow, 2020).

Psychostimulants

The 2023 National Survey on Drug Use and Health reported 1.8% of Americans over the age of 12 had used cocaine and 0.9% used methamphetamine within the past month of being surveyed and 0.4% of Americans had suffered from a cocaine use disorder and 0.6% from a methamphetamine use disorder in the past year (Administration, 2024). Three drugs are mainly categorized and referred to as psychostimulants: cocaine, methamphetamine, and amphetamine. Psychostimulants act on the dopamine, serotonin, and norepinephrine systems (to varying degrees) (Koob et al., 2014f). All three inhibit the reuptake of dopamine, serotonin, and norepinephrine from the synapse. Methamphetamine and amphetamine additionally increase the amount of monoamine in the synapse through reversing the reuptake transporter and binding to vesicular transporters. Current pharmacological treatments for psychostimulant use disorder: none.

Treatment Approaches

While there are many approaches to combating SUD, most approaches can be classified into two main categories: Abstinence Only Based Approaches and Harm Reductionist Approaches.

Abstinence Only Based Approaches

In relation to SUD treatment, abstinence is typically defined as the complete and sustained cessation of all psychoactive drug use. As the name suggests, abstinence only based approaches prioritize abstinence from drug use, with abstinence being a requirement for receiving treatment and maintaining abstinence being the only goal of treatment (Marlatt et al., 2001). Abstinence only based approaches are often driven by the moral model of addiction which views consuming any amount of psychoactive drugs, particularly illicit drugs, as immoral and a failing of the individual (Philip et al., 1982). Most rehabilitation centers in the United States utilize an abstinence only approach. Entry into these facilities requires complete cessation of drug use, continuation of treatment is contingent on remaining abstinent, and success of treatment is evaluated by the longevity of abstinence after leaving the facility. Under this model, relapse is viewed as a complete set-back and failure of treatment. Another prime example of an abstinence only based approach is the 12-step model of Alcoholics Anonymous / Narcotics Anonymous (AA / NA). At the center of these approaches is an all-or-nothing model where there is no room for moderate improvement, you either are abstinent (treatment is working / you are acting morally) or you are not abstinent (treatment failed / you are immoral). Abstinence only based approaches have certainly been helpful for a number of individuals, but they have a high cost of entry (abstinence), can result in cessation of treatment after only minor relapse, and may not align with the current goals of individuals at the time (being completely abstinent). These factors result in a treatment barrier too high for many individuals who use drugs to overcome (Marlatt et al., 2001).

Harm Reductionist Approaches

As the name suggests, harm reductionist approaches are wholly centered on reducing harm. Programs under a harm reductionist approach to SUD meet individuals where they are and help them achieve their own goals without judgement. In contrast to the all-or-nothing approach of abstinence only based programs, harm reduction programs approach SUD treatment as an ongoing journey of reducing harm seeing any steps towards harm reduction as a success and do not penalize individuals for backslides (Marlatt et al., 2001). While abstinence can be a goal in a harm reductionist approach if abstinence is the goal of the individual using drugs, harm reductionist approaches do not require abstinence for treatment and do not force abstinence as a goal. Reducing harm can be achieved in a plethora of ways and includes reducing both harm to the individual and society. Importantly, reducing harm is not synonymous with reducing drug consumption although the two can be correlated at times. Syringe exchange programs are just one example of harm reduction that does not inherently involve reduction in drug consumption. Syringe exchange programs give individuals who inject drugs easy access to sterile syringes with the goal of reducing the risk of HIV and hepatitis transmission/infection. Other common examples of harm reduction approaches to SUD include drug safety testing services, Narcan distribution, housing first, safe injection sites, and substitution therapy (such as methadone). Harm reductionist approaches strive to work with the community it serves and have a low barrier to entry allowing more individuals to receive and maintain treatment than with abstinence only based approaches (Klein, 2020; Paquette et al., 2022).

Theoretical Frameworks of SUD

Similar to the mystery of the neurobiological underpinnings of SUD, the psychological and overall driving factors of SUD (regardless of their neurobiological basis) are also still unclear / debated. As new evidence emerges, data is typically contextualized under a specific theoretical framework or used to support / not support specific theoretical frameworks. Realistically, SUD is a complex human condition within a population of complex, nonhomogeneous individuals. For

one individual, the combination of multiple theoretical frameworks with varying weights may be able to accurately describe the motivating factors behind their SUD and predict their future behavior. Alternatively, SUD in some individuals may be well described using a single theoretical framework while the SUD in a different set of individuals may be well described using a different theoretical framework. Below, I will briefly detail two of the main theoretical frameworks for SUD (note this list is not exhaustive).

Negative Reinforcement

The negative reinforcement framework of SUD is present at the core of many conceptualizations of SUD, such as: opponent process theory, allostasis, casual to compulsive drug taking, spiraling distress, etc. Naturally, these ideas have a lot of overlap and build on top of one another. Overall, the negative reinforcement framework of SUD postulates that over the course of the development of SUD an individual's motivation for drug consumption transitions from positive reinforcement to negative reinforcement (Koob and Moal, 2008). Initially, an individual consumes drugs for the euphoric, pleasurable effects of the drug. Over time and after repeated drug use, the individual begins to consume drugs to escape the negative withdrawal effects that results in not consuming drugs. In the spiraling distress hypothesis, this is described as the preoccupation/anticipation, binge/intoxication, withdrawal/negative affect cycle of SUD (Koob and Moal, 2001). The negative reinforcement framework often presumes that continued drug-taking in SUD is driven primarily through withdrawal mechanisms.

The opponent process theory is often utilized to describe the potential underlying biological processes of the negative reinforcement model (Solomon, 1980). In opponent process theory, as is relevant to addictive drugs, the drug evokes a positive α-process. In attempt to maintain homeostasis, biological systems then induce a β-process to counteract the drug-induced α-process. Over repeated drug exposures, the α-process remains the same (assuming the same amount of drug), while the β-process grows in magnitude. This is also sometimes used as a biological conceptualization for the phenomenon of tolerance to drug effects. Additionally, the β-

process outlasts the time of the α-process, producing negative affect / withdrawal effects. The allostasis theory extends the opponent process theory by positing a change in the homeostatic setpoint (termed allostatic point) that is outside the normal homeostatic range and maintained by constant active processes and altered setpoints of several biological systems (Koob and Moal, 2001). Generally, the opposing processes described under this theory are canonical stress systems, such as dynorphin and corticotropin releasing factor. Under the allostasis theory, individuals suffering from SUD are in a constant reward-deficient state that motivate continued and increased drug consumption to compensate and relieve withdrawal symptoms. Typically, negative reinforcement frameworks view SUD being driven by a hypodopaminergic state. One of the main criticisms of this framework is its inability to explain relapse behavior that occurs long after withdrawal symptoms have subsided.

Incentive Sensitization

Robinson and Berridge first introduced the incentive sensitization theory of SUD in 1993 (Robinson and Berridge, 1993). Incentive sensitization posits that SUD is a result of the sensitization of neural systems that are activated during drug consumption. More specifically, incentive sensitization theory focuses on the sustained increase in the incentive salience of drug-associated cues, even long after a period of drug consumption, resulting in increased drug-taking and chronic relapse. This theory relies on a few key principles: 1) addictive drugs increase dopamine and this increase is conferred to drug-associated cues, 2) repeated drug use evokes sensitization, including sensitization of dopamine release, and 3) dopamine mediates the psychological phenomenon of ‘wanting’ and not the perception of ‘liking’ / hedonic impact of addictive drugs (Robinson and Berridge, 2025). The incentive sensitization theory describes a change in the ratio of ‘wanting’ to ‘liking’ based on increases in ‘wanting’ without any assumptions on the changes of ‘liking.’ Therefore, this framework does not disagree with the concept brought up by the negative reinforcement framework that individuals may initially consume drugs because they enjoy the euphoria and that this is later dissipated after chronic drug use. Furthermore, this

theory offers a biological framework to describe the human experience of being unable to abstain from or decrease drug consumption despite a real desire. Additionally, in accordance with various human accounts that state drugs are still enjoyable even after chronic use, the perceived liking of a drug does not need to decrease to support the incentive sensitization theory of SUD. In regard to relapse, this framework details how drug-associated cues maintain strong motivational power / incentive salience even after long periods of abstinence to ultimately result in relapse behavior without the need for withdrawal symptoms. Overall, this framework also suggests the SUD is driven by a hyperdopaminergic state, in contrast to the negative reinforcement framework.

Behavioral Models

Working with human subjects has a myriad of limitations especially when it comes to studying the neurobiology of SUD and drug consumption. While clearly human research is important and necessary to further understand the population of interest and assess the effectiveness of treatment, non-human research allows scientists more control in their experimentation and offers a wider range of experimental tools to employ. Additionally, using non-human subjects allows for research that would otherwise be considered unethical, such as studying the ability of factors to cause relapse. There is a multitude of animal models utilized in SUD research including c. elegans, drosophila melanogaster, rodents, and non-human primates. Different animal models serve a unique purpose in the study of addictive drugs and SUD. For the purpose and scope of this dissertation, I will focus primarily on rodent models of SUD.

In order to uncover the neurobiological mechanisms underlying SUD, scientists develop and utilize behavioral models. SUD is complex with a wide range of phenotypes. Focusing on one or a few SUD phenotypes, as described by the DSM-V, when designing a behavioral model or experimental design can increase the interpretability of the results. Since a single SUD phenotype does not warrant a SUD diagnosis and defining what SUD looks like in non-humans can be ambiguous, I will refer to phenotypes that are studied to mimic human SUD as “SUD-like

phenotypes." However, multiple behavioral assays measuring multiple SUD-like phenotypes can be employed in the same experimental design to bolster the belief that an animal is exhibiting a SUD. Below, I will briefly detail several common behavioral models used to study addictive drugs. Note that this list is non-exhaustive, and the focus biases towards behavioral models that are commonly utilized to study psychostimulants (although not exclusively) as those are most relevant here.

Experimenter-Administered Drug

Experimenter-administered drug refers to experiments in which an addictive drug is administered to an animal by the experimenter involuntarily and typically non-contingently. While experimenter-administered drug does not refer to a behavioral model inherently, it is often used in combination with behavioral assays and has been pivotal in understanding the pharmacological effects of addictive drugs as well as basic neurobiological effects. Behavioral assays that may be used in combination include open field, elevated plus maze, rotarod, forced swim, etc. Experimenter-administered drug is also often used to systematically facilitate drug dependency in animals as well as precipitated withdrawal following drug dependency. In both cases, behavioral and/or neurobiological metrics can be measured to understand the behavioral/neurobiological impact. Another common behavior studied with this method is behavioral sensitization/tolerance.

This method of drug administration offers a lot of experimental control (such as amount and timing of drug intake). However, involuntary drug intake is not representative of most human drug consumption and even less representative of SUD. Therefore, while this method is very useful for basic pharmacological understanding, there are strong limitations to its translatability to SUD. Additionally, there is strong evidence showing differences in the neurobiological changes in animals that underwent non-contingent versus contingent (such as self-administration) drug administration (Jacobs et al., 2003).

Conditioned Place Testing

Conditioned place testing (CPT) is a behavioral assay to measure valence of a substance and can also be used to measure relative changes in the magnitude of the valence. Typically, CPT is performed in a 2-chamber box with each chamber having distinct attributes (flooring texture, wall patterning, etc). The procedure consists of many sessions, typically 1-2 sessions per day. During the first session, the animal is allowed to explore the entire box and time spent in each chamber is measured. This session has two main purposes: 1) to habituate the animal to the box and 2) to determine if the animal has an inherent bias toward one chamber or the other. Afterwards, one chamber is assigned the “substance chamber” (a substance such as an addictive drug) and the other chamber is assigned the “vehicle chamber” (vehicle such as saline). The animal is then put through one or more pairings of the substance/vehicle and its associated chamber. During pairing sessions, the animal is injected with the substance/vehicle and then placed in the box with its access restricted to only the associated chamber (substance or vehicle chamber). Finally, the animal undergoes a test session during which the animal is placed back in the box again with free access to all chambers. The amount of time spent in each chamber is measured and evaluated for a preference for one side or another. If the animal spends more time in the substance chamber than the vehicle chamber, the substance is deemed to produce conditioned place preference (CPP) and be rewarding [positive valence]. If the animal spends less time in the substance chamber than the vehicle chamber, the substance is deemed to produce conditioned place aversion (CPA) and be aversive [negative valence]. If the animal spends equal amounts of time in the substance and vehicle chamber, the substance is deemed neither rewarding nor aversive [neutral valence]. The magnitude of CPP – as measured by time spent in the substance chamber – can also be used to assess and compare reward magnitude following intervention.

CPT involves experimenter-administered drug and therefore has many of the same limitations as described in the above section. An additional limitation of CPT is the reliance on the

animal's learning and memory during the task. Interventions that impact learning or memory will provide results indistinguishable from the results of interventions that impact drug reward.

Self-Administration

Drug self-administration (SA) is the current gold standard for studying SUD with animal models (Kuhn et al., 2019). Drug SA is an operant task where the animal has volitional control over how much drug they take and when they take it – within the parameters of the specific experimental design. Typically, for a given drug SA session, the animal is placed into an operant chamber where they have access to a manipulandum (such as a nose-poke port or lever) that when interacted with (operant response) results in the delivery of drug. The method of drug delivery varies including intravenous, intraoral, and through a pellet or liquid dispenser. Typically, drug delivery also coincides with cues (audio, visual, or both) and a time-out period during which additional operant responses do not result in additional drug. Other cues, such as discriminative cues signaling drug availability, are also often present. There is also usually at least one other manipulandum that has no programmed consequences available for the animal to interact with. Within this behavioral assay, there are multiple experimental design aspects that can be altered to impact the animal's performance or to measure different underlying behaviors/motivations. This includes but is not limited to changing: the total length of the task, the ratio of operant responses to drug, the time-out period, the number of different operant responses necessary to receive drug, and the timing of availability to drug.

There are two main variations of drug SA that differ in their total length of the task: short access and long access. Short access is typically about 1 hour long while long access is typically about 6 or more hours long. Everything else about the assay is identical. Long access drug SA is often used to study the SUD-like phenotype of escalation of drug consumption. In cocaine SA, rats undergoing short access conditions maintain stable rates of responding for cocaine over sessions, while rats undergoing long access conditions begin to increase in their responding for cocaine over sessions (Koob and Ahmed, 1998). Additionally, it has been demonstrated that

under long access conditions, only a subset of rats escalate in their cocaine intake, displaying an SUD-like phenotype – similar to the fact that not all humans that consume addictive drugs ultimately develop a SUD (Willuhn et al., 2014). Another variation of drug SA that is used for studying escalation is intermittent access. Intermittent access is argued by some to be more resemblant of human drug-taking patterns (Zimmer et al., 2011; Allain et al., 2015). In this variation, the timing of drug availability is altered. Animals have access to the drug in short bouts (about 5 minutes) interleaved with longer bouts of no drug availability (about 25 minutes). The total task lasts around 3 hours typically. Intermittent access has also been demonstrated to induce escalation of drug intake during the drug available time windows.

The ratio of operant responses to drug can also be altered in drug SA. In a fixed ratio schedule, a fixed number of responses are necessary for every fixed amount of drug. For instance, an FR5 means that the animal needs to perform the operant action five times to get one volume of drug. In a fixed interval schedule, a fixed interval of time exists between each drug availability period following an operant response. For instance, an FI10 could mean that the animal can only get one drug delivery every ten minutes. Alternatively, you can also have a non-fixed ratio. One standard example is a progressive ratio design. In progressive ratio, the number of operant responses necessary for one drug delivery increases in a semi-logarithmic fashion over the course of the session. Unlike a fixed ratio, a progressive ratio schedule is utilized to measure changes in motivation for the drug by measuring the maximum ratio the animal is willing to perform for reward (termed breakpoint). Another, more complex, version of drug SA that measures motivation is called behavioral economics.

The number of types of operant responses can also be altered in drug SA. All of the previous examples assumed a first-order operant task – where operant responses on only one manipulandum was necessary for drug delivery. However, there is also second-order (or more) operant conditioning. In second-order conditioning, an operant response on one manipulandum results in access to a second manipulandum that when interacted with results in the delivery of

drug. This design is typically used to separate out the appetitive and consummatory responses for intravenous delivery of drugs or to separate out the drug-seeking and drug-taking responses. In second-order drug SA, the first operant response is the appetitive / drug-seeking response, and the second operant response is the consummatory / drug-taking response.

Electric Barrier

Electric barrier is an addition to drug SA that aims to study the SUD-like phenotype of putting oneself in harm to obtain drug. An electric barrier drug SA task is the same as the drug SA behavioral design described above with the addition of an electric barrier separating a safe platform and the operant response. The animals have to cross the barrier and be shocked in order to respond for and receive the drug.

Punished Responding

Punished responding is an addition to drug SA that aims to study the SUD-like phenotype of continuing drug use in spite of negative consequences. This task is the same as the drug SA task described above with the addition of an electric foot shock (or other negative consequence) coinciding with operant response / drug delivery at varying probability.

Reinstatement

Reinstatement aims to study the SUD-like phenotype of relapse. Reinstatement models begin with drug SA as described above. After drug SA, the animal goes through extinction sessions which are the same as drug SA sessions but without the delivery of drug. Over extinction sessions, animals stop responding on the manipulandum that previous resulted in drug delivery demonstrating “extinguished responding.” After the animal extinguishes their responding, the animal undergoes a reinstatement session. The reinstatement session is the same as an extinction session with the addition of a prime that incites reinstatement of drug-seeking. There are three main ways to elicit reinstatement: non-contingent delivery of the drug, non-contingent

delivery of the drug-associated cue, and stress (pharmacological, such as yohimbine, or physical, such as foot shock) (Shaham et al., 2003).

Choice Models

Choice models are argued to better represent the world of an individual suffering from SUD, where drugs are not the only rewarding option available to them to consume. Choice models are also operant tasks similar to drug SA, however now the animal can perform operant responses on various manipulanda to choose different rewards such as drug, social interaction with a cage-mate, or palatable food. The introduction of choice has also been used as an alternative way to extinguish responding for drug in reinstatement assays (Venniro et al., 2018).

RELEVANT NEUROBIOLOGICAL BACKGROUND

Canonical Reward Pathway

It is postulated that SUD of any addictive drug is driven by similar underlying mechanisms. Therefore, understanding SUD as a whole should produce treatments effective for all types of SUD. This is particularly pertinent with the prevalence of polysubstance use (Crummy et al., 2020). While the direct actions of addictive drugs vary across pharmacodynamic class, most addictive drugs interact with the canonical reward circuitry in the brain (CHIARA and IMPERATO, 1988). Additionally, while this pathway is commonly and historically seen as a reward pathway, it is also heavily implicated in reinforcement and overall activation of motivated behaviors – all of which are essential and common among all SUDs. Below, I will briefly detail the main players in the canonical reward pathway (biased towards areas most relevant to this dissertation) and their implications in addictive drugs and SUD.

Ventral Tegmental Area

The ventral tegmental area (VTA) is a midbrain region and one of the two primary sources of dopamine in the brain. The cellular population consists of 60-65% dopamine-containing cells,

30-35% GABA-containing cells, and 2-3% glutamate-containing cells (Sesack and Grace, 2010). A subset of cells co-express and co-release more than one of these neurotransmitters (Morales and Margolis, 2017). The VTA is heterogenous in its inputs and outputs. Inputs to the VTA include the ventral pallidum, dorsal raphe nucleus, bed nucleus of the stria terminalis, prefrontal cortex, lateral habenula, rostromedial tegmental nucleus, nucleus accumbens, and periaqueductal grey, among others. Outputs from the VTA include the nucleus accumbens, lateral habenula, amygdala, hippocampus, bed nucleus of the stria terminalis, and prefrontal cortex, among others.

Nucleus Accumbens

The nucleus accumbens (NAc) is a brain region in the ventral striatum. The cellular population consists of 90% GABA-ergic medium spiny projection neurons (Meredith et al., 1992). Interneurons in the NAc are primarily cholinergic or GABA-ergic parvalbumin expressing neurons. There are two main populations of medium spiny neurons: D1 receptor-expressing and D2 receptor-expressing. The two populations have differences in their projection patterns and expression patterns of other receptors (Yager et al., 2015). The NAc can be split into the core and shell subdomains (including medial and lateral shell). These subdomains are distinct but overlapping in their inputs, outputs, and implications in behavioral dynamics (Heymann et al., 2020). Overall, inputs to the NAc include excitatory inputs from the prefrontal cortex, basolateral amygdala, and hippocampus, dopaminergic inputs from the ventral tegmental area and substantia nigra, and serotonergic inputs from the dorsal raphe nucleus, among others (Sesack and Grace, 2010). Overall, outputs from the NAc include the ventral pallidum, ventral tegmental area, substantia nigra, hypothalamus, and brainstem, among others.

Dopamine

Dopamine is a neurotransmitter produced by neurons with cell bodies located in the VTA and substantia nigra and is known to play a major role in reward, reinforcement, associative learning, and voluntary movement. Activation of VTA dopamine neurons supports intracranial self-stimulation procedure suggesting that dopamine neuron activity is rewarding and/or reinforcing

(Witten et al., 2011; Wolff and Saunders, 2024). A large subset of VTA dopamine neurons increase in activity in response to unpredicted rewarding stimuli, such as sucrose (Schultz et al., 1997). These neurons also increase in activity in response to cues that predict rewarding stimuli. When an expected reward is omitted, dopamine signaling decreases. Additionally, the magnitude of the phasic dopamine response is correlated to the size of the expected reward (Tobler et al., 2005). This pattern of signaling is known as reward prediction error (RPE) and is believed to act as a teaching signal to guide reward-related behaviors (Schultz et al., 1997). The ability of phasic dopamine release to extend back to reward-associated / reward-predictive cues, as also detailed by temporal difference model, is imperative to the incentive sensitization theory described earlier (Sutton and Barto, 1990). In an operant task such as drug self-administration, initially the delivery of drug evokes a phasic dopamine response in the NAc (unexpected reward) but over time as the association between the cue and the drug reward is developed, the drug-associated cue elicits a phasic dopamine response on its own(Phillips et al., 2003). This process is believed by some to imbue the cue with incentive salienc e(Robinson and Berridge, 2008). Additionally, stimulation of dopamine release into the NAc during drug self-administration induces drug-taking (Phillips et al., 2003).

In relation to addictive drugs specifically, dopamine is one of the systems that all addictive drugs impact, whether directly or indirectly, regardless of their pharmacodynamic class. All addictive drugs that have been tested elicit an increase in dopamine in the NAc and this increase is believed to confer addictive drugs their addiction liability (CHIARA and IMPERATO, 1988). During drug self-administration, levels of NAc dopamine can be used to predict drug-taking behavior as animals try to maintain a set desired dopamine concentration (Wise et al., 1995). Intra-NAc injection of a dopamine D1 antagonist increases cocaine self-administration on an FR1 schedule and decreases breakpoint on a progressive ratio schedule demonstrating the necessity of dopamine to the rewarding effects of cocaine (Maldonado et al., 1993; McGregor and Roberts, 1993).

In particular relevance to the work presented in this dissertation, previous research demonstrated that the phasic dopamine response in the NAc following a contingent cue delivery during long access cocaine self-administration decreases across behavioral sessions in direct correlation to the amount of cocaine escalation (Willuhn et al., 2014). Blocking this decrement in dopamine response through pretreatment with L-DOPA, the dopamine precursor, blocked escalation of cocaine consumption and treatment with L-DOPA following escalation of cocaine consumption reversed escalation. Additionally, replacing the contingent cue phasic dopamine response with optogenetic stimulation of VTA terminals in the NAc in animals that escalated their cocaine consumption resulted in decreases in consumption (Burgeno et al., 2023). These data demonstrate that the decrement of contingent cue elicited phasic dopamine release in the NAc is causally linked to the SUD-like phenotype of escalation of drug consumption.

Dynorphin / Kappa Opioid Receptor System

Kappa opioid receptors (KOR) are one of the three classes of opioid receptors (κ, kappa; μ, mu; δ, delta). KOR is a GPCR coupled to Gi and has a distinct pattern of expression in the central nervous system compared to the other opioid receptors (Mansour et al., 1994; Bruchas et al., 2010). The assumed endogenous ligand for KOR is the neuropeptide dynorphin (Chavkin et al., 1982). Dynorphin has affinity for all three classes of opioid receptors, but its affinity is strongest for KOR. Dynorphin-containing cells are expressed throughout the central nervous system including the canonical reward pathway (Watson et al., 1982; Koob, 2008).

The dynorphin/KOR system has been implicated in general negative affect and stress-induced behaviors (Bruchas et al., 2010). Systemic KOR agonism produces conditioned place aversion – suggesting negative valence (Mucha and Herz, 1985). Stressful stimuli modulate dynorphin levels in various brain regions (Nabeshima et al., 1992). Activation of the KOR system is necessary for stress-induced real time place avoidance (Land et al., 2008).

Implications in Substance Use Disorder

Drugs of abuse and the KOR system interact with each other. Cocaine increases prodynorphin mRNA in humans and rodent NAc (Daunais and McGinty, 1994; Hurd and HERKENHAM, 1995). Changes in dynorphin levels through under/overexpression of CREB in the NAc shell impacts cocaine reward, where underexpression of CREB results in higher levels of dynorphin and cocaine CPP and overexpression of CREB results in lower levels of dynorphin and cocaine CPA, which is blocked via a KOR antagonist (Carlezon et al., 1998). Blocking KOR activity via a long-lasting antagonist decreases consumption for several drugs of abuse including methamphetamine, heroin, and alcohol (Walker et al., 2011; Schlosburg et al., 2013; Whitfield et al., 2015). Additionally, KOR activation is capable of decreasing stimulated dopamine release in the NAc (Ehrich et al., 2015). These data demonstrate a relationship between drugs of abuse, KOR activation, and subsequent drug-taking behavior.

KOR has also been implicated in changes in drug-taking behavior resulting from stress and withdrawal. KOR activation increases ethanol consumption and blockade of KOR decreases anxiety-like behaviors during ethanol withdrawal (Rose et al., 2016). Blocking KOR during chronic experimenter-administered cocaine injections blocks the increase in intracranial self-stimulation threshold commonly seen during cocaine withdrawal (Chartoff et al., 2012). Additionally, KOR activation is necessary and sufficient for stress-induced cocaine CPP (McLaughlin et al., 2003, 2006; Abraham et al., 2022). Taken together with the KOR system's general involvement in negative affect, these data suggest the dynorphin/KOR system to potentially contribute to the β-process of opponent process theory (Wee and Koob, 2010).

Corticotropin-Releasing Factor / CRF Receptor System

Corticotropin-Releasing Factor (CRF), also known as corticotropin-releasing hormone, is a neuropeptide commonly referred to as a stress hormone. The CRF system is composed of two main categories of receptors (CRF-R1 and CRF-R2) and a family of ligands (CRF and the

urocortin family – UcnI, UcnII, and UcnIII) (Bale and Vale, 2004). Both CRF-R1 and CRF-R2 are G-protein coupled receptors predominantly coupled with G_s. CRF has a higher affinity to CRF-R1, UcnI has equal affinity for CRF-R1 and CRF-R2, and UncII and UncIII have higher affinity for CRF-R2. The remainder of this discussion will focus primarily on CRF and CRF-R1/R2. The expression pattern of CRF-R1 and CRF-R2 mRNA is largely non-overlapping, with CRF-R1 mRNA expression mainly in neocortical, cerebellar, and sensory structures and CRF-R2 mRNA expression mainly restrained to the lateral septum, ventromedial hypothalamus, and mesencephalic raphe nuclei (Pett et al., 2000). Areas of overlapping expression include the extended amygdala, hypothalamus, hippocampus, olfactory areas, and brainstem. CRF-expressing neurons are found in the paraventricular nucleus of the hypothalamus (hypothalamic CRF) as well as the brain regions in the basal telencephalon, brain stem, and cerebral cortex (extra-hypothalamic CRF) (Swanson et al., 1983).

CRF-expressing neurons in the paraventricular nucleus of the hypothalamus are stimulated by stressful stimuli resulting in the release of CRF and activation of the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis ultimately results in the increase in cortisol/corticosterone and activation of glucocorticoid systems to suppress the stress-response in a negative-feedback loop. As such, the CRF system has been strongly linked to stress and anxiety-like behaviors (Bale and Vale, 2004; Chen et al., 2012). Relatedly, CRF is often seen as producing negative valence due to systemic CRF administration resulting in conditioned place aversion (Cador et al., 1992). However, local injections of CRF in specific brain regions can result in either conditioned place aversion or preference, and this valence can be dependent on prior experience (Sahuque et al., 2006; Land et al., 2008; Lemos et al., 2012).

Implications in Substance Use Disorder

Most drugs of abuse activate hypothalamic and extrahypothalamic CRF. In humans, cocaine increases cortisol levels (HEESCH et al., 1995). In rats, cocaine increases corticosterone levels via activation of the CRF system in the hypothalamus (Sarnyai et al., 1992). Additionally,

cocaine self-administration results in changes in CRF system activity in several brain regions (Goeders et al., 1990). Chronic cocaine has been shown to impact the ability of CRF to modulate dopamine in the lateral septum and ventral tegmental area (Hahn et al., 2009; Sotomayor-Zárate et al., 2013; Oliva et al., 2021). CRF has also been shown to impact drug consumption directly. Delivery of a CRF-R1 antagonist intracerebrally blocks cocaine CPP and decreases the ability of a cocaine injection to increase dopamine in the NAc and VTA (Lu et al., 2003). Systemic CRF-R1 antagonist injection reduces cocaine and heroin consumption, particularly in long access animals (Goeders and Guerin, 2000; Specio et al., 2008; Greenwell et al., 2009). These studies demonstrate an inherent interplay between drugs of abuse and the CRF systems – both having an impact on each other.

Combining the role of CRF in stress and drug consumption, CRF has also been implicated in stress-induced drug behaviors. Stress-induced enhancement of cocaine reward, measured via CPT, is contingent on CRF-R1 activation during the stressor (Kreibich et al., 2009). Stress potentiates cocaine acquisition, and this effect is correlated with corticosterone levels (Goeders and Guerin, 1993). Stress-induced reinstatement of cocaine and heroin seeking is dependent on CRF activation (Shaham et al., 2000). These studies underline the potential for CRF to be involved in promoting drug consumption when an individual is faced with adverse life events.

CRF has also been implicated in mediating withdrawal symptoms. Anxiety-like withdrawal symptoms following chronic experimenter-administered cocaine is dependent on CRF (Sarnyai et al., 1995). CRF levels in the central amygdala increase during acute withdrawal following cocaine self-administration (RICHTER and WEISS, 1999). Increases in brain reward threshold following nicotine withdrawal are blocked by local CRF-R antagonism in the nucleus accumbens shell (Marcinkiewcz et al., 2009). Taken together with CRF's role in stress, these data indicate the CRF system as being a possible main player in the β-process in negative reinforcement models of SUD (Koob, 2010).

Notably, CRF has also been implicated in promoting incentive salience. Local injection of CRF into the medial nucleus accumbens shell increases incentive salience for sucrose in a Pavlovian instrumental transfer task (Peciña et al., 2006). CRF-expressing cells in the nucleus accumbens and central amygdala support intracranial self-stimulation (Baumgartner et al., 2021). Additionally, stimulations of these cells promote sucrose and cocaine pursuit and increases motivation demonstrated through increased breakpoint on a progressive ratio schedule (Baumgartner et al., 2021, 2022). These data highlight the heterogeneity of the CRF system to potentially underline several mechanisms of operant behavior and SUD.

OVERALL THESIS

This dissertation investigates the neurobiological mechanisms underlying the development and sustainment of escalation of drug intake utilizing a rat long access cocaine self-administration model. Previous work has demonstrated a relationship between escalation and the phasic dopamine signal in the NAc in response to a contingent cue, where a decrement in the dopamine signal drives escalation (Willuhn et al., 2014; Burgeno et al., 2023). The systems at play to result in this decrement in dopamine signaling is unknown. I present in this dissertation two possible candidates: dynorphin and corticotropin releasing factor systems. Both systems are capable of modulating dopamine in the NAc (Lemos et al., 2012; Ehrich et al., 2015); addictive drugs activate both systems (Daunais and McGinty, 1994; HEESCH et al., 1995; Hurd and HERKENHAM, 1995); both systems can impact drug-taking and/or drug reward (Carlezon et al., 1998; Lu et al., 2003); and both systems have been proposed to play a role in the β-process of opponent process theory due to their role in withdrawal symptoms (Koob et al., 2014g). Additionally, evidence suggests interactions between the two systems. Activation of CRF receptors increases pCREB in neurons (Kreibich et al., 2009) and increases in CREB are known to increase dynorphin (Carlezon et al., 1998), thus suggesting activation of CRF receptors leading to increases in dynorphin production which when released results in increases in phosphorylated

KOR. Additionally, intracerebral CRF increases phosphorylated KOR, and this activation of KOR is necessary for intracerebral CRF to evoke CPA (Land et al., 2008). The overarching hypothesis of this dissertation is that increases in CRF in the NAc leads to the increase of dynorphin release in the NAc to act on KOR on VTA terminals in the NAc to inhibit dopamine release and result in escalation of drug intake. Importantly, however, while there is evidence to suggest CRF and KOR may mediate escalation of cocaine consumption, the necessity of these systems in the NAc for these behavioral effects – irrespective of the potential system interactions with each other and with dopamine– have not been studied and must first be addressed. Chapter 2 investigates the necessity of KOR activation in the NAc for the development of escalation. Chapter 3 investigates the necessity of CRF-R1 in the development and sustainment of escalation. Additionally, a majority of the literature has been performed in only male animals. Chapter 4 presented here will detail differences (or lack there-of) in cocaine self-administration behavior as a whole between sex through an analysis of control animals found elsewhere in this dissertation. Overall, the work presented here furthers our understanding of the neurobiology driving escalation of drug consumption to inform future harm reductionist therapeutic approaches to SUD.

FIGURES

Criteria for SUD
Hazardous Use
Social/Interpersonal Problems Related to Use
Neglected Major Roles to Use
Used Larger Amounts / for Longer Period of Time
Unsuccessful Repeated Attempts to Quit or Control Use
Large Amount of Time Spent Using
Activities Given up to Use
Physical/Psychological Problems Related to Use
Withdrawal
Tolerance
Craving

Table 1. Criteria for Substance Use Disorder

List of the 11 behavioral (purple) and physiological (blue) criteria for substance use disorder.

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Chapter 2:

Kappa Opioid Receptors in Mesolimbic Terminals Mediate Escalation of Cocaine Consumption

L. Gordon-Fennell*, R.D. Farero*, L.M. Burgeno*, N.L. Murray, A.D. Abraham, M.E. Soden, M Trzeciak, G.D. Stuber, C. Chavkin, L.S. Zweifel, P.E.M. Phillips. *Kappa Opioid Receptors in Mesolimbic Terminals Mediate Escalation of Cocaine Consumption*. bioRxiv 2023.12.21.572842; doi: https://doi.org/10.1101/2023.12.21.572842

* authors contributed equally

ABSTRACT

Increases in drug consumption over time, also known as escalation, is a key behavioral component of substance use disorder (SUD) that is related to potential harm to users, such as overdose. Studying escalation also allows researchers to investigate the transition from casual drug use to more SUD-like drug use. Understanding the neurobiological systems that drive this transition will inform therapeutic treatments in the aim to prevent increases in drug use and the development of SUD. The kappa opioid receptor (KOR) system is typically known for its role in negative affect, which is commonly found in SUD as well. Furthermore, the KOR system has also been implicated in drug use and importantly, modulating the negative effects of drug use. However, the specific neuronal subpopulation expressing KOR involved has not been identified. Here, we first demonstrated that pharmacologically inhibiting KOR in the nucleus accumbens core (NAcC), as a whole, blocks cocaine escalation under long-access self-administration conditions. We then demonstrated that KOR expressed on ventral tegmental area (VTA) neurons but not

NAcC neurons is sufficient for blocking cocaine escalation by utilizing a novel virally-mediated CRISPR-SaCas9 knock-out of the *oprk1* gene. Together, this suggests that activation of KOR on VTA terminals in the NAcC drives the transition to the SUD-like phenotype of escalation of cocaine consumption.

INTRODUCTION

Substance use disorder (SUD) and harmful drug consumption is an ongoing epidemic, with currently no treatment options for cocaine use disorder specifically. One key component of SUD is the transition from casual to compulsive drug-taking, often coupled with an increase in overall drug consumption which leads to a higher likelihood of harm to the user. This SUD-like phenotype, termed escalation, can be modelled in animals utilizing a long-access (six-hour) drug self-administration (SA) paradigm, allowing researchers to probe the neurobiological processes underlying this transition to inform future therapeutics (Koob and Ahmed, 1998).

The kappa opioid receptor (KOR) / dynorphin system is implicated in components of SUD including stress (McLaughlin et al., 2003, 2006; Abraham et al., 2022), withdrawal (Chartoff et al., 2012; Rose et al., 2016), and overall negative affect (Koob, 2013). Cocaine itself increases prodynorphin mRNA in the nucleus accumbens (NAc) (Daunais and McGinty, 1994; Hurd and HERKENHAM, 1995), a brain region implicated in SUD that receives dense dopaminergic innervation from the ventral tegmental area (VTA). Dopamine dynamics, particularly in the NAc, are associated with the rewarding and reinforcing properties of most addictive substances (CHIARA and IMPERATO, 1988; Wise et al., 1995; Phillips et al., 2003). Activation of KOR, a Gi-coupled receptor, inhibits dopamine release in the NAc (Ehrich et al., 2015). Both medium spiny neurons in the NAc and VTA dopamine terminals express KOR (George et al., 1994; Mansour et al., 1994; Rosin et al., 1999; Svingos et al., 2001). The ability of KOR to modulate dopamine release in the NAc and KOR's role in aversive phenotypes underlines its potential to be a key system in the transition to compulsive drug-taking. Prior studies have already demonstrated that

pharmacological inhibition of KOR systemically and locally in the NAc shell blocks escalation of heroin (Schlosburg et al., 2013) and methamphetamine (Whitfield et al., 2015) consumption. However, the subpopulations of KOR within the NAc responsible for this effect have yet to be studied.

In this paper, we examined the role of KOR in the NAc core (NAcC) in the progression of cocaine escalation. We utilized the long-lasting KOR antagonist, norBNI, to pharmacologically inhibit KOR in the NAcC during long-access cocaine SA. To determine if KOR mediates effects through pre-synaptic or post-synaptic activation, we also utilized a novel CRISPR-SaCas9 construct targeting the oprk1 gene to knock-out KOR from NAcC medium spiny neurons or VTA neurons prior to long-access cocaine SA. Through these experiments, we found that KOR activation on VTA terminals in the NAcC, but not medium spiny neurons of the NAcC, is necessary for escalation of cocaine consumption.

METHODS

Subjects

Male and female Wistar rats were purchased from Charles River and singly housed on a 12-hr light-dark cycle (lights on at 7:00) with chew-toy enrichment and *ad libitum* access to laboratory chow and water. Upon arrival, male rats weighed 250-275g and female rats weighed 200-225g. All behavioral experiments were performed during the light cycle. The pharmacological inhibition of KOR with norBNI was performed solely in male rats. All other experiments were performed in both female and male rats. All animal use and procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Surgical Procedures

Animals were acclimated in the housing facility for at least one week before surgical procedures were performed. For rats that underwent multiple surgeries, there was a minimum of

one week recovery period between the surgeries. All surgeries were performed utilizing aseptic technique. Anesthesia was induced and maintained with isoflurane (1-5%). An NSAID (Meloxicam, 1mg/mL/kg, in 3mL total saline, s.c.) was administered on the day of surgery and post-operative day 1. Surgical areas were shaved and cleaned with alternating ethanol and betadine.

Cannula Implantation

Custom 26G bilateral guide cannulas with a 2.6mm center-to-center distance and 7mm projection were purchased from Plastics One (8IC235G26XXC). The rat was fixed in a stereotaxic frame (Kopf), the head was leveled, and burr holes were drilled overlaying our target region. We lowered the guide cannula 1mm dorsal to the target region (**NAcC** angle: 0˚, AP: 1.3mm, ML: +/- 1.3mm, DV: -7.2mm) and secured the implant to the skull with dental cement. A dummy cannula with 0mm projection (Plastics One) was inserted and a dustcap (Plastics One) was screwed onto the guide cannula to block contaminates.

Viral Injection

The rat was fixed in a stereotaxic frame (Kopf), the head was leveled, and burr holes were drilled overlaying our target regions. A Hamilton syringe (7105KH 5.0ul SYR (24/2.75”/3), ref#: 88000) operated by Micro4 Microsyringe Pump was used to deliver the viral vector (1µL per site at 250nL/min). We injected a 1:8 viral cocktail of AAV1-Cre-GFP and AAV1-FLEx-SaCas9-U6-HA-sgOprk1 (oprk1 knock-out CRISPR) or AAV1-FLEx-SaCas9-U6-HA-sgGabrg2 (control CRISPR). We lowered the Hamilton syringe 0.2mm ventral to the target region (**NAcC** angle: 0˚, AP: 1.3mm, ML: +/- 1.3mm, DV: -7.2mm ; **VTA** angle: 0˚, AP: -6.35mm, ML: +/- 0.5mm, DV -8.5mm; distances measured from bregma), waited for 2min, began the 4min injection while raising the syringe to 0.2mm dorsal to the target DV over the first minute, and then waited 8min post-injection before slowly raising the syringe out of the brain. At the conclusion of the surgery, incisions were closed with sutures and cleaned with betadine. For behavioral experiments, there

was a minimum of four weeks between viral surgery and baseline short-access cocaine self-administration to allow for sufficient removal of KOR from transfected neurons.

Catheter Implantation

Indwelling catheters were assembled in-house using custom 26G guide cannulas with a 5mm up/bent projection on one side and a 6mm projection cut below the pedestal purchased from Plastics One (8IC315GFLX03), Liveo laboratory tubing (cat 508-001), and silicone. Catheters were cut at different lengths for males (10cm) and females (8cm) to accommodate for differences in size.

If multiple surgeries, the catheter implantation surgery was performed second to reduce amount of time necessary to maintain patency. The catheter tubing was inserted into the right jugular vein, secured with sutures, and the catheter pedestal was secured onto the dorsal side for back-mount access. At the conclusion of surgery, the catheter was back-filled with a 60% polyvinylpyrrolidone-40 solution in saline containing gentamicin (20mg/mL) and heparin (1,000units/mL) and capped with an in-house fabricated dust-cap (PE20 tubing). Rats were allowed to recover for 7-9d before starting flushing their catheters daily with approximately 0.05mL of 0.9% saline or heparinized saline (80units/mL) and beginning cocaine self-administration.

Drugs and Viral Vectors

Norbinaltorphimine (norBNI) was suspended in ACSF (5µg/0.3µL) and injected bilaterally into the NAc (0.3µL/hemisphere) two days prior to the first long-access SA session. U69,593 (Sigma Aldrich) was dissolved in solvent 0.01% DMSO and aCSF for a final concentration of 1µM. Cocaine hydrochloride was dissolved in physiological saline (5mg/mL), filtered, and administered at a dose of 0.5mg/kg.

The following viral constructs were manufactured by the University of Washington Center for Excellence in Neurobiology of Addiction, Pain, and Emotion's Molecular Genetics Resource

Core: AAV1-FLEx-ChR2-eYFP, AAV1-FLEx-SaCas9-U6-HA-sgOprk1, AAV1-FLEx-SaCas9-U6-HA-sgGabrg2 Control, and AAV1-Cre-GFP. Titer for all viruses range from 0.5 to $5x10^9$ particles.

Cocaine Self-Administration

All behavioral experiments were performed in Med Associates modular operant chambers outfitted with a liquid swivel attached to a syringe pump, two nosepoke ports with internal cue lights, a houselight, a tone generator, and a white noise generator. The houselight and white noise turned on to mark the beginning of the session and availability of cocaine. Operant responding into the assigned active nosepoke port resulted in one cocaine infusion (FR1, 5mg/mL/kg at 0.02755mL/sec) and the start of a 20-second time-out period during which the houselight and white noise are off, an audiovisual cue is on (tone + nosepoke port cue light), and further operant responses are recorded but do not result in additional cocaine infusions. The second nosepoke port (referred to as inactive nosepoke) does not have any programmed consequences and is used to assess general locomotion and responding behavior.

Rats are first trained on short-access self-administration (SA) sessions that last for one hour per session until they reach our learning criterion (ten active responses per session for three consecutive sessions). After reaching criterion, rats continue short-access SA for five additional days to establish a baseline of cocaine consumption. Then, rats enter two five-day blocks of long-access (six-hour sessions) SA. Between each block (short-access block, 1st long-access block, and 2nd long-access block), the rats receive a 0- to 2-day break from SA. At the conclusion of behavioral experiments, we performed a linear regression on active responses during the first hour of each session across sessions and classified rats with a significant, positive correlation as escalators and rats with a non-significant or significant, negative correlation as non-escalators, as previously described (Willuhn et al., 2014).

Microinjections

We injected norBNI (5µg/mL) or vehicle bilaterally into the NAcC two days prior to the rats' first long-access SA session and at least one day post their last short-access SA session. We inserted a bilateral injector with 1mm projection (Plastics One, part#: 8IC235ISPCXC) into the guide cannula and used two Hamilton syringes (1801RN 10ul SYR (26s/2"/2), ref#: 84877) coupled with a kdScientific injector (model 210) to inject 0.3µL/hemisphere followed by a two-minute wait period before removing the injector.

Ex Vivo Fast-Scan Cyclic Voltammetry

For slice voltammetry experiments animals were euthanized with pentobarbital (150mg/kg) and perfused with a cold sucrose solution. The brain was removed and 300µm coronal slices containing the NAcC were prepared in an oxygenated sucrose solution. Slices were held in a recovery chamber containing oxygenated aCSF for at least 45min at 32°C before any recordings took place. To record dopamine transients the slices were placed in a recording chamber, and continually perfused with oxygenated aCSF at 33°C. Carbon-fiber microelectrodes (CFM) were fabricated with a fused-silica capillary (Clark et al., 2010) and cut to have a recording surface of ~175µm. Transients were evoked by either a single pulse of a blue LED through the microscope objective or bipolar electrical stimulation. Recordings were accomplished by applying a triangular waveform in which, the CFM potential is ramped from -0.4V (versus Ag/AgCl) to +1.3V and then back -0.4V at a rate of 10 Hz. The experiment did not begin until dopamine recordings were stable for 10min. Evoked transients during baseline and experimental recordings took place every two minutes. Following stable recordings an additional 5 recordings were taken for baseline, then 1µM of U69,593 within oxygenated ACSF was washed on and an additional ten recordings were performed. Waveform generation, data acquisition, and analysis were carried out using two PCI cards and software written in LabVIEW 7.1 (National Instruments, Austin, TX).

Histology

For all behavioral experiments, rats were euthanized with pentobarbital (150mg/kg) and transcardially perfused with saline followed by 4% paraformaldehyde. For cannulated rats, 0.3µL of blue dye was injected into the NAcC prior to perfusion. Their brains were removed and submerged in ascending concentrations of sucrose, ending with 30% sucrose in PBS, before being frozen and sliced into 40µm coronal sections on a cryostat. Slices from cannulated rats were mounted and the location of the blue dye was imaged and analyzed to confirm location of pharmacological manipulations. Slices from virally-injected animals underwent immunohistochemistry for HA-tag to confirm location of viral transduction of the CRISPR virus. Rats with placements outside of the tar were excluded.

Statistical Analysis and Data Visualization

Statistical analyses and data visualization for all behavioral experiments were performed in R (version 4.0.5). Mixed effects models were computed with the “lme4” package (version 1.1.27.1). ANOVA was computed with the “afex” package (version 1.0.1). HSD was computed with the “emmeans” package (version 1.7.0). Linear regression was computed with base R. Data visualization was computed with “ggplot2” (version 3.3.5). Figure compilation was performed in Adobe Illustrator (CS4).

RESULTS

Pharmacological Inhibition of KOR in the NAcC Blocks Cocaine Escalation

To test the necessity of KOR activation in the NAcC for escalation of cocaine consumption, we pharmacologically inhibited KOR via local microinjection of the long-lasting KOR antagonist, norBNI, prior to transitioning rats from short-access SA to long-access SA (Fig. 1b). A mixed effects model analysis revealed an interaction between treatment (vehicle vs norBNI) and time (SA sessions) demonstrating differential cocaine consumption patterns over SA across the two

groups (Fig. 1c). As a group, vehicle-treated rats escalated in their cocaine consumption over long-access SA sessions, as expected, while norBNI-treated rats did not escalate in their consumption (Fig. 1d). Analyzing average cocaine consumption per block, vehicle-treated rats show increased responding in the first and second block of long-access SA (LgAb1 and LgAb2, respectively) in comparison to their short-access baseline (ShA) while norBNI-treated rats showed no significant difference in responding in LgAb1 or LgAb2 compared to ShA (Fig. 1e). Categorizing rats as escalators or non-escalators based on a linear regression analysis of drug consumption across SA session where significant, positive correlations are deemed escalators, demonstrated a trend toward a lower proportion of escalators in the norBNI-treated group (Fig. 1f). Overall, pharmacologically inhibiting KOR in the NAc prior to long-access SA blocked (or at least prolonged) the development of the SUD-phenotype of escalation.

Virally-mediated CRISPR Knock-Out of oprk1 in the VTA Eliminates KOR-mediated Inhibition of Dopamine in the NAcC

To knock-out KOR on specific neurons/terminals in the NAcC, we developed a viral CRISPR-SaCas9 construct targeting the *oprk1* gene (encoding for KOR) to knock-out KOR in specific neuronal populations (Fig. 2a). To confirm the knock-out of functional KOR in neurons, we injected the viral CRISPR-SaCas9 construct into the VTA and, after incubation, measured the effect of KOR agonist U69,593 on stimulated dopamine release in the NAcC with *ex vivo* fast-scan cyclic voltammetry (FSCV). We additionally injected channelrhodopsin into the VTA to enable light-activation of VTA projections in the NAcC with simultaneous recording. Bath application of U69,593 had a differential effect on evoked dopamine release across groups (Fig. 2c). An unpaired t-test revealed a significant decrease in the effect of U69,593 on evoked dopamine release in VTA-oprk1-KO rats compared to control rats (Fig. 2c). Our virally-mediated CRISPR knock-out of oprk1 was sufficient to significantly reduce the ability of a KOR agonist to

modulate dopamine release in the NAcC when expressed in the VTA demonstrating the functional validity of our construct.

Virally-mediated CRISPR Knock-Out of oprk1 in the VTA but not the NAcC Blocks Cocaine Escalation

To delineate which KOR-expressing neurons in the NAcC are necessary for cocaine escalation, we knocked-out (KO) KOR in NAc neurons or VTA neurons independently using virally-mediated CRISPR-SaCas9 technology targeting the *oprk1* gene prior to training the rats on the cocaine SA. A control CRISPR virus was injected either into the NAcC or VTA (Fig. 3a). There was no difference in cocaine consumption between the two groups of control-virus rats (supp fig. 1), and therefore, the two groups were pooled. All the rats, regardless of treatment, learned cocaine SA and there was no significant difference in baseline cocaine consumption during short-access SA (supp fig. 2). A mixed effects model analysis revealed a significant interaction between group (virus/location) and time (SA sessions) demonstrating differential cocaine consumption patterns over SA across the groups (Fig. 3c). As a group, control-virus rats and NAcC-oprk1-KO rats escalated in their cocaine consumption over long-access SA sessions, while VTA-oprk1-KO rats did not escalate in their consumption (Fig. 3d). Analyzing average cocaine consumption per block (Fig. 3e), control-virus rats showed increased responding from ShA to LgAb2; NAcC-oprk1-KO rats showed increased responding from ShA to LgAb1; VTA-oprk1-KO rats did not show any significant differences across all three blocks. Examining the proportion of escalators vs non-escalators across groups demonstrated a significant reduction in the proportion of escalators in VTA-oprk1-KO rats compared to control-virus rats, with no significant difference in the proportions of escalators in NAcC-oprk1-KO rats compared to control-virus rats (Fig. 3f). Additionally, there was no interaction between session and sex for any group, although control-virus and VTA-oprk1-KO groups did have a main effect of sex (supp fig. 3). Overall, the knock-out of *oprk1* from VTA neurons (including their presynaptic terminal in the

NAcC), but not from medium spiny neurons in the NAcC, was sufficient to completely block escalation of cocaine consumption. This demonstrates that activation of KOR on VTA neurons is necessary specifically in the transition from casual to SUD-like cocaine consumption. The ability of KOR on VTA neurons to produce escalation may be a result of KOR's ability to modulate dopamine, a phenomenon observed both prior to and after escalation of cocaine consumption (supp fig 4) and demonstrated to be disrupted in VTA-oprk1-KO rats.

DISCUSSION

Here, we demonstrated that local inhibition of KOR via a long-lasting antagonist, norBNI, in the NAcC blocks cocaine escalation in a long-access model of cocaine SA. To probe which KOR in the NAcC are responsible for this effect, we validated a novel virally-mediated CRISPR targeting the *oprk1* gene and utilized this technique to knock-out KOR of NAcC medium spiny neurons and VTA neurons. We demonstrated that knocking-out KOR in VTA neurons, but not NAcC neurons, completely blocked cocaine escalation. We did not observe effects on learning or baseline cocaine consumption when KOR was knocked out of VTA neurons indicating that this effect is specific to escalation behavior. Therefore, the development of escalation (a SUD-like phenotype), but not cocaine consumption in general, requires activation of KOR on VTA terminals in the NAcC.

It is important to note that KORs are also expressed on VTA cell bodies and presumably VTA projections to regions other than the NAcC (Mansour et al., 1994; Margolis et al., 2006). The viral technique used in this paper was not specific to the VTA-NAcC pathway and there is yet a well-validated technique to knock-out receptors selectively on terminals. Therefore, it is possible that our effect could be due to knocking out KOR in areas other than VTA terminals in the NAcC. However, in combination with the pharmacological data, which only affects KORs present in the NAcC, the data is highly suggestive that it is KOR on VTA pre-synaptic terminals in the NAcC necessary our observed effect on escalation of cocaine consumption.

The projection from the VTA to the NAcC mostly consists of dopamine neurons (Morales and Pickel, 2012). As demonstrated here and in previous literature (Ehrich et al., 2015), activation of KOR inhibits dopamine release. We additionally demonstrated here that this effect persists after cocaine experience. Knocking out KOR from VTA neurons blocks dynorphin activation of KOR in the NAcC and the resulting decrement in dopamine transmission. Previous work has demonstrated that decreases in response-contingent cue-evoked dopamine release across long-access cocaine SA is causal to escalation of cocaine consumption (Willuhn et al., 2014). Therefore, it is possible that in these experiments, blocking the activation of KOR on VTA neurons through pharmacology or gene-editing inhibited KOR from decreasing dopamine responses ultimately resulting in a lack of escalation.

The results of this paper provide evidence for KOR activation on VTA terminals in the NAcC to drive escalation of cocaine consumption, potentially working upstream of dopamine through KOR-activated inhibition of dopamine release. KOR has been implicated in SUD of various addictive substances including cocaine (Maisonneuve et al., 1994; Kuzmin et al., 1998; Thompson et al., 2000; Schenk et al., 2001; McLaughlin et al., 2006; Veer et al., 2013), methamphetamine (Whitfield et al., 2015), heroin (Schlosburg et al., 2013), and alcohol (Walker and Koob, 2008; Karkhanis et al., 2016; Rose et al., 2016). Therefore, these results will likely be relevant to more than cocaine use disorder alone. Uncovering the neural mechanisms behind SUD will inform therapeutic strategies to combat the current drug epidemics.

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FIGURES

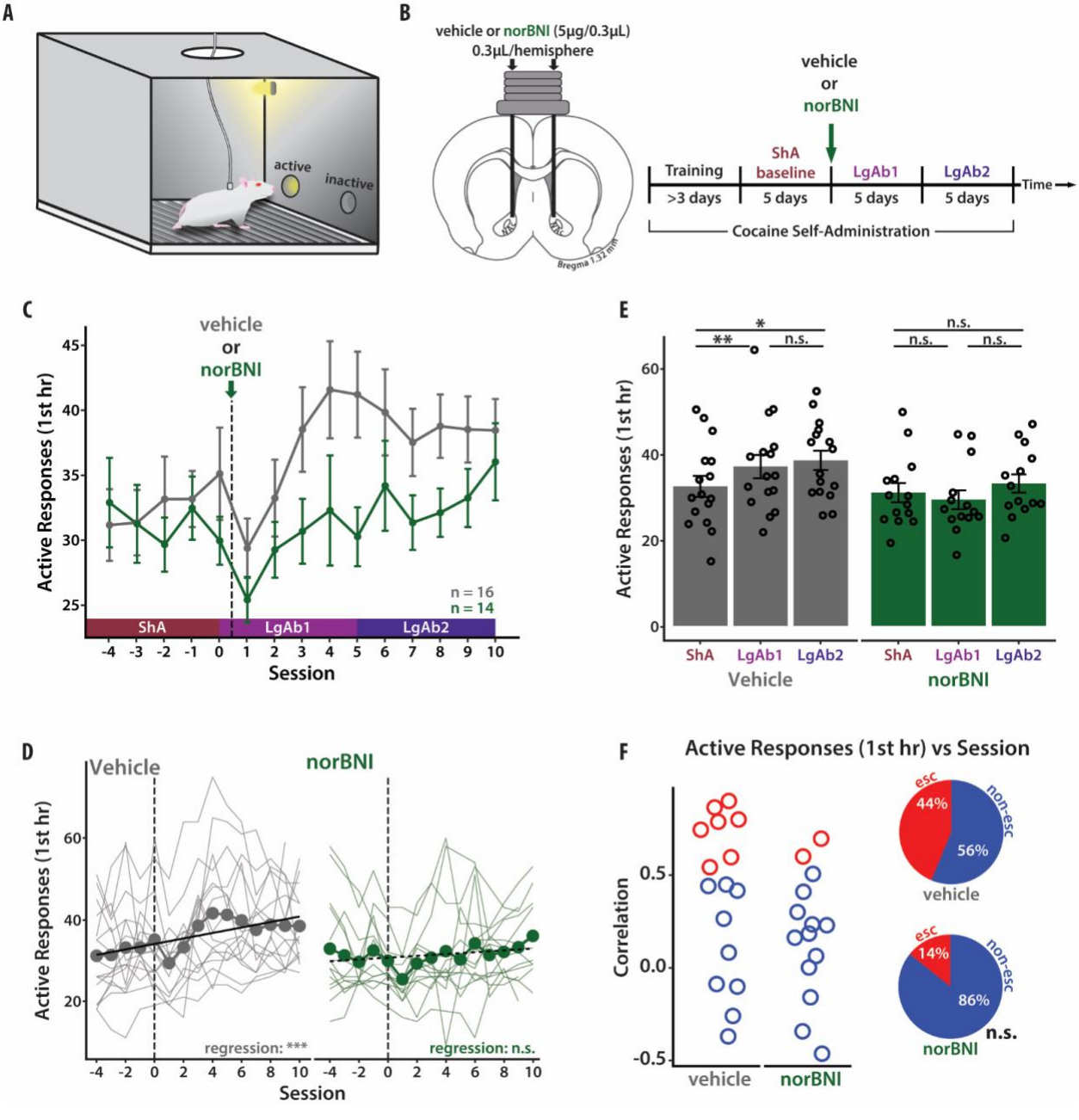


Fig 1. Pharmacological Inhibition of KOR in the NAcC Blocks Cocaine Escalation.

(A) Cocaine self-administration setup. **(B)** experimental design. **(C)** Local NAcC infusion of long-lasting KOR antagonist, norBNI, prior to long-access SA results in differential drug-taking across sessions compared to control (main effect of session $F(14,368)=4.78$, p-value < 0.001; main effect of group $F(1,28)=2.53$, p-value > 0.05; interaction $F(14,368)=2.00$, p-value < 0.05). Active

responses for only the first hour of SA and error bars show SEM. **(D)** Thinner lines represent individual rats. Black line indicated line of best fit from a linear regression. As a group, vehicle-treated rats showed an increase in active responses across session (r^2=0.058, p-value < 0.001) whereas norBNI-treated rats did not (r^2=0.009, p-value > 0.05). **(E)** Active responses during the first hour of SA averaged into three 5-session blocks: short-access (ShA), long-access block 1 (LgAb1), and long-access block 2 (LgAb2). A two-way anova revealed a significant interaction between group and block ($F(2,56)=4.49$, p-value < 0.05). Post-hoc Tukey test showed a significant increase between ShA and LgAb1 for vehicle-treated rats (p-value < 0.01) and between LgAb1 and LgAb2 for vehicle-treated rats (p-value < 0.05). No significant changes were detected in norBNI-treated rats. **(F)** Each rat was classified as an escalator or a non-escalator based on a linear regression analysis of active responses across session. Rats with a significant, positive regression are classified as escalators and rats with a non-significant or a significant, negative regression are classified as non-escalators. (left) correlation values of escalators and non-escalators in the two groups. (right) percentage of escalators to non-escalators in both groups. A Chi-squared goodness of fit test showed no significant difference between the two groups (p-value > 0.05).

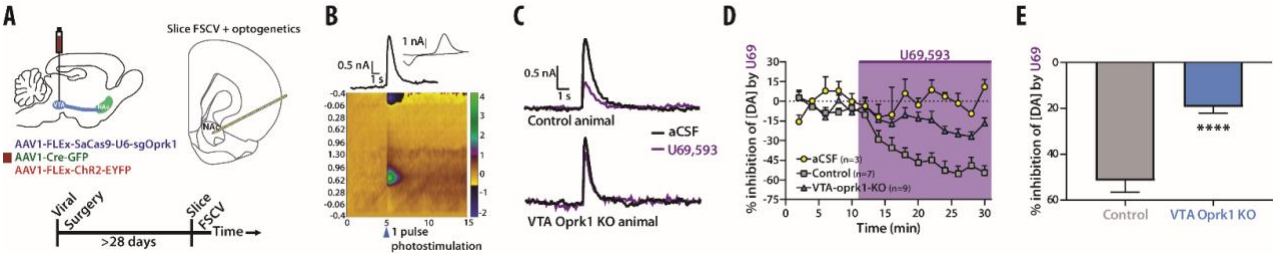


Fig 2. Virally-mediated CRISPR-SaCas9 Targeting *oprk1* Effectively Knocks Down KOR Function.

(A) Experimental setup. **(B)** Expression of ChR2 was sufficient in stimulating dopamine transients following a pulse of blue light onto a coronal slice. Current vs time (top left), the corresponding CV (top right), and a color plot with current (color), time (x-axis), and potential (y-axis). **(C)** Representative traces of stimulated dopamine release before and after washing the KOR agonist U69,593, in a control animal (top) and VTA-oprk1-KO animal (bottom). **(D)** Time series of stimulated dopamine release under various conditions. Five stimulations occurred as baseline prior to U69 application (purpled shaded region) for control or VTA-oprk1-KO. A subset of slices were aCSF only. A mixed-effects model revealed there was a main effect of group ($F(2,16)=12.81$, p-value <0.001), and there was an interaction of group by time ($F(28,216) = 6.299$, $p<0.0001$). A post-hoc Tukey test showed there was a significant difference between VTA-oprk1-KO and control slices (p-value <0.0001), and a difference between the VTA-oprk1-KO and aCSF-only slices (p-value < 0.0001). **(E)** Bar plot of percent inhibition of dopamine concentration by application of treatment. The last 5 stimulations before experimental end were averaged and used to identify the % inhibition from baseline. We observed a significant differences in the effect a KOR agonist had on inhibition of dopamine release (unpaired t-test ($t(14) = 5.87$, $p<0.0001$).

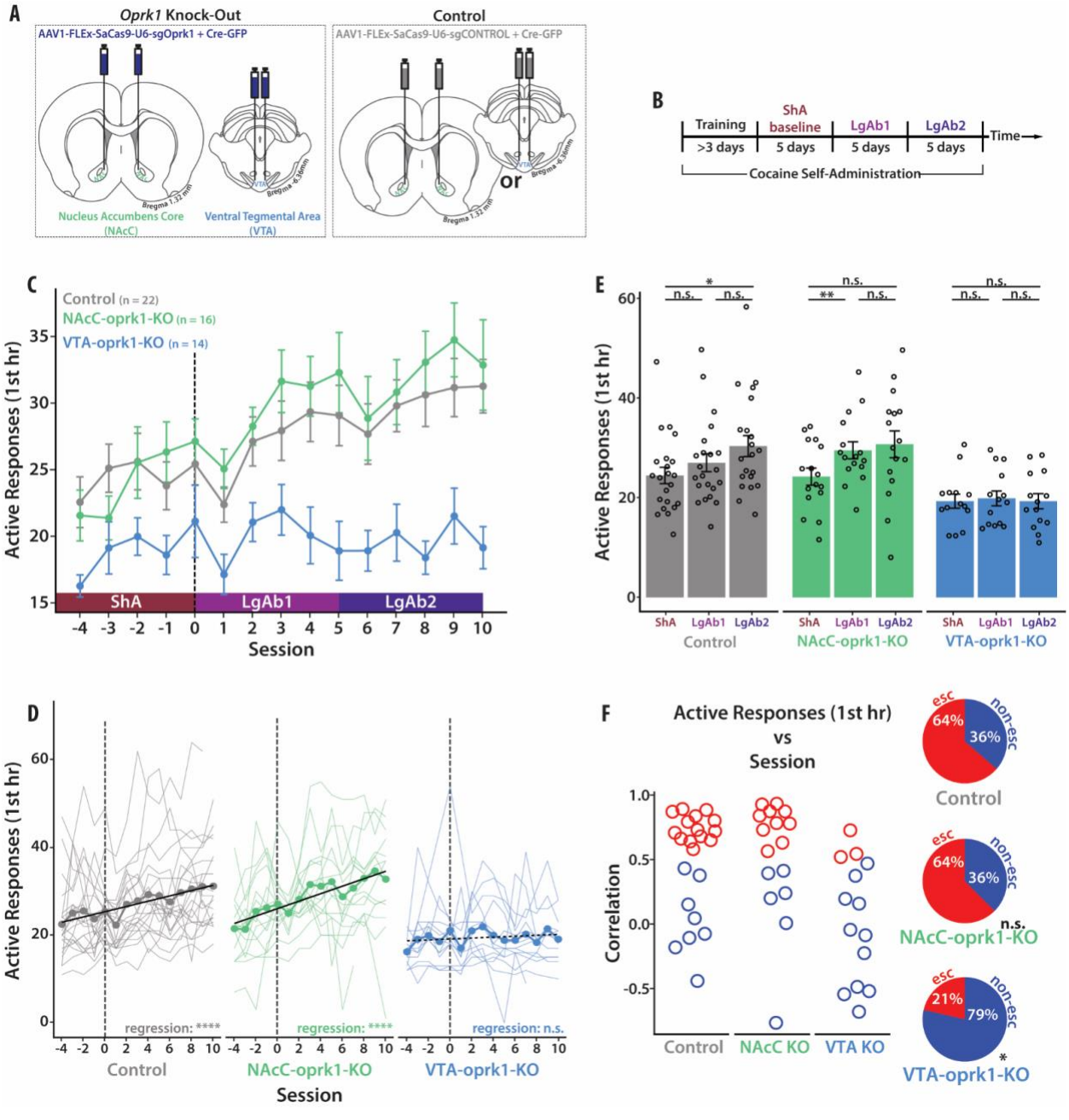
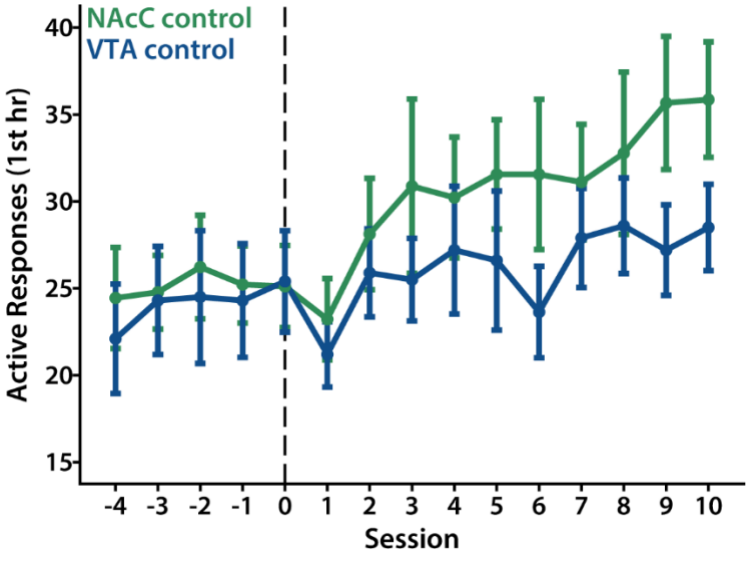


Fig 3. CRISPR-SaCas9 Knock-Out of KOR in VTA Neurons but not in NAcC Neurons Blocks Cocaine Escalation.

(A) Viral strategy. **(B)** Behavioral timeline. **(C)** A mixed effects model on active responses in the first hour across session revealed a significant main effect of session ($F(14,657)=10.60$, p-value < 0.001), main effect of group ($F(2,49)=7.47$, p-value < 0.01), and an interaction ($F(28,657)=2.10$, p-value < 0.001). **(D)** Thinner lines represent individual rats. Black line indicated line of best fit from a linear regression. As a group, control-virus rats and NAcC-oprk1-KO rats showed an

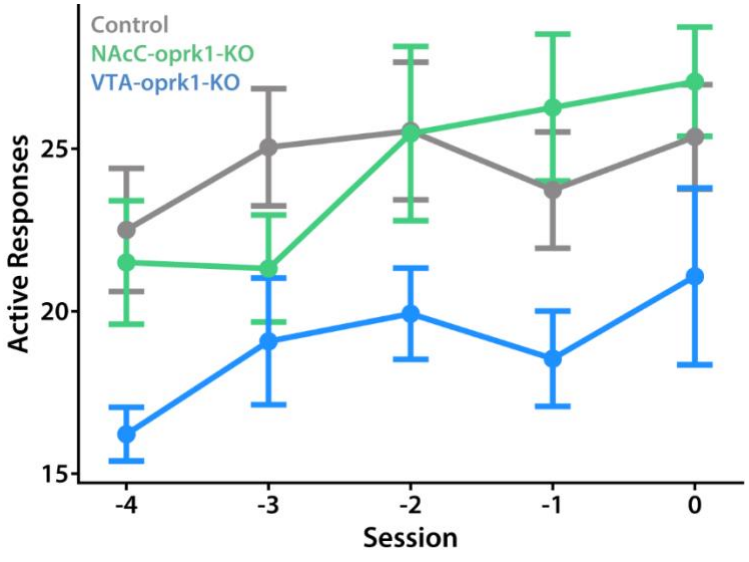
increase in active responses across session (control-virus: r^2=0.07, p-value < 0.001; NAcC-oprk1-KO: r^2=0.14, p-value < 0.001;) whereas VTA-oprk1-KO rats did not (r^2=0.004, p-value > 0.05). **(E)** Active responses during the first hour of SA averaged into three 5-session blocks: short-access (ShA), long-access block 1 (LgAb1), and long-access block 2 (LgAb2). A two-way anova revealed a significant interaction between group and block (F(4,98)=2.97, p-value < 0.05). Post-hoc Tukey analysis demonstrated the following effects: (control-virus) a significant increase between ShA and LgAb2 (p-value < 0.05); (NAcC-oprk1-KO) a significant increase between ShA and LgAb1 (p-value < 0.01); (VTA-oprk1-KO) no significant differences. **(F)** Each rat was classified as an escalator or a non-escalator based on a linear regression analysis of active responses across session. Rats with a significant, positive regression are classified as escalators and rats with a non-significant or a significant, negative regression are classified as non-escalators. (left) correlation values of escalators and non-escalators in the two groups. (right) percentage of escalators to non-escalators in both groups. A Chi-squared goodness of fit test showed a significant difference between the control-virus and VTA-oprk1-KO (p-value < 0.05) but not NAcC-oprk1-KO (p-value > 0.05).

SUPPLEMENTAL FIGURES



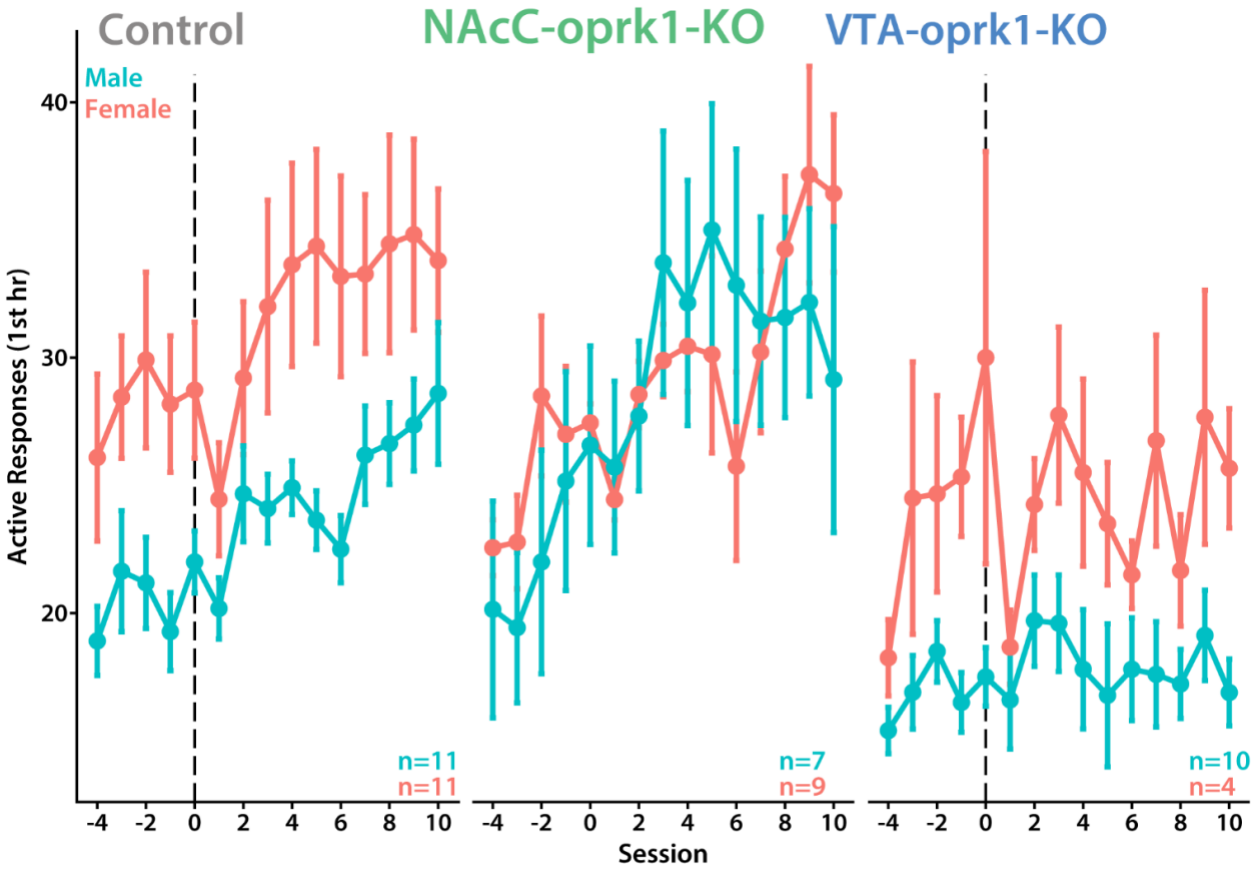
Supp Fig 1. Location of CRISPR Control Virus Injection Does Not Impact Cocaine SA.

Active responses across session for all control-virus animals, divided by their injection region. A mixed effect model analysis revealed no main effect of group nor interaction.



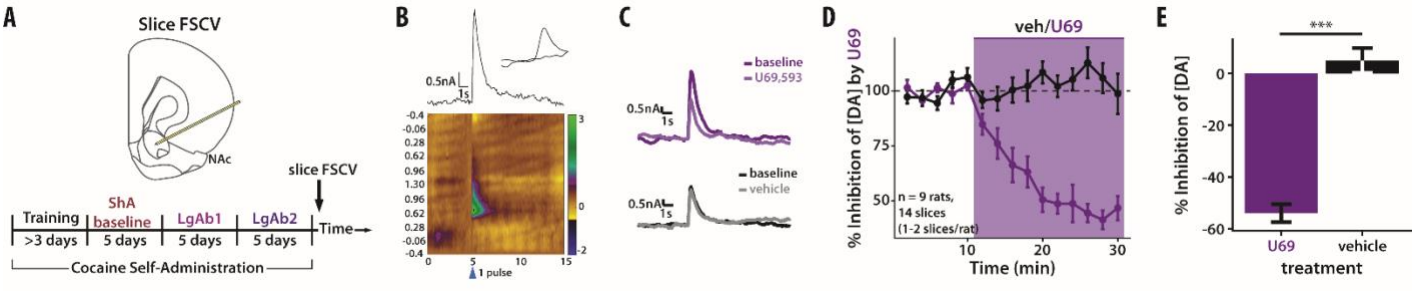
Supp Fig 2. CRISPR KO or KOR does not Affect Baseline Cocaine Consumption.

Active responses during 5 baseline short-access (ShA) cocaine SA sessions. A mixed effects model showed no significant main effect of group (p-value > 0.05) nor interaction (p-value > 0.05).



Supp Fig 3. CRISPR KO of KOR Effect on Cocaine SA in Males and Females

For control-virus rats, a mixed effects model revealed a main effect of session ($F(14,275)=7.67$, p-value < 0.001) and a main effect of sex ($F(1,20)=6.20$, p-value < 0.05), but no interaction. For NAcC-oprk1-KO rats, a mixed effects model revealed only a main effect of session ($F(14,183)=6.70$, p-value < 0.001). For VTA-oprk1-KO rats, a mixed effects model revealed only a main effect of sex ($F(1,12)=10.4$, p-value < 0.01).



Supp Fig 4. KOR Activation Decreases Stimulated Dopamine Release after Cocaine SA

(A) Experimental design. **(B)** Electrical stimulation evokes dopamine release. Current vs time (top left), corresponding CV (top right), and a color plot with current (color), time (x-axis), and potential (y-axis). **(C)** Representative traces of stimulated dopamine release at baseline and after washing on U69,593 (U69) / vehicle in the same slice. U69 is a KOR agonist. **(D)** Time series of stimulated dopamine release under various conditions. Five stimulations occurred as baseline prior to U69/vehicle application. Mixed effects model revealed a significant main effect of time ($F(14, 382) = 9.32$, p-value < 0.001), significant main effect of treatment ($F(1, 382) = 273.4$, p-value < 0.001), and a significant interaction ($F(14, 382) = 14.5$, p-value < 0.001). **(E)** Bar plot of percent inhibition of dopamine concentration by application of treatment. The last 5 stimulations before experimental end were averaged and used to identify the % inhibition from baseline. Paired t-test revealed a significant difference between U69 and vehicle treatment on the percent inhibition of dopamine (***, p-value < 0.001).

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Chapter 3:

Corticotropin-Releasing Factor in the Nucleus Accumbens Does Not Drive High Levels of Cocaine Consumption

L. Gordon-Fennell, R.D. Farero, J.D. Jones, L.S. Zweifel, P.E.M. Phillips, *Corticotropin-Releasing Factor in the Nucleus Accumbens Does Not Drive High Levels of Cocaine Consumption.* bioRxiv 2025.02.25.640140; doi: https://doi.org/10.1101/2025.02.25.640140

ABSTRACT

Uncovering the neurobiological processes underlying substance use disorder informs future therapeutic interventions. Prior research implicates the corticotropin releasing factor (CRF) system as a major player in a wide variety of substance use disorder -like phenotypes. However, the complexity of the CRF system in regard to brain region specific effects and experience-dependent changes in activity is poorly understood. Employing a cocaine self-administration paradigm that induces escalation of cocaine consumption in a subset of subjects, we investigated the role of CRF activity in the Nucleus Accumbens (NAc) in cocaine-taking patterns both before and after chronic cocaine experience. Our results showed that pharmacologically inhibiting CRF-R1 in the NAc did not reduce cocaine consumption following escalation and genetically deleting CRF-R1 from cells in the NAc did not prevent escalation. Overall, this suggests that any effect of CRF activity driving escalation or high levels of cocaine consumption is not through its actions on CRF-R1 in the NAc.

INTRODUCTION

Substance Use Disorder (SUD) is a brain disease characterized by a plethora of behavioral and physiological phenotypes. The current epidemic of SUDs has a major toll on individuals and society today, with cocaine SUD specifically still having no current pharmaceutical interventions. Revealing the underlying neurobiology of SUD and its various phenotypes is a necessary step toward the development of effective therapeutics. The SUD phenotype characterized by increases in drug consumption over time – termed escalation – is an ideal candidate for employing harm-reductionist approaches to combat the toll of SUD. Preventing or reversing escalation would result in lower levels of drug consumption, decreasing the risk of overdose, loss of opportunity due to frequency of use, likelihood of infection, and more. We can model escalation of drug consumption using rodent self-administration under long-access (~6hr) conditions (Koob and Ahmed, 1998).

Corticotropin-releasing factor (CRF) is a neuropeptide most known for its role in stress-related behaviors and has been widely implicated in a variety of SUD phenotypes (Koob, 2010). Stressful stimuli, such as drug withdrawal, are believed to be a factor in escalation of drug consumption which may be a result of CRF activity (Sarnyai et al., 1995; Land et al., 2008). However, CRF activity is not a monolith – its activity results in different behavioral and neurobiological effects based on brain region and prior experience of the subject (Lemos et al., 2012; Sotomayor-Zárate et al., 2013; Oliva et al., 2021; Baumgartner et al., 2022). Brain-region specificity of CRF activity in relation to cocaine escalation is poorly understood.

The nucleus accumbens (NAc) is a basal forebrain brain region that receives dense innervation from midbrain dopamine neurons. Dopamine activity, particularly in the NAc, is related to the rewarding and reinforcing properties of drugs of abuse (CHIARA and IMPERATO, 1988; Wise et al., 1995; Phillips et al., 2003). Related to cocaine escalation, a decrement of the dopamine phasic response in the NAc to contingent delivery of drug-associated cues drives

increases in cocaine consumption (Willuhn et al., 2014; Burgeno et al., 2023). Systems that are activated during drug consumption and can modulate dopamine in the NAc would be key candidates for further understanding the neurobiology of SUD. The majority of drugs of abuse stimulate hypothalamic and extra-hypothalamic CRF release (Goeders et al., 1990; Sarnyai et al., 1995; Zorrilla et al., 2012). Increases in CRF-R1 expression has been shown to increase nicotine self-administration (Uribe et al., 2020). Additionally, previous literature demonstrates that CRF positively modulates dopamine activity in the NAc under certain conditions and this effect is modulated by cocaine experience and stress (Lemos et al., 2012; Oliva et al., 2021).

In this paper, we aim to dissect the brain-region and broad temporal specificity of the role of CRF-R1 in escalation of cocaine consumption. To investigate the role of NAc CRF-R1 in later stages of this SUD-like phenotype, we utilized pharmacological approaches to block NAc CRF-R1 after undergoing an escalation paradigm and measured changes in cocaine consumption. To investigate the role of NAc CRF-R1 in the development of this SUD-like phenotype, we utilized a novel CRISPR-SaCas9 viral vector to knock-out NAc CRF-R1s prior to cocaine self-administration. Overall, we demonstrate a lack of necessity of NAc CRF-R1 activity in the development and sustainment of cocaine escalation.

METHODS

Subjects

Female (200-250g) and male (250-350g) Wistar rats were procured from Charles River Laboratories and singly housed in a temperature and humidity-controlled environment with access to enrichment and *ad libitum* laboratory chow and water. Rats were allowed at least one week of acclimation to their new environment before undergoing any experimentation, such as surgery. The housing room was on a 12hr on/off light cycle (lights on at 7:00am), and all behavioral experiments were performed during the light cycle. All animal use and procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Surgical Procedures

All surgical procedures were performed under isoflurane (1-5%) using aseptic technique. Alternating swabs of betadine and alcohol (3x/each) were used to sterilize surgical sites. Rats were given an NSAID (meloxicam, 1mg/mL/kg in 3mL of saline, s.c.) on the day of surgery and post-operative day 1. Rats were allowed at least one week of recovery in between surgeries, if undergoing multiple surgical procedures.

Cannula Implantation

For cannula implantation, rats were affixed onto a stereotaxic frame (Kopf) and major landmarks were measured (lambda and bregma) and utilized to flatten the skull. Four or more skull screws were secured into the skull in areas surrounding the site of interest to help reinforce the headcap. Burr holes were drilled overlaying the site of interest (NAc, angle: 0˚, AP: 1.3mm, ML: +/- 1.3mm, DV: -7.2mm). We lowered the custom bilateral guide cannula to 1mm dorsal to the target (-6.2mm) and secured the implant to the skull with dental cement (guide cannula details: 26G, 2.5mm center-to-center distance, 7mm projection, purchased from Plastics One, cat: 8IC235G26XXC). To prevent contaminants, we inserted a dummy cannula with 0mm projection (Plastics One) into the guide cannula and secured a dust cap (Plastics One) onto the top of the guide cannula.

Viral Injection

For viral injection, rats were affixed onto a stereotaxic frame (Kopf) and major landmarks were measured (lambda and bregma) and utilized to flatten the skull. Burr holes were drilled overlaying the site of interest (NAc, angle: 0˚, AP: 1.3mm, ML: +/- 1.3mm, DV: -7.2mm). A Hamilton syringe (7105KH 5.0ul SYR (24/2.75”/3), ref#: 88000) containing the viral cocktail was lowered to 0.2mm ventral the site of interest (-7.4mm). After a 2-minute pre-injection wait period, we utilized a Micro4 Microsyringe Pump to inject the viral vector into the site of interest at a rate of 250nL/min for a total of 1µL (4 min injection). Over the course of the first minute of the injection,

the Hamilton syringe was slowly raised to 0.2mm dorsal to the site of interest (-7.0mm) where it remained for the rest of the injection time. We waited 8 minutes post-injection before raising the Hamilton syringe out of the brain. Viral injections were a 1:8 viral cocktail of AAV1-Cre-GFP and either AAV1-CMV-SaCas9-sgCrhr1 (*crhr1* KO) or AAV1-CMV-SaCas9-sgRNA (control). After both injections were completed, we sutured the scalp with 4-0 silk suture and applied betadine to the incision. For all behavioral experiments, there was a minimum of four weeks between viral injection surgery and the short-access baseline cocaine self-administration sessions to allow for sufficient knock-out of the CRF-R1s.

Catheter Implantation

Catheters were made in-house using: a custom 26G guide cannula with a 5mm up/bent projection on one side and a 6mm projection cut below the pedestal on the other side (Plastics One, cat: 8IC315GFLX03), Liveo laboratory tubing (cat: 508-001), and DAP silicone. Male catheters measured a total length of 10cm, and female catheters measured a total length of 8cm. Final catheter length, specifically the length inserted into the vein, was determined during surgery. Catheters were sterilized in betadine for at least 24hrs before implantation.

Catheter implantation surgery was always performed second (if multiple surgeries) to reduce attrition due to loss of patency. During surgery, two surgical sites were prepared: the rat's dorsal side, anterior and posterior to the scapulae, and the area surrounding the right peck. The catheter was placed with the access-port secured to the dorsal side in between the scapulae and the tubing going over the right shoulder and down into the jugular vein. We used 4-0 silk sutures to secure the catheter onto the vein, secure the pedestal into place, and close incisions. We used a locking solution of 60% polyvinylpyrrolidone-40 solution in saline containing gentamicin (20mg/mL) and heparin (1,000units/mL) at the conclusion of the surgery and capped the port with an in-house fabricated dust-cap (PE20 tubing). The locking solution remained in the catheter for 7-9 days before daily flushing began (flushing solutions: 0.9% saline or heparinized saline

(80units/mL)). Rats were allowed at least 7 days of recovery before beginning cocaine self-administration.

Drugs and Viral Vectors

Antalarmin hydrochloride (Sigma, cat: A8727) was prepared in 0.5% (w/v) carboxymethylcellulose (Sigma, cat: C5678) saline solution and injected intraperitonially 80 minutes before a cocaine self-administration session (Specio et al., 2008). CP 154,526 (Sigma, cat: PZ0100) was prepared in aCSF with 1% DMSO and 5% Cremophor and injected bilaterally into the NAc (0.5µL/hemisphere) 20 minutes before a cocaine self-administration session. Cocaine hydrochloride was dissolved into 0.9% sodium chloride (5mg/mL) and filtered into 10mL syringes. Cocaine hydrochloride was obtained through the NIDA drug supply program.

The following viral constructs were manufactured by the University of Washington Center for Excellence in Neurobiology of Addiction, Pain, and Emotion's Molecular Genetics Resource Core: AAV1-CMV-SaCas9-sgCrhr1, AAV1-CMV-SaCas9-sgRNA, and AAV1-Cre-GFP. Titer for all viruses range from 0.5 to $5x10^9$ particles.

Cocaine Self-Administration

Cocaine self-administration (SA) began a minimum of 7 days post catheter implantation. Rats were handled for 1-3 days prior to starting behavioral training. Prior to each cocaine SA session, rats' catheters were flushed with either saline or heparinized saline (80units/mL).

Cocaine SA was performed in modular operant chambers (Med Associates) equipped with two nose-poke ports (each with their own nose-poke light), a white noise generator, a tone generator, a houselight, and a liquid swivel mounted on a weighted balance arm. Syringe pumps were used to deliver cocaine at a rate of 5RPM. Upon the start of the session, the white noise and houselight turn on, signaling availability of cocaine. One nose-poke port was designated the "active" port and the other nose-poke port the "inactive." Active and inactive designations switched

between cohorts to control for chamber side preferences, but a given designation was always the same for a specific rat. One operant response in the active port (FR1 schedule) resulted in an infusion of cocaine (0.5mg/kg/inf), the cessation of the discriminative stimuli for 20-seconds (white noise and houselight), and a 20-second audiovisual cue (nose-poke cue light and tone). This 20-second period was also the length of the time-out period during which additional operant responses into the active port were recorded but did not result in additional cocaine infusions. Operant responses in the inactive port were recorded but had no programmed consequence. Short-access sessions (ShA) were 1 hour in length and long-access sessions (LgA) were 6 hours in length.

Rats were trained on cocaine SA under daily ShA conditions. To reach criteria of learning the task, rats had to obtain 10 or more infusions per session for three consecutive sessions. Therefore, training was a minimum of three days. In the unlikely event that an animal could not reach this criterion or were not stable in their drug consumption above 10 infusions, they were dropped from the study. After training, rats began the 'baseline ShA' block, consisting of five days of ShA sessions (1x/day), followed by two five-day blocks of LgA (LgAb1 and LgAb2). When investigating reversal of escalation, rats continued LgA past LgAb2 for about five days (LgAb3). Between blocks, rats had 0-3 days off from cocaine SA. To classify rats as escalators or non-escalators, we performed a linear regression analysis on active responses in the first hour of a session across the sessions of baseline ShA, LgAb1, and LgAb2. Rats with a significant, positive regression were classified as escalators, and rats with a non-significant or significant, negative regression were classified as non-escalators, as previously described (Willuhn et al., 2014).

Microinjections

We microinjected CP-154,526 (2µg/µL) or vehicle bilaterally into the NAc (0.5µL/hemisphere) 20 minutes prior to a LgA cocaine SA session. Rats had one day of normal LgA cocaine SA in between injection days. For the microinjection, we equipped a kdScientific

injector (model 210) with two Hamilton syringes (1801RN 10µL SYR (26s/2"/2), ref#: 84877). We used a bilateral injector with 1mm projection (Plastics One, part#: 8IC235ISPCXC) to insert into the guide cannula and waited 2 minutes post-injection before removing the injector. The same setup was used to microinject Blue Sky dye during euthanasia to confirm injection placements.

Histology

For rats that underwent neural manipulations (viral injections and intra-NAc microinjections), we euthanized with pentobarbital (150mg/kg) and performed transcardial perfusion of 0.9% sodium chloride followed by 4% paraformaldehyde. Their brains were removed, additionally post-fixed in 4% paraformaldehyde for 24 hours, and then submerged in ascending percentages of sucrose (ending with 30% sucrose in PBS). The brains were then frozen and sliced into 40µm slices on a cryostat. To determine the location of our neural manipulations, we analyzed the location of the blue dye and/or cannula implant for microinjections and the location of GFP fluorescence for viral manipulations.

Immunohistochemistry

For the CRISPR *crhr1* KO validation, neural tissue was collected in the same manner as all other rats that underwent neural manipulations. After collection, brain sections containing the cannula implant track were used to perform immunohistochemistry against HA-tag (for the CRISPR virus) and c-fos. Fluorescence images were taken using an apotome microscope.

Statistical Analysis and Data Visualization

Statistical analyses and data visualization were performed in R (version 4.4.1). The following statistical analyses were performed with the corresponding package: Mixed effects model – "lme4" (version 1.1.35.5); ANOVA – "afex" (version 1.4.1); HSD – "emmeans" (version 1.10.4); linear regression – base R. In all plots, error bars represent standard error of the mean. Data visualization was performed with "ggplot2" (version 2.0.0). Data visualization and unpaired

t-test for the CRISPR *crhr1* KO validation was performed in Prism. Adobe Illustrator (CS4) was used for final figure compilation.

RESULTS

Global CRF-R1 Activity is Necessary to Sustain High Levels of Cocaine Consumption

Previous work has demonstrated that systemic antagonism of CRF-R1 reduces cocaine consumption in male rats undergoing long-access cocaine self-administration (Specio et al., 2008). We validated this effect with our behavioral setup in male and female rats (Fig 1A). Rats underwent a baseline of 5 short-access cocaine SA sessions followed by 10 long-access sessions before undergoing within-subjects systemic injection of a CRF-R1 antagonist (antalarmin, 25mg/kg, s.c.) 80 minutes prior to a long-access session (Fig 1B). As a group, rats increased in their cocaine consumption across behavioral sessions as determined by a mixed effects model showing a main effect of session (Fig 1C). Systemic injection of antalarmin reduced the total cocaine consumption during the first hour of the task relative to vehicle injection day (Fig 1E). This effect appeared minor, with the change in mean active responses being only a reduction of 3.4 responses from vehicle to antalarmin injection days, which may not be a biologically relevant change. Possibly, the small effect seen in the first hour could be masking a larger, more transient effect earlier in the task, such as would be the case if only the load-up phase was affected. To analyze this, we broke down the first hour active responses into ten-minute bins. As expected, rats consume the most cocaine in the first ten-minute bin (load-up) and then sharply reduce consumption to be maintained at lower levels (Fig 1F). While there is a main effect of injection drug, post-hoc analyses did not reveal any significant differences between antalarmin and vehicle days for any given ten-minute bin, similar to previously described (Specio et al., 2008).

The overall minor effect of antalarmin could also be a result of differential effects on different populations of rats. The ability of a CRF-R1 antagonist to reduce drug consumption in long-access paradigms is believed to be due to CRF activity underlying cocaine escalation. Therefore, we sought to determine if systemic CRF-R1 activity is necessary to sustain high levels of cocaine consumption selectively in rats that escalated in their cocaine consumption. Rats were classified as escalator or non-escalator based on a linear regression of their active responses in the first hour across behavioral sessions (see methods for more details). When grouping the rats by escalation status, we observed that escalators have a different pattern of cocaine consumption compared to non-escalators, where escalators overtake non-escalators by the end of LgAb2, as demonstrated by a significant interaction between session and escalation group (mixed effects model) (Fig 1G). Analyzing the behavioral data on injection days, while there is a main effect of injection drug, post-hoc analyses did not show a significant difference between antalarmin- and vehicle- treated sessions when rats were split up into their escalation group (Fig 1I). This lack of effect persisted when looking at the data broken into ten-minute bins (Fig 1J). Similar lack of effects were observed when dividing by sex (Supp Fig 1). It is possible that the lack of effects seen when grouping the data by escalation group and/or sex is simply due to reducing the group size below what is necessary to observe this seemingly biologically-small effect.

CRF-R1 Activity in the Nucleus Accumbens is Not Necessary to Sustain High Levels of Cocaine Consumption

To delineate a specific brain region underlying the effect seen with systemic CRF-R1 antagonism, we used the same behavioral paradigm and blocked CRF-R1 receptors only in the Nucleus Accumbens (NAc) through local microinjections of a CRF-R1 antagonist (CP 154,526 (CP), 2μg/μL, 0.5 μL/hemi) 20 minutes prior to a long-access session (Fig 2A). As a group, rats increased in their cocaine consumption across behavioral sessions as determined by a mixed effects model showing a main effect of session (Fig 2B). Local blockade of CRF-R1 in the NAc

did not decrease cocaine consumption in the first hour (Fig 2D). Similarly, there was no significant differences between CP- and vehicle- treated days when the data was broken into ten-minute bins (Fig 2E).

We sought to determine if there was a differential effect of intra-NAc CRF-R1 antagonism based on escalation status. Escalators and non-escalators showed different patterns of cocaine consumption across the sessions as demonstrated by a significant interaction between session and escalation group (mixed effects model). Non-escalators were stable in the cocaine consumption across sessions whereas escalators started low and increased their consumption over sessions (Fig 2F). When separated by escalation status, there was no effect of CP on cocaine consumption during the first hour of cocaine SA (Fig 2H) nor during any ten-minute bin (Fig 2I).

CRF-R1 Activity in the Nucleus Accumbens is Not Necessary for Escalation of Cocaine Consumption

There is evidence to suggest that the CRF system may be altered by chronic cocaine exposure (Sotomayor-Zárate et al., 2013; Oliva et al., 2021) and therefore, even though NAc CRF-R1 activity was not necessary to sustain high levels of cocaine consumption following long-access, NAc CRF-R1 activity may still be necessary for the establishment of cocaine taking patterns. To reduce the activity of CRF-R1 for a sustained period, we developed a novel virally mediated CRISPR-SaCas9 targeting *crhr1* (the gene encoding for CRF-R1) to completely knock-out CRF-R1 in cells. To functionally validate this technique, we unilaterally knocked-out *crhr1* in the NAc of rats before microinjecting CRF, euthanizing the rats, and assessing neuronal activation via c-fos immunostaining (Supp Fig 2). The virally transfected hemisphere had a significantly smaller number of c-fos positive cells than the control hemisphere, demonstrated a reduction in the functionality of CRF-R1 in those cells.

To determine if CRF-R1 activation is necessary for the development of escalation of cocaine consumption, we knocked-out (KO) *crhr1* in the NAc prior to the start of cocaine SA (Fig 3A). As a group, there was no difference in cocaine consumption across behavioral sessions between control and *crhr1* KO rats as demonstrated by a lack of a main effect of group and interaction (mixed effects model) (Fig 3B). Performing linear regression analysis, both control and *crhr1* KO groups showed a significant, positive correlation – demonstrating escalation as a group (Fig 3D). Looking at individual rats and their escalation status, there was no change in the proportion of escalators versus non-escalators (Fig 3E). There were no sex differences in cocaine consumption (Supp Fig 3).

DISCUSSION

In alignment with previous literature (Goeders and Guerin, 2000; Specio et al., 2008), we showed here that systemic antagonism of CRF-R1 modestly reduces levels of cocaine self-administration. However, this effect was not maintained when analyzing subgroups of rats based on escalation status or sex. Since CRF is known to have differential effects based on brain region, the small magnitude of this effect may be a result of simultaneously activating opposing systems via a systemic injection. Therefore, we sought to investigate specific brain region effects of CRF-R1 antagonism on cocaine consumption through local microinjection. We demonstrated that local CRF-R1 blockade in the NAc does not reduce cocaine consumption following long-access cocaine SA. Considering that the effects of CRF, such as its ability to modulate dopamine in the NAc, are dependent on experience, we investigated the role of NAc CRF-R1 during an earlier timepoint of the task before the SUD-like phenotype of escalation emerges. We demonstrated that knock-out of CRF-R1 in the NAc does not reduce the magnitude of cocaine escalation nor the proportion of rats that escalate in their cocaine intake. Overall, this data suggests that CRF-R1 activation in the NAc is not necessary for the development or maintenance of escalation of cocaine intake.

CRF has long been implicated in SUD due to its involvement in stress-induced changes in drug-related behaviors such as potentiation of drug reward (Kreibich et al., 2009), reinstatement of drug seeking (Shaham et al., 2000), and potentiation of drug acquisition (Goeders and Guerin, 1993). Additionally, CRF systems have been shown to be necessary for general negative affective behaviors resulting from stress and drugs of abuse (Sarnyai et al., 1995; Land et al., 2008; Marcinkiewcz et al., 2009; Refojo et al., 2011; Chen et al., 2012; Grieder et al., 2014). These data position CRF to be a key player in the development or maintenance of the beta-process of opponent process theory. Elevations of CRF due to drugs of abuse or the stress of acute withdrawal could influence future drug taking behavior. Indeed, overexpression of CRF in the NAc leads to increases in CRF-R1 expression and nicotine SA in female rats (Uribe et al., 2020). However, the data presented here suggests that for cocaine, CRF-R1 activity in the NAc may not play a role in drug taking patterns.

Appreciation for the diversity of the NAc and the roles of each subregion (such as: NAc core and NAc shell) has grown in the previous years (Heymann et al., 2020). The techniques employed here did not allow for subregion specificity. While our targeting mainly impacts the NAc core, the close proximity of the NAc shell and the changes in subregion medial-lateral and dorsal-ventral location throughout the anterior-posterior axis result in the considerable potential for volumes of the NAc shell to also be impacted. Therefore, similar to the systemic injection, these results may still be clouded by the manipulation of potentially opposing systems. Alternatively, the NAc shell has also been implicated in SUD and drug-taking behavior with relation to CRF activity (Peciña et al., 2006; Marcinkiewcz et al., 2009; Chen et al., 2012) and therefore, potentially targeting our manipulation more towards the NAc shell could reveal changes in cocaine consumption.

In this paper, we only investigated the role of CRF-R1. However, CRFR antagonists are known to not be entirely selective to CRF-R1 or CRF-R2 especially at high concentrations. Additionally, CRF-R2 have also been implicated in SUD and negative components of stress (Land

et al., 2008; Uribe et al., 2020). It is believed that the majority of CRF receptors in the NAc are CRF-R1, however, even with a smaller population of receptors, CRF-R2 could still play a role in modulating activity in the NAc to result in changes in drug-taking behavior (Pett et al., 2000). Utilizing pharmacology with higher affinity to CRF-R2 and CRISPR targeting *crhr2* would provide additional evidence of the role (or lack therefore) of NAc CRF system in cocaine consumption and SUD-related behaviors.

FIGURES

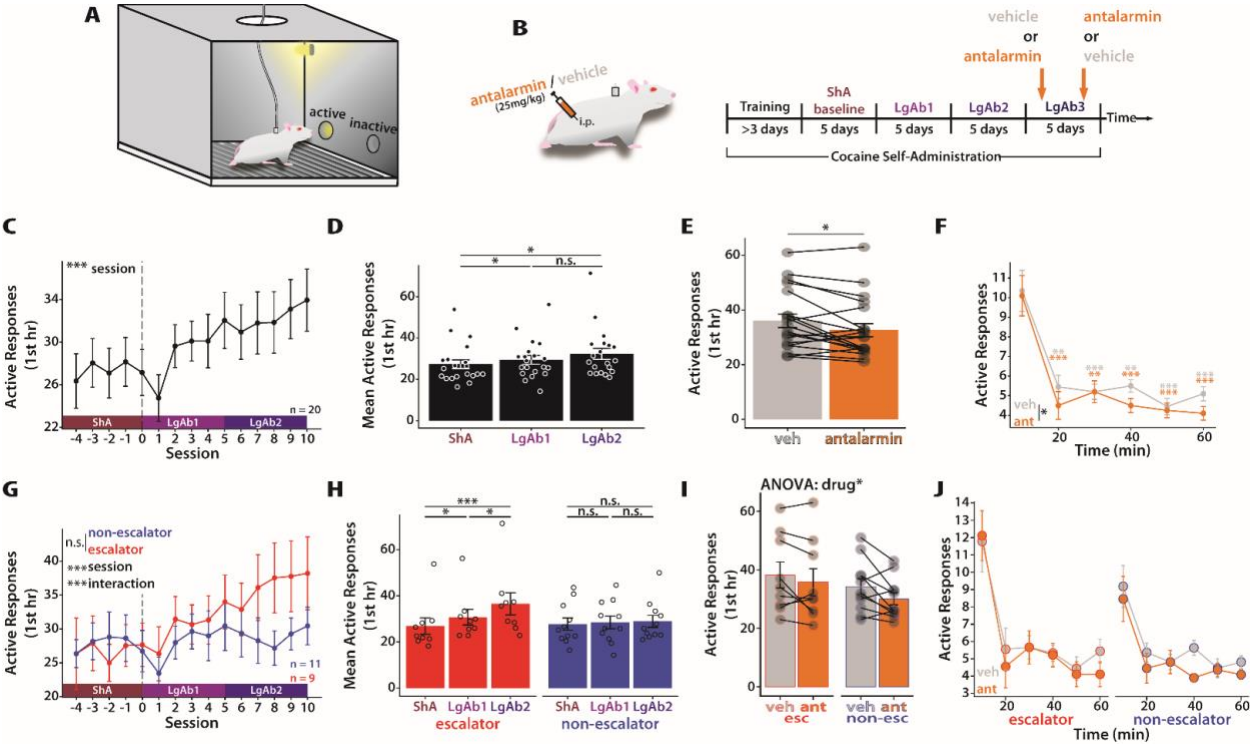


Figure 1. Systemic CRF-R1 Antagonism Reduces Cocaine Consumption Regardless of Escalation Status

(A) Behavioral setup. **(B)** Experimental timeline. Antalarmin is a CRF-R1 antagonist. **(C & G)** Number of operant responses in the active hole during the first hour of cocaine SA across behavioral sessions. **(C)** A mixed effects model showed drug consumption changes across behavioral sessions (main effect of session $F(14,264)=5.23$, p-value < 0.001). **(G)** A mixed effects model revealed a significant main effect of session ($F(14,250) = 6.47$, p-value < 0.001), no main effect of escalation status ($F(1,18) = 0.45$, p-value = 0.51), and an significant interaction ($F(14,250) = 3.24$, p-value < 0.001). **(D & H)** Mean number of responses in active hole during the first hour of cocaine SA across behavioral blocks. **(D)** A repeated-measures ANOVA revealed a main effect of block (p-value < 0.001). **(H)** A repeated-measures ANOVA revealed a significant main effect of block (p-value < 0.001), no main effect of escalation status (p-value = 0.51), and a significant interaction (p-value < 0.001). **(E & I)** Number of operant responses in the active hole

in the first hour of cocaine SA following vehicle and antalarmin injection. **(E)** A paired t-test revealed a significant difference between active responses in the first hour following vehicle vs antalarmin injection. **(I)** A repeated-measures ANOVA revealed a significant main effect of injection drug (p-value < 0.05) and no main effect of escalation status (p-value = 0.21) nor interaction (p-value = 0.51). **(F & J)** Number of operant responses in the active hole in ten-minute bins across the first hour of cocaine SA for sessions following vehicle and antalarmin injection. **(F)** A repeated-measures ANOVA revealed a significant main effect of time (p-value < 0.001) and a main effect of injection drug (p-value < 0.05), but no interaction (p-value = 0.62). Statistics shown on plot are post-hoc Tukey of the given time bin compared to time bin = 10 for each injection drug. **(J)** A repeated-measures ANOVA revealed a significant effect of time (p-value < 0.001), injection drug (p-value < 0.05), and escalation status X time (p-value < 0.05). All other comparisons were non-significant (escalation status, p-value = 0.32; escalation status X injection drug, p-value = 0.51; time X injection drug, p-value = 0.64; escalation status X time X injection drug, p-value = 0.71). **(G-J)** Plots are grouped by escalation status ('escalators' vs 'non-escalators'), see methods for definition of escalation. **(all)** *** = p-value < 0.001 ; ** = p-value < 0.01 ; * = p-value < 0.05

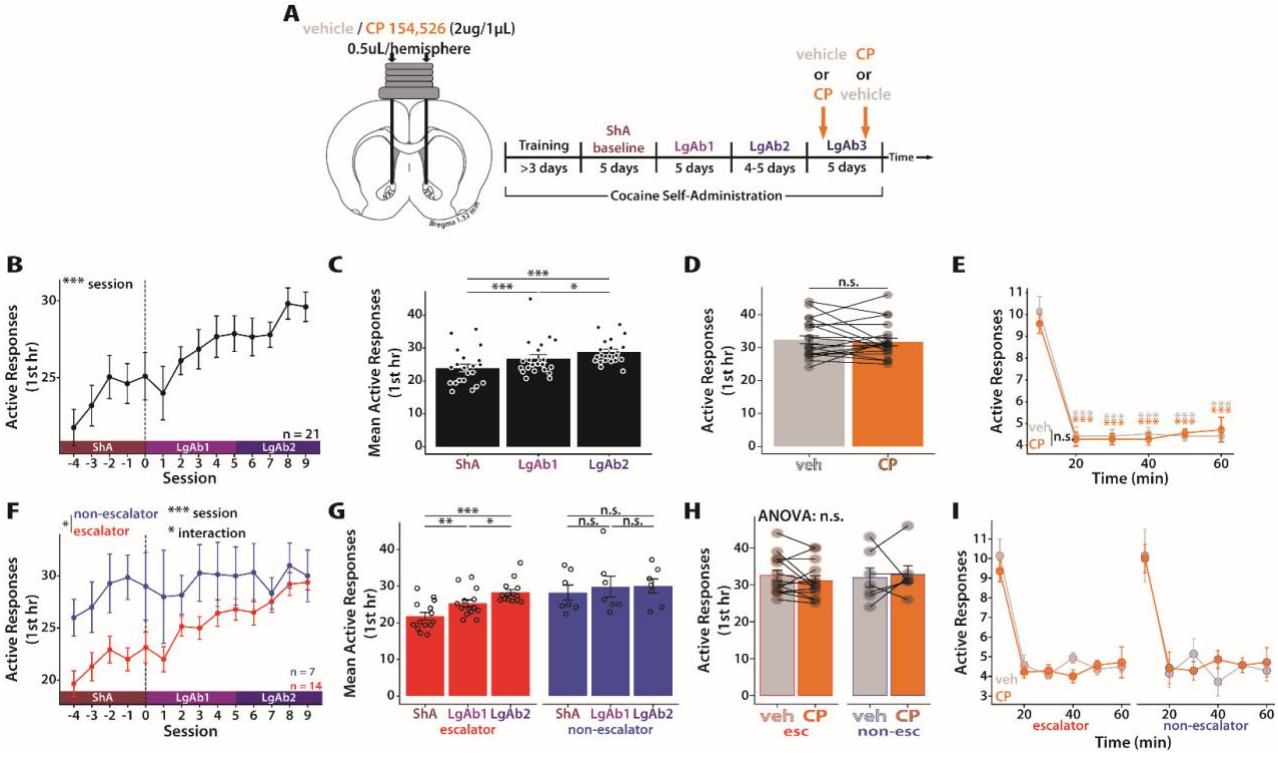


Figure 2. CRF-R1 Activity in the NAc is Not Necessary to Sustain High Levels of Cocaine SA

(A) Experimental timeline. CP 154,526 (CP) is a CRF-R1 antagonist. **(B & F)** Number of operant responses in the active hole during the first hour of cocaine SA across behavioral sessions. **(B)** A mixed effects model showed drug consumption changes across behavioral sessions (main effect of session $F(13,253)=9.89$, p-value < 0.001). **(F)** A mixed effects model revealed a significant main effect of session ($F(13,240) = 7.17$, p-value < 0.001), main effect of escalation status ($F(1,19) = 4.74$, p-value < 0.05), and an interaction ($F(13,240) = 2.06$, p-value < 0.05). **(C & G)** Mean number of responses in active hole during the first hour of cocaine SA across behavioral blocks. **(C)** A repeated-measures ANOVA revealed a main effect of block (p-value < 0.001). **(G)** A repeated-measures ANOVA revealed a main effect of block (p-value < 0.001), a main effect of escalation status (p-value < 0.05), and an interaction (p-value < 0.01). **(D & H)** Number of operant responses in the active hole in the first hour of cocaine SA following vehicle and CP injection. **(D)** A paired t-test revealed no significant difference between active responses

in the first hour following vehicle vs CP injection (p-value = 0.55). **(H)** A repeated-measures ANOVA revealed no significant effect of injection drug (p-value = 0.81), escalation status (p-value = 0.81), nor interaction (p-value = 0.35). **(E & I)** Number of operant responses in the active hole in ten-minute bins across the first hour of cocaine SA for sessions following vehicle and CP injection. **(E)** A repeated-measures ANOVA revealed a significant main effect of time (p-value < 0.001), but no significant effect of injection drug (p-value = 0.56) nor interaction (0.82). Statistics shown on plot are post-hoc Tukey of the given time bin compared to time bin = 10 for each injection drug. **(I)** A repeated-measures ANOVA revealed a significant effect of time (p-value < 0.001) and all other comparisons non-significant (escalation status, p-value = 0.81; injection drug, p-value = 0.81; escalation status X injection drug, p-value = 0.35; time X injection drug, p-value = 0.86; escalation status X time X injection drug, p-value = 0.31). **(F-I)** Plots are grouped by escalation status ('escalators' vs 'non-escalators'), see methods for definition of escalation. **(all)** *** = p-value < 0.001 ; ** = p-value < 0.01 ; * = p-value < 0.05

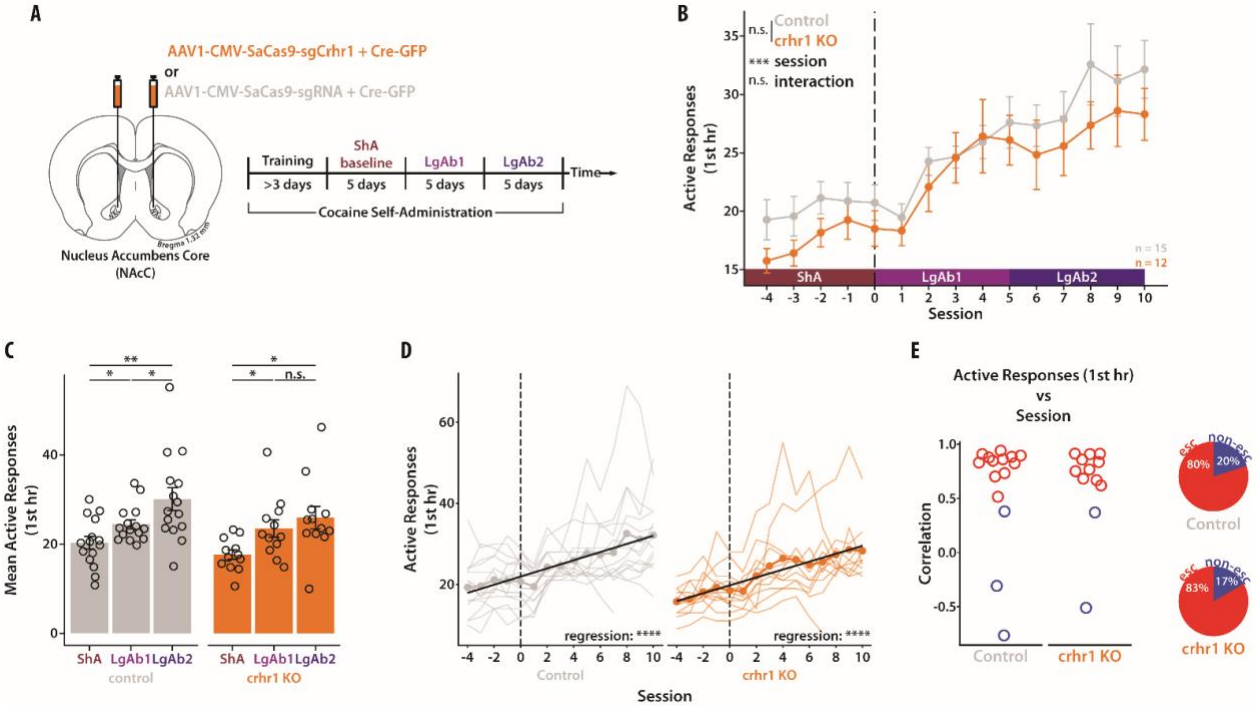
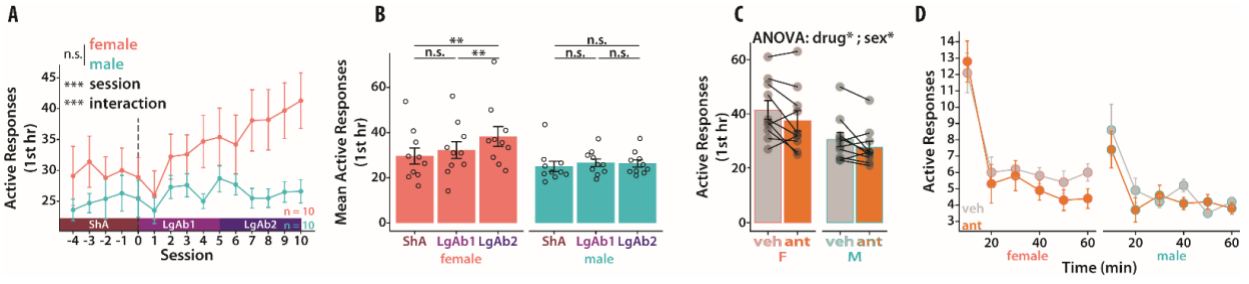


Figure 3. CRF-R1 Activity is Not Necessary for Escalation of Cocaine Consumption

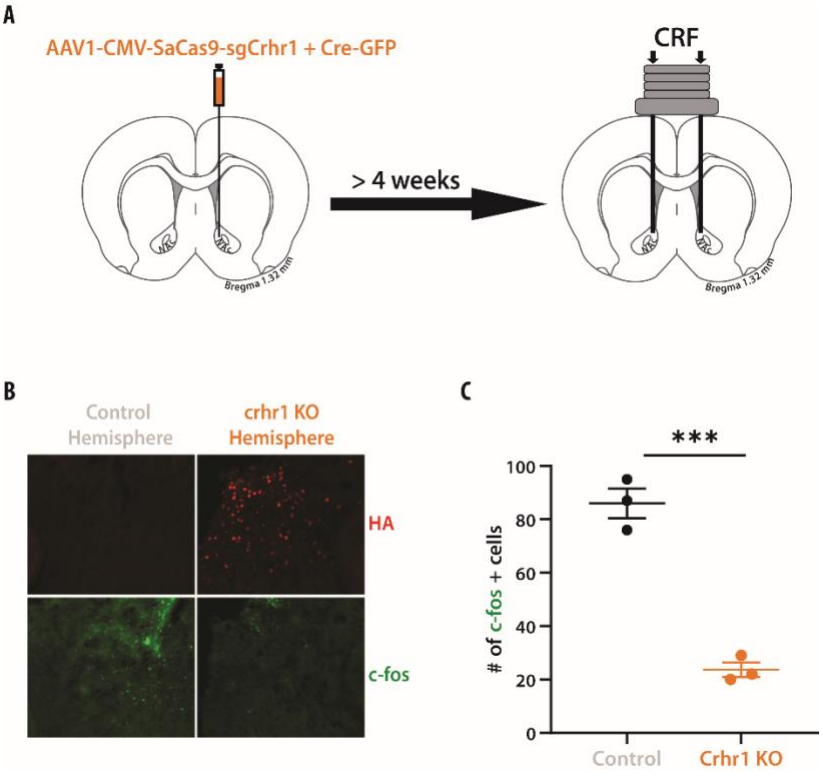
(A) Experimental design. A minimum four-week incubation period occurred between viral surgery and ShA baseline. **(B)** Active responses in the first hour of cocaine SA across behavioral sessions. A mixed effects model revealed a significant main effect of session ($F(14,339) = 19.02$, p-value < 0.001), but not of group ($F(1,25) = 1.17$, p-value = 0.29) nor an interaction ($F(14,339) = 0.46$, p-value = 0.95). **(C)** Mean active responses in the first hour for each block. A repeated-measures ANOVA revealed a significant main effect of block (p-value < 0.001), but not of group (p-value = 0.25) nor an interaction (p-value = 0.49). **(D)** Same data as shown in B with the addition of individual subjects (thinner lines). The solid black line represents the best fit from a linear regression. Both control and *crhr1* KO groups showed a positive, significant regression demonstrating overall escalation of drug consumption as a group. **(E, left)** Correlation values (ρ) for individual subjects colored by escalation status (red, escalator; blue, non-escalator). **(E, right)** Pie charts showing percentage of escalators vs non-escalators for control and *crhr1* KO groups. The proportion of escalators and non-escalators in the *crhr1* KO group is not significantly different from control (p-value = 0.82). **(all)** *** = p-value < 0.001 ; ** = p-value < 0.01 ; * = p-value < 0.05

SUPPLEMENTAL FIGURES



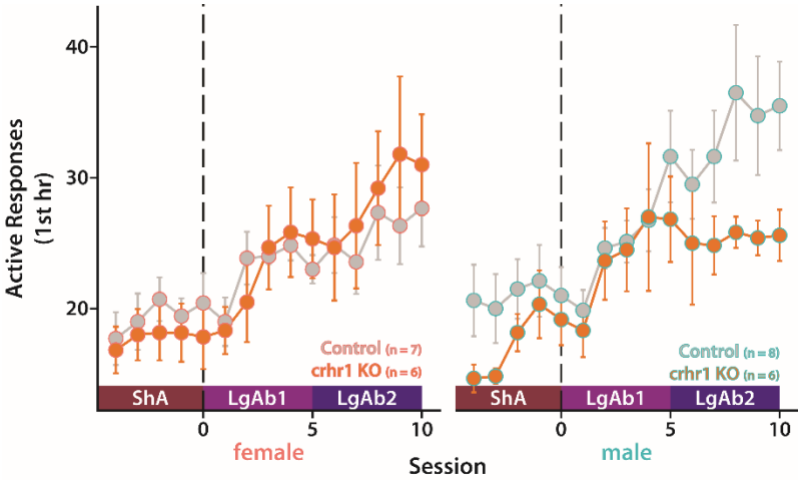
Supp Figure 1. Antalarmin Effect on Cocaine SA in Females vs Males

(A) Number of active responses in the first hour of cocaine SA across sessions separated by sex. Mixed effects analysis revealed a significant main effect of session (p-value < 0.001) and a significant interaction (p-value < 0.001), but no main effect of sex (p-value = 0.09). **(B)** Mean number of active responses in the first hour of cocaine SA for each block separated by sex. A repeated-measures ANOVA revealed a significant main effect of block (p-value < 0.001) and a significant interaction (p-value < 0.01), but no main effect of sex (p-value = 0.09). **(C)** Number of active responses in the first hour of cocaine SA following vehicle and antalarmin (ant) injection. A repeated-measures ANOVA revealed a significant main effect of injection drug (p-value < 0.05) and sex (p-value < 0.05), but no interaction (p-value = 0.65). All relevant post-hoc Tukey were non-significant. **(D)** Number of operant responses in the active hole in ten-minute bins across the first hour of cocaine SA for sessions following vehicle and antalarmin injection separated by sex. A repeated-measures ANOVA revealed a significant main effect of time (p-value < 0.001), sex (p-value < 0.05), injection drug (p-value < 0.05), and interaction between sex and time (p-value < 0.01). All other comparisons were nonsignificant: sex X injection drug (p-value = 0.65), time X injection drug (p-value = 0.61), sex X time X injection drug (p-value = 0.20). **(all)** *** = p-value < 0.001 ; ** = p-value < 0.01 ; * = p-value < 0.05



Supp Figure 2. *crhr1* CRISPR Functionally Removes CRF-R1s

(A) Experimental Design. **(B)** Representative image of control and *crhr1* KO hemispheres immunostained against HA for the virus and c-fos as a proxy for neural activity. **(C)** Number of c-fos positive cells on control and *crhr1* KO hemisphere. Unpaired t-test (p-value < 0.001).



Supp Figure 3. CRISPR KO of *crhr1* Effect on Cocaine SA in Females vs Males

Number of active responses in the first hour of cocaine SA across sessions separated by sex. Mixed effects analysis revealed a significant main effect of session (p-value < 0.001) and non-significant effects for all other comparisons (group, p-value = 0.33; sex, p-value = 0.39; session X group, p-value = 0.98; session X sex, p-value = 0.85; group X sex, p-value = 0.32; session X group X sex, p-value = 0.21).

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Chapter 4:

Females and Males Similarly Escalate in their Cocaine Consumption

Gordon-Fennell L., Phillips PEM.

ABSTRACT

To discover and develop effective treatments for any disease or disorder, sex as a biological factor needs to be considered. However, many fields still lack an abundance of sex differences data necessary to inform effective experimental designs and contextualize interpretations. We compiled cocaine self-administration data across many experiments, utilizing only control or non-manipulated rats, and examined sex differences in cocaine self-administration broadly and in escalation of cocaine consumption under long access conditions. While female rats consumed more cocaine during the first hour of cocaine self-administration than male rats, the overall pattern of cocaine-taking behavior and ability of the paradigm to produce escalation was not significantly different between females and males. Therefore, under this behavioral paradigm, both female and male rats similarly demonstrate the substance use disorder –like phenotype of escalation of cocaine consumption, further validating its utility as a paradigm to study substance use disorder in both females and males.

INTRODUCTION

The importance of sex as a biological factor has increasingly been acknowledged leading to an increase in studies involving female subjects. Conducting research on both males and females is necessary to ensure findings, such as potential therapeutic targets, will generalize across sex and be effective for both males and females. Typically, for many studies, while both

sexes are included in the research, the number of subjects of each sex is not sufficient to identify sex differences. This of course does not apply to studies that are intentionally designed to study sex differences. While including both males and females in the experimental design does amplify the confidence that findings may generalize across sex, there are still many unknowns regarding sex differences that may lessen the impact of the findings. Without adequate numbers to study sex differences, it is unclear in many studies whether the experimental design/question is equally relevant to both sexes. The more data available to researchers on sex differences the better researchers can design experiments and interpret their results in the context of sex as a biological factor.

Drug self-administration is a common behavioral paradigm utilized to study substance use disorder (SUD) of many addictive drugs, including cocaine (Kuhn et al., 2019). Increasing the amount of access to cocaine self-administration is known to produce the SUD-like phenotype known as escalation of drug consumption (Koob and Ahmed, 1998). Previous studies on sex differences in cocaine self-administration with varying access amounts (30min to 6hr) have inconsistent results. Several studies have demonstrated that female rats consume more cocaine during longer access cocaine self-administration paradigms (Roth and Carroll, 2004; Algallal et al., 2020; Templeton-Jager et al., 2023; Guglielmo et al., 2024). However, other studies demonstrate that females consume similar amounts to males during shorter (Haney et al., 1995; Roth and Carroll, 2004) and longer access times (Roberts et al., 1989; Lynch et al., 2000). Similarly, some studies have demonstrated that females acquire cocaine self-administration faster than males (Lynch and Carroll, 1999; Guglielmo et al., 2024) while others demonstrate that there is no difference between females and males in acquisition speed (Algallal et al., 2020; Templeton-Jager et al., 2023). The variance in experimental design and inconsistencies in overall behavioral differences between males and females found in the literature warrants further examination of potential sex differences in cocaine self-administration and escalation behavior.

Here, we compiled control and non-manipulated Wistar rats across many behavioral cohorts to examine sex differences in cocaine self-administration and escalation behavior. We analyzed several behavioral outputs for differences between males and females. Additionally, we analyzed differences in the behavior of rats classified as escalators and non-escalators, both respective and irrespective of biological sex. Overall, we found that while female rats acquire cocaine self-administration faster and consume more cocaine than male rats, our specific behavioral paradigm is able to produce an escalation phenotype in both males and females to a similar degree.

METHODS

Subjects

Subjects included 50 female and 56 male Wistar rats procured from Charles River Laboratories. Upon arrival, female rats weighed 200-250g and male rats weighed 250-350g, and rats were group housed (two/cage) until their first surgery (minimum one week post-arrival). Rats were housed in a temperature- and humidity-controlled vivarium on a 12hr on/off light schedule (lights on at 7:00am) with chew bone enrichment and *ad libitum* access to laboratory chow and water. All behavioral experiments were performed during the light cycle. All cohorts included in these analyses were mixed-sex cohorts. All animal use and procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Surgical Procedures

Surgical procedures were performed under isoflurane (1-5%) using aseptic technique. Surgical sites were sterilized with 3x alternations of alcohol and betadine. During surgery and on post-surgery day one, rats received an injection of an NSAID (meloxicam, 1mg/kg/mL in 3mL of saline, s.c.). If undergoing multiple surgeries, there was a minimum of 7 days between each surgery.

Cranial Surgery

Out of the 106 rats, 78 of them (37 female and 41 male) underwent a cranial surgery. All cranial surgeries were performed on a stereotaxic frame. Of the 78 rats, 53 of them (26 female and 27 male) underwent a viral injection surgery and 25 of them (11 female and 14 male) underwent a cannula implantation surgery.

For viral injection surgeries, a Hamilton syringe (7105KH 5.0ul SYR (24/2.75”/3), ref#: 88000) coupled to a Micro4 microsyringe pump delivered 1µL of control virus bilaterally either in the nucleus accumbens (NAc, angle: 0˚, AP: 1.3mm, ML: +/- 1.3mm, DV: -7.2mm) and/or ventral tegmental area (VTA, angle: 0˚, AP: -6.35mm, ML: +/- 0.5mm, DV -8.5mm). Of the 53 viral injection rats, 37 of them (18 female and 19 male) had one bilateral viral injection either into the NAc or VTA. These animals received one of the following virus cocktails into the target region: 1:8 cocktail of AAV1-Cre-GFP + AAV1-FLEx-SaCas9-U6-HA-sgGabrg2 CONTROL into the NAc; 1:8 cocktail of AAV1-Cre-GFP + AAV1-FLEx-SaCas9-U6-HA-sgGabrg2 CONTROL into the VTA; 1:8 cocktail of AAV1-Cre-GFP + AAV1-CMV-SaCas9-sgRNA into the NAc. Of the 53 viral injection rats, 16 of them (8 female and 8 male) had two bilateral viral injections, one set into the NAc and one set into the VTA. For these animals, we injected CAV2-Cre into the NAc and a 1:8 cocktail of AAV1-HSyn-DIO-mCherry + AAV1-FLEx-SaCas9-sgslc32a1 CONTROL or a 1:8 cocktail of AAV1-HSyn-DIO-mCherry + AAV1-FLEx-SaCas9-sgGabrg2 CONTROL into the VTA. For more details on viral injection surgery, see (Gordon-Fennell et al., 2023) and (Gordon-Fennell et al., 2025).

For cannula implantation surgeries, a bilateral guide cannula was implanted in the nucleus accumbens (NAc, angle: 0˚, AP: 1.3mm, ML: +/- 1.3mm, DV: -6.2mm), secured 1mm above the target region. Note that the guide cannula was never utilized during the behavioral time periods analyzed here. For more details on cannula implantation surgery, see (Gordon-Fennell et al., 2025).

Catheter Implantation Surgery

All 106 rats underwent catheter implantation surgery. Catheters were constructed in-house using custom 26G guide cannula with a 5mm up/bent projection on one side and a 6mm projection cut below the pedestal on the other side (Plastics One, cat: 8IC315GFLX03), Liveo laboratory tubing (cat: 508-001), and DAP silicone. Female and male catheters were constructed at different lengths (female: 8cm and male: 10cm) to account for differences in body size. Final catheter length was cut during surgery. In-dwelling catheters were implanted into the right jugular vein with a back-mounted port for access. Custom catheter caps were constructed from PE20 tubing. If multiple surgeries, the catheter implantation surgery was always performed second. There was a minimum of 7 days between catheter implantation surgery and the start of cocaine self-administration training. After 7-9 days post-surgery, catheters were flushed daily with alternating saline and heparinized saline (80 units/mL). For more details on catheter implantation surgery see (Gordon-Fennell et al., 2023) and (Gordon-Fennell et al., 2025).

Drugs and Viral Vectors

Cocaine hydrochloride was dissolved into 0.9% sodium chloride (5mg/mL) and filtered into 10mL syringes. Cocaine hydrochloride was obtained through the NIDA drug supply program.

The following viral constructs were manufactured by the University of Washington Center for Excellence in Neurobiology of Addiction, Pain, and Emotion's Molecular Genetics Resource Core: AAV1-Cre-GFP, AAV1-FLEx-SaCas9-U6-HA-sgGabrg2 CONTROL, AAV1-CMV-SaCas9-sgRNA, Cav2-Cre, AAV1-HSyn-DIO-mCherry, and AAV1-FLEX-SaCas9-sgslc32a1 CONTROL. Titer for all viruses range from 0.5 to $5x10^9$ particles.

Cocaine Self-Administration

Cocaine self-administration (SA) was performed in modular Med Associates operant chambers equipped with two nose-poke holes each with their own nose-poke cue light, a

houselight, a tone generator, and a white noise generator. One nose-poke hole was designated the ‘active hole’ and the other the ‘inactive hole.’ The designation switched between cohorts but was always consistent for a given rat throughout their behavioral experiment. Upon the start of the session, the houselight and white noise turn on. Operant responses on the active hole (AH) resulted in the cessation of houselight and white-noise, a 20-second audiovisual cue (light in the nose-port and tone), and infusion of cocaine (0.5mg/kg/inf). Additionally, this activated a 20-second time-out period during which additional responses into the active hole were measured but did not deliver additional infusions of cocaine (counted as “active hole time-out” (AHTO)). After the time-out period, the audiovisual cue ended and the houselight and white-noise returned. Operant responses on the inactive hole (IH) had no programmed consequences. Operant responses on the inactive hole during the time-out period were counted as “inactive hole time-out” (IHTO) responses.

Rats were trained on cocaine SA under short-access (ShA, 1hr long) conditions. Learning criteria was 10 infusions per session for three consecutive sessions. Therefore, training was a minimum of three sessions long. After training, rats underwent 5 additional days of ShA – ShA baseline. Then, rats transitioned to long-access (LgA, 6hr long) conditions. Rats underwent 5 days of LgA block 1 (LgAb1) and then 5 days of LgA block 2 (LgAb2). The conditions in LgAb1 and LgAb2 were identical. Rats received between 0-3 days off of cocaine SA in between blocks (training, ShA baseline, LgAb1, and LgAb2).

Data Analysis

To classify rats as an escalator vs non-escalator, a linear regression was performed on active responses during the first hour of cocaine SA across sessions (not including training) (Willuhn et al., 2014). If the analysis revealed a significant, positive linear regression, the rat was classified as an escalator. If the analysis revealed a non-significant, positive or significant/non-significant negative linear regression, the rat was classified as a non-escalator.

In analysis of the first hour of cocaine SA, percent of baseline used the mean of ShA baseline days as the baseline. In the analysis of the total operant responses of cocaine SA, percent of baseline used the first day of long access (session 1) as the baseline.

Discrimination was calculated as number of operant responses in the active hole divided by the number of operant responses in the active or inactive hole. Operant responses made during the time-out period were not used in the calculation.

Statistical Analysis and Data Visualization

Statistical analyses and data visualization was performed in R (version 4.4.1). The following statistical analyses were performed with the corresponding package: Mixed effects model – “lme4” (version 1.1.35.5); ANOVA – “afex” (version 1.4.1); HSD – “emmeans” (version 1.10.4); linear regression – base R; unpaired t-test – base R. In all plots, error bars represent standard error of the mean. Data visualization was performed with “ggplot2” (version 2.0.0). Adobe Illustrator (CS4) was used for final figure compilation.

RESULTS

Sex Differences (or lack thereof) in Cocaine Self-Administration

We compiled control rats from several experiments to analyze potential sex differences in cocaine self-administration (SA) behavior. Control animals included: rats with cannulas (no microinjections), rats with control virus transfection, and rats that did not undergo any experimental manipulation until after session 10. There is no main effect or interaction of experimental design when analyzing operant responses in the active hole during the first hour of cocaine SA (raw values or percent of baseline) between sexes (Supp Fig 1).

When looking at just the first hour of each behavioral session, overall, both females and males as a group increase in their cocaine consumption across sessions with females

demonstrating overall higher levels of consumption illustrated by a significant main effect of session, significant main effect of sex, and no interaction (Fig 2A). However, looking at each block individually, female ShA, LgAb1, and LgAb2 are not significantly different from male ShA, LgAb1, and LgAb2 (Fig 2B). To account for possible baseline differences, we also computed active responses in the first hour as a percent baseline, with the ShA sessions constituting the baseline behavioral sessions (Fig 2C-D). Looking at percent baseline over all behavioral sessions, the significant main effect of session remains but there is no longer a main effect of sex and still no interaction (Fig 2C). This suggests that female rats consume more cocaine in the first hour compared to males, but their cocaine consumption trajectory is not different. When looking at the total six hours of behavior for long access session, there is still a significant main effect of session but no main effect of sex or interaction whether the data is presented as raw values or percent of baseline (Fig 2E-H). For long access sessions, the first day of long access was used as the baseline (session 1). This demonstrates that any potential differences between females and males occurs in the first hour of cocaine SA.

Examining the first hour of cocaine SA separated into ten-minute bins allows for the visualization of the load-up and maintenance phase. For sessions -4, 1, 6, and 10 – representing sessions at the start and end of each block – both females and males had significantly higher operant responding on the active hole during the first ten minutes than any other time-bin (Fig 3A). In none of the sessions was the responding in the first ten-minute time bin significantly different between females and males. In session 10, female rats had more active responses in the first ten-minute bin than in session -4. The mean inter-trial-interval (ITI) in each behavioral session was not significantly different between females and males in the first ten minutes but was significantly different in the last fifty minutes of the first hour (Fig 3B-C). There was additionally an interaction between sex and session for mean ITI in the last fifty minutes of the first hour. However, this effect seems to be driven by a higher mean ITI for females during ShA baseline, in line with

the overall higher level of taking during ShA baseline. This suggests a lack of differences in the load up phase between females and males.

When examining the other operant behaviors available to the rats, only operant responses in the inactive hole during the time-out of the first hour had a significant main effect of sex (Fig 4C). However, overall these numbers of responses were very low and any difference is likely not biologically relevant. There was no main effect of sex for operant responses in the inactive hole, operant responses in the active hole during the time-out, discrimination between active and inactive hole, nor latency to first active hole response (Fig 4). All of the above also showed no interaction except for operant responses in the active hole during time-out. However, this was likely driven by a small subset of animals that may have gotten stuck in a stereotyped movement in the active hole (Supp Fig 3). Overall, females took a smaller number of sessions to reach criteria than males demonstrated by a significant unpaired t-test (Fig 4F). Note, however, that the difference in number of sessions to criteria means is only 1.42 sessions.

Differences in Escalators and Non-Escalators in Cocaine SA

Without considering sex, we examined differences between rats classified as escalators versus non-escalators (see methods for escalation definition). Overall, when looking at the first hour of cocaine SA, escalators and non-escalators are not different in their overall cocaine consumption but they show differences in their consumption over time, demonstrated by a significant main effect of session and significant interaction but no main effect of escalation status (Fig 5A). Escalators have lower levels of cocaine consumption during ShA baseline compared to non-escalators, and escalators almost surpass the cocaine consumption level of non-escalators by LgAb2 (esc LgAb2 vs non-esc LgAb2, p-value = 0.07) (Fig 5B). Escalators show an increased mean active responses in the first hour from ShA to LgAb1 and LgAb2 and from LgAb1 to LgAb2. Non-escalators show no significant differences between any of the blocks. When computing a percent baseline, the significant main effect of session and interaction is still present, and a

significant main effect of escalation status is revealed (Fig 5C). Escalators have a higher percent baseline of active responses in LgAb1 and LgAb2 than non-escalators in LgAb1 and LgAb2, respectively (Fig 5D). Similar trends are observed in the six-hour data, with the exception of any differences in baseline (Fig 5E-H). Overall, these data demonstrate that escalators have a difference in their cocaine consumption pattern, but not necessarily the amount of cocaine consumption, compared to non-escalators.

To examine load-up and maintenance, we again looked at active responses in the first hour binned into ten-minute bins (Fig 6A). For sessions -4, 1, 6, and 10, both escalators and non-escalators showed significantly more active responses in the first ten minutes than any other time bin. In none of the sessions was the amount of active responses in the first ten minutes significantly different between escalators and non-escalators. The amount of active responses in the first ten minutes did not change across behavioral sessions for non-escalators. However, escalators demonstrated an increased number of active responses in the first ten minutes for sessions 6 and 10 compared to session -4. The mean ITI for the first ten minutes and last fifty minutes of the first hour demonstrated no main effect of escalation status but a signification main effect of session and an interaction (Fig 6B-C).

When examining the other operant behaviors available to the rats, no other operant behavior demonstrated a significant main effect of escalation status nor interaction (Fig 7A-E). Additionally, there was no significant difference in number of sessions to criteria between escalators and non-escalators (Fig 7F).

Differences Female versus Male Escalators and Female versus Male Non-escalators

We wanted to investigate differences in escalation behavior between females and males. Overall, the proportion of escalators versus non-escalators was not different between females and males (Fig 8A). Additionally, there was no significant difference between the escalation slope

of female versus male escalators nor female versus male non-escalators (Fig 8B). As expected, there is a significant main effect of escalation slope between escalation status. Female escalators have a higher escalation slope than female non-escalators, and male escalators have a higher escalation slope than male non-escalators. This suggests that as a population, females and males have a similar proportion of escalators and those escalators escalate in their cocaine consumption to a similar magnitude.

When looking at the number of operant responses in the active hole during the first hour of cocaine SA, there is no significant interaction of sex and escalation status nor sex, escalation status, and session (Fig 9A). However, qualitatively, it appears that female escalators are up-shifted in their cocaine consumption compared to male escalators but this trend is not present between female/male non-escalators. Analyzing the mean active responses in the first hour, both female and male escalators increase in their mean cocaine consumption across blocks whereas female and male non-escalators do not change in their mean cocaine consumption across blocks (Fig 9B). For escalators and non-escalators, there are no significant differences in mean active responses in the first hour for each block between sex. Male escalators demonstrated a lower mean active responses in the first hour during ShA baseline compared to male non-escalators. When computing percent baseline, there is still no significant interaction between escalation status and sex nor escalation status, sex, and session (Fig 9C). Similar to the raw values, female and male escalators both increase in their mean active responses in the first hour across blocks while female and male non-escalators do not change across blocks. Consistent with the raw values, there are no significant differences in mean active responses in the first hour for each block between sex. In contrast to the raw values, with percent baseline there is a significant difference in mean active responses in the first hour for each block between escalation status. Similar trends are seen with the six-hour active responses (Fig 9E-H).

Looking at load-up and maintenance, there are no significant differences between escalator females versus males and non-escalator females versus males (Fig 10A). The mean

ITI during the first ten minutes showed no significant interaction between escalation status and sex nor escalation status, sex, and session (Fig 10B). The mean ITI during the last fifty minutes of the first hour showed a significant interaction between escalation status and sex but no interaction between escalation status, sex, and session (Fig 10C).

Examining other operant responses revealed no significant interaction between escalation status and sex nor escalation status, sex, and session for any other operant responses (Fig 11). There was also no difference in number of sessions to criteria between escalator females versus males nor non-escalator females versus males.

DISCUSSION

The limited studies on sex differences in cocaine self-administration and, more specifically, escalation of cocaine consumption in rats are inconsistent in their conclusions and generally have low subject numbers. Here, we compiled 106 control/non-manipulated rats across several experiments all with the same behavioral design to examine potential differences between sex (female versus male), SUD-like phenotypic status (escalator versus non-escalator), and the combination of the two.

There were two main differences observed between females and males: 1) during the first hour of cocaine SA sessions, females consume more cocaine than males and 2) females acquire cocaine SA faster than males. Further analyzing differences in overall consumption, while there is a difference during the first hour of cocaine SA across sessions, there is no difference in consumption when sessions are averaged into blocks, there is no difference in total (6hr) consumption, and there is no difference in the first ten minutes (load-up phase). Additionally, the overall pattern of consumption between females and males appears qualitatively similar, and when we normalize to the short-access baseline, the sex difference disappears. This demonstrates that the pattern of cocaine consumption over sessions is similar between females and males.

We classified rats as escalators or non-escalators based on a linear regression of their active responses in the first hour across sessions. Overall, as expected, we saw that the escalator group increased in their cocaine consumption across sessions while the non-escalator group maintained steady levels of cocaine consumption across sessions, particularly in the first hour of cocaine SA. In accordance with the SUD-like phenotype of escalation, we saw that the escalator group increased in their cocaine consumption specifically in the first ten minutes across sessions, demonstrating an increase in load-up, which was not observed in the non-escalator group. The main unexpected difference that we observed was the escalator group had lower levels of cocaine consumption in the first hour of cocaine SA during short-access compared to the non-escalator group.

To assess our behavioral paradigm's ability to effectively produce the SUD-like phenotype of escalation similarly across sex, we examined potential difference between females and males in the escalator and non-escalator groups. Importantly, we observed similar percentages of escalators to non-escalators in both females and males and the degree of escalation was similar between females and males. Overall, there were no significant interactions between sex and escalation status. This demonstrates that this behavioral paradigm is able to produce this SUD-like phenotype similarly between females and males.

It is important to note that a lack of sex differences reported here is strictly discussing behavioral differences. While this paradigm produces similar behavioral phenotypes across sex, it is still unknown if this behavioral phenotype is driven by the same neural mechanisms in females and males. However, this data suggests that this behavioral paradigm can be utilized to study escalation of cocaine consumption in both females and males in order to further investigate the underlying neurobiology and therapeutic interventions.

FIGURES

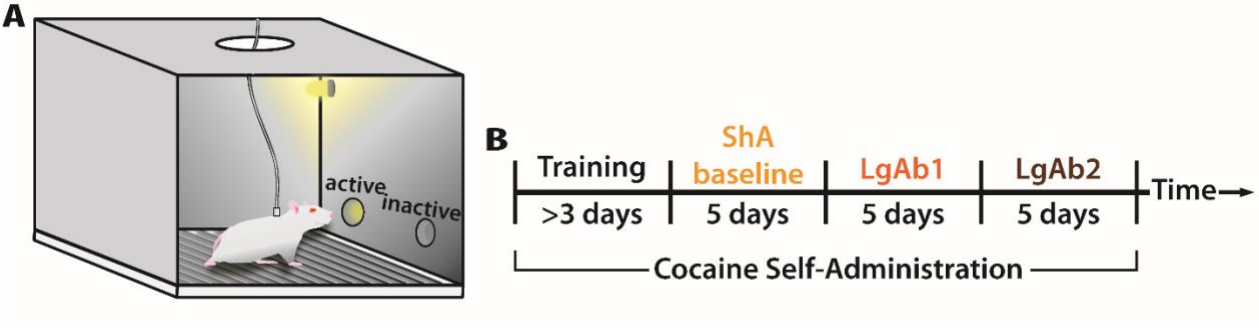


Fig 1. Overarching Experimental Design

(A) Behavioral Setup. Cocaine dose is 0.5 mg/kg/infusion. Fixed Ratio schedule 1. **(B)** Common experimental timeline. There was a minimum of 7 days between the last surgery and the start of training. Short Access (ShA) sessions are 1hr and Long Access (LgA) sessions are 6hr long.

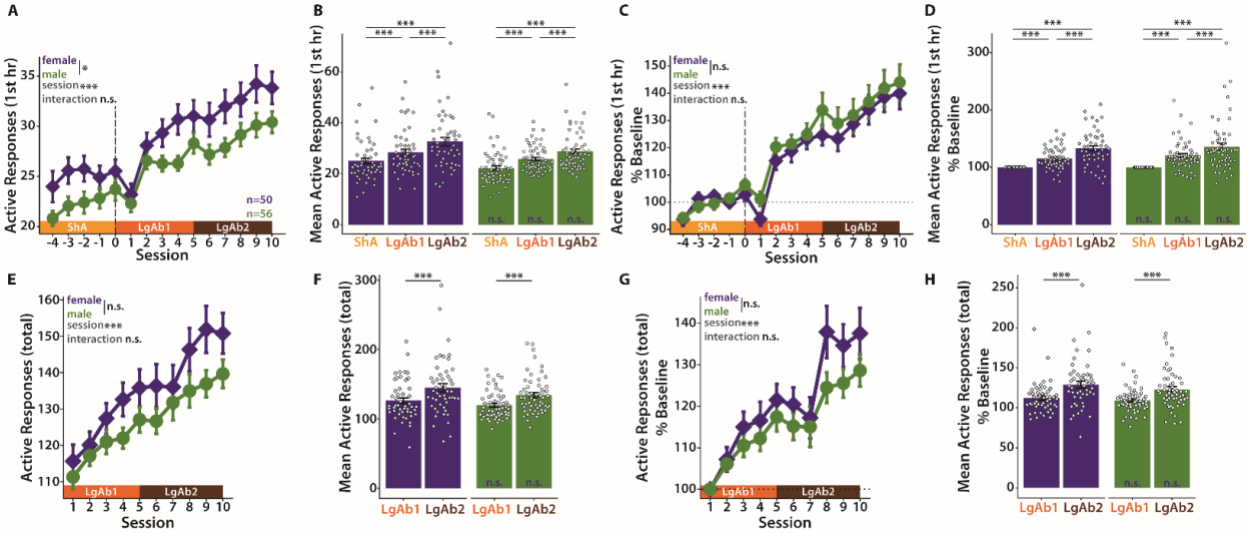


Fig 2. Cocaine Self-Administration across Behavioral Sessions

(A) Number of operant responses in the active hole during the first hour of cocaine SA across behavioral sessions. A mixed effects model revealed a significant main effect of session ($F(14,1425) = 46.6$, p-value < 0.001), a significant main effect of sex ($F(1,104) = 4.6$, p-value < 0.05), and no interaction ($F(14,1425) = 0.9$, p-value = 0.60). **(B)** Mean number of responses in active hole during the first hour of cocaine SA across behavioral blocks. A repeated-measures ANOVA revealed a significant main effect of block (p-value < 0.001), a significant main effect of sex (p-value < 0.05), and no interaction (p-value = 0.45). **(C)** Number of operant responses in the active hole during the first hour of cocaine SA computed as a percent baseline (ShA sessions used as baseline) across behavioral sessions. A mixed effects model revealed a significant main effect of session ($F(14,1425) = 49.9$, p-value < 0.001), no effect of sex ($F(1,104) = 0.6$, p-value = 0.45), and no interaction ($F(14,1425) = 0.5$, p-value = 0.92). **(D)** Mean number of responses in active hole during the first hour of cocaine SA computed as a percent baseline (ShA sessions used as baseline) across behavioral blocks. A repeated-measures ANOVA revealed a significant main effect of block (p-value < 0.001), no main effect of sex (p-value = 0.48), and no interaction (p-value = 0.66). **(E)** Total number of operant responses in the active hole during cocaine SA

across long-access behavioral sessions. A mixed effects model revealed a significant main effect of session ($F(9,902) = 41.9$, p-value < 0.001), no effect of sex ($F(1,104) = 2.6$, p-value = 0.11), and no interaction ($F(9,902) = 1.1$, p-value = 0.38). **(F)** Mean total number of responses in active hole during cocaine SA across long-access behavioral blocks. A repeated-measures ANOVA revealed a significant main effect of block (p-value < 0.001), no effect of sex (p-value = 0.11), and no interaction (p-value = 0.31). **(G)** Total number of operant responses in the active hole during cocaine SA computed as a percent baseline (long-access session 1 used as baseline) across long-access behavioral sessions. A mixed effects model revealed a significant main effect of session ($F(9,929) = 33.7$, p-value < 0.001), no effect of sex ($F(1,104) = 1.56$, p-value = 0.22), and no interaction ($F(9,929) = 1.2$, p-value = 0.30). **(H)** Mean total number of responses in active hole during cocaine SA computed as a percent baseline (long-access session 1 used as baseline) across long-access behavioral blocks. A repeated-measures ANOVA revealed a significant main effect of block (p-value < 0.001), no main effect of sex (p-value = 0.29), and no interaction (p-value = 0.40). **(all)** *** = p-value < 0.001 ; ** = p-value < 0.01 ; * = p-value < 0.05

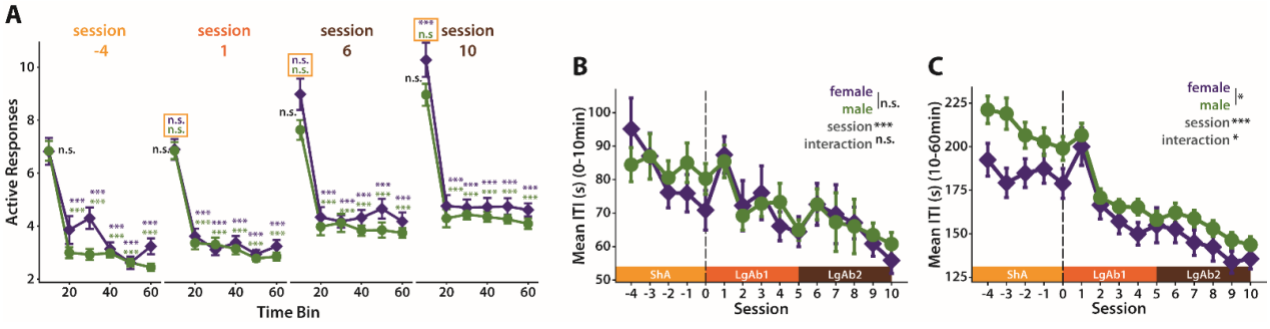


Fig 3. Load up and Maintenance of Cocaine Self-Administration

(A) Number of operant responses on the active hole during cocaine SA broken down into ten-minute bins for the first hour. The first day of ShA (session -4), first day of LgAb1 (session 1), first day of LgAb2 (session 6), and the last day of LgAb2 (session 10) are shown. A repeated-measures ANOVA revealed a significant main effect of time bin (p-value < 0.001), a significant main effect of session (p-value < 0.001), no main effect of sex (p-value = 0.09), a significant interaction between time bin and session (p-value <0.001), no interaction between sex and time bin (p-value = 0.81), no interaction between sex and session (p-value = 0.39), and no interaction between sex, time bin, and session (p-value = 0.7). Statistics shown on time bins 20-60 of the plot are post-hoc Tukey of the given time bin compared to time bin = 10 for the given sex/session. Statistics shown in the boxes above time bin = 10 on the plot are post-hoc Tukey of the time bin = 10 for the given session compared to time bin = 10 for session -4 for the given sex. Statistics shown in black to the side of time bin = 10 for each session is post-hoc Tukey comparing female versus male for time bin = 10 of the given session. **(B)** Mean inter-trial interval (ITI) during the first ten minutes of cocaine SA across behavioral sessions. A repeated-measures ANOVA revealed a significant main effect of session (p-value < 0.001), no main effect of sex (p-value = 0.97), and no interaction (p-value = 0.76). **(C)** Mean inter-trial interval (ITI) during the 10-60 minute period of each cocaine SA session. A repeated-measures ANOVA revealed a significant main effect of session (p-value < 0.001), a significant main effect of sex (p-value < 0.05), and a

significant interaction (p-value < 0.05). **(all)** *** = p-value < 0.001 ; ** = p-value < 0.01 ; * = p-value < 0.05

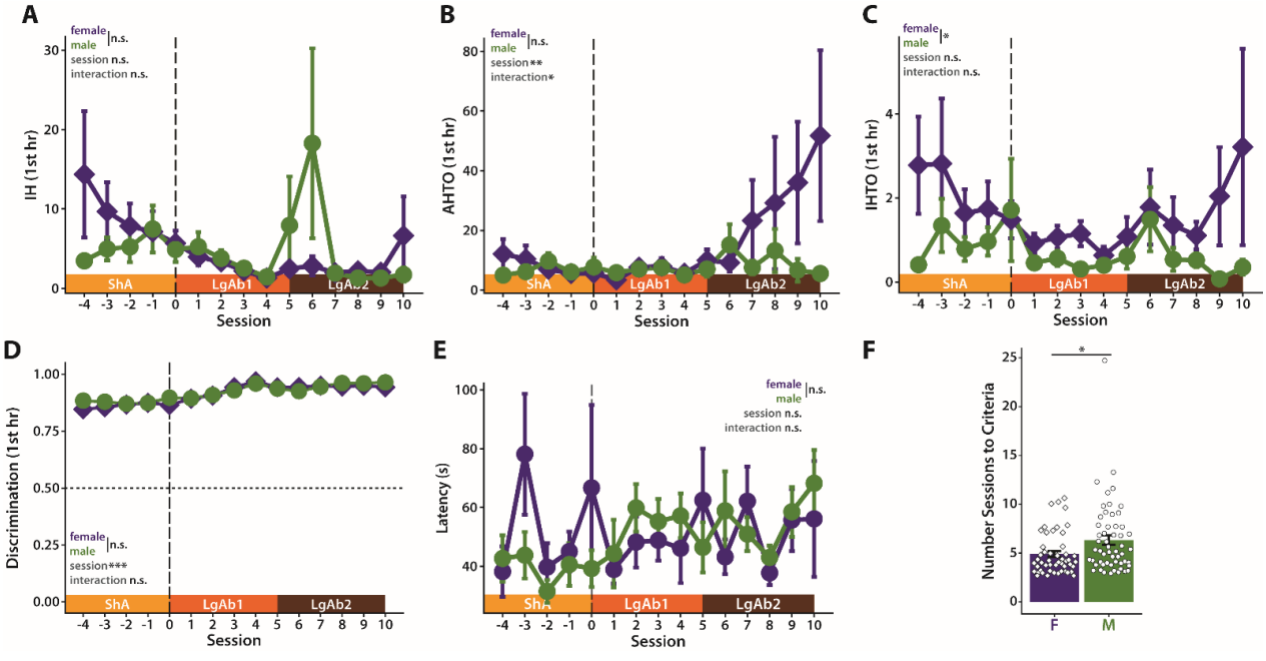


Figure 4. Other Operant Responses during Cocaine Self-Administration

(A) Operant responses in the inactive hole (IH) during the first hour of cocaine SA across behavioral sessions. A mixed effects model revealed no main effect of session ($F(14,1427) = 1.6$, p-value =0.07), no main effect of sex ($F(1,105) = 0.007$, p-value = 0.93), and no interaction ($F(14,1427) = 1.6$, p-value = 0.08). **(B)** Operant responses in the active hole during the time-out period (AHTO) during the first hour of cocaine SA across behavioral sessions. A mixed effects model revealed a significant main effect of session ($F(14,1426) = 2.1$, p-value < 0.01), no main effect of sex ($F(1,104) = 1.7$, p-value = 0.20), and a significant interaction ($F(14,1426) = 2.1$, p-value < 0.05). **(C)** Operant responses in the inactive hole during the time-out period (IHTO) during the first hour of cocaine SA across behavioral sessions. A mixed effects model revealed no main effect of session ($F(14,1425) = 1.0$, p-value = 0.40), a significant main effect of sex ($F(1,103) = 4.1$, p-value < 0.05), and no interaction ($F(14,1425) = 0.85$, p-value = 0.61). **(D)** Discrimination during the first hour of cocaine SA across behavioral sessions. Discrimination was calculated as the number of operant responses on the active hole divided by the number of operant responses on either active or inactive hole (note: only responses during cocaine available periods). A mixed effects model revealed a significant main effect of session ($F(14,1426) = 11.2$, p-value < 0.01),

no main effect of sex ($F(1,104) = 0.19$, p-value = 0.67), and no interaction ($F(14,1426) = 0.56$, p-value = 0.90). **(E)** Latency to first operant response in the active hole across behavioral sessions. A mixed effects model revealed no main effect of session ($F(14,1424) = 1.3$, p-value = 0.18), no main effect of sex ($F(1,104) = 0.10$, p-value = 0.76), and no interaction ($F(14,1424) = 1.1$, p-value = 0.34). (F) Number of days to meet criteria for females and males. Minimum number of days to meet criteria necessary is 3 days (see methods for more details on criteria). A t-test revealed a significant difference in number of days to meet criteria (p-value < 0.05). **(all)** *** = p-value < 0.001 ; ** = p-value < 0.01 ; * = p-value < 0.05

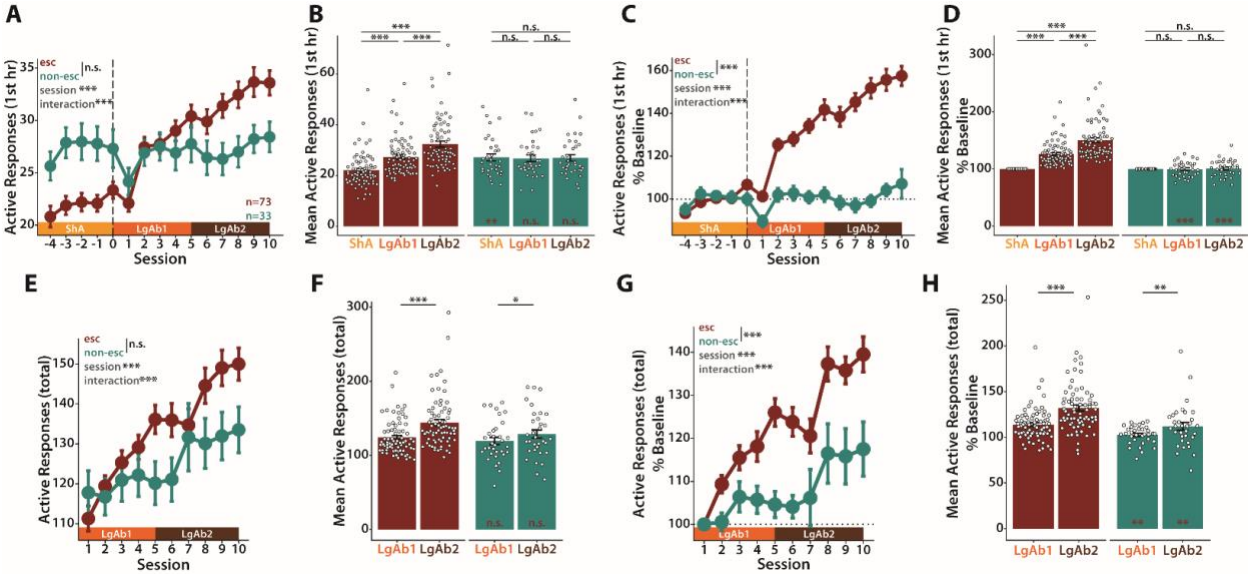


Figure 5. Cocaine Self-Administration Across Behavioral Sessions Separated by Escalation Status

(A) Number of operant responses in the active hole during the first hour of cocaine SA across behavioral sessions. A mixed effects model revealed a significant main effect of session ($F(14,1425) = 27.3$, p-value < 0.001), no main effect of escalation status ($F(1,104) = 0.01$, p-value = 0.91), and a significant interaction ($F(14,1425) = 20.9$, p-value < 0.001). **(B)** Mean number of responses in active hole during the first hour of cocaine SA across behavioral blocks. A repeated-measures ANOVA revealed a significant main effect of block (p-value < 0.001), no main effect of escalation status (p-value = 0.87), and a significant interaction (p-value < 0.001). **(C)** Number of operant responses in the active hole during the first hour of cocaine SA computed as a percent baseline (ShA sessions used as baseline) across behavioral sessions. A mixed effects model revealed a significant main effect of session ($F(14,1425) = 29.9$, p-value < 0.001), a significant main effect of escalation status ($F(1,104) = 58.9$, p-value < 0.001), and a significant interaction ($F(14,1425) = 22.1$, p-value <0.001). **(D)** Mean number of responses in active hole during the first hour of cocaine SA computed as a percent baseline (ShA sessions used as baseline) across behavioral blocks. A repeated-measures ANOVA revealed a significant main effect of block (p-value < 0.001), a significant main effect of escalation status (p-value < 0.001), and a significant

interaction (p-value < 0.001). **(E)** Total number of operant responses in the active hole during cocaine SA across long-access behavioral sessions. A mixed effects model revealed a significant main effect of session ($F(9,902) = 28.3$, p-value < 0.001), no effect of escalation status ($F(1,104) = 2.7$, p-value = 0.10), and a significant interaction ($F(9,902) = 5.7$, p-value < 0.001). **(F)** Mean total number of responses in active hole during cocaine SA across long-access behavioral blocks. A repeated-measures ANOVA revealed a significant main effect of block (p-value < 0.001), no effect of escalation status (p-value = 0.10), and a significant interaction (p-value < 0.001). **(G)** Total number of operant responses in the active hole during cocaine SA computed as a percent baseline (long-access session 1 used as baseline) across long-access behavioral sessions. A mixed effects model revealed a significant main effect of session ($F(9,929) = 22.5$, p-value < 0.001), a significant main effect of escalation status ($F(1,104) = 12.2$, p-value < 0.001), and a significant interaction ($F(9,929) = 3.4$, p-value < 0.001). **(H)** Mean total number of responses in active hole during cocaine SA computed as a percent baseline (long-access session 1 used as baseline) across long-access behavioral blocks. A repeated-measures ANOVA revealed a significant main effect of block (p-value < 0.001), a significant main effect of escalation status (p-value < 0.001), and a significant interaction (p-value < 0.05). **(all)** *** = p-value < 0.001 ; ** = p-value < 0.01 ; * = p-value < 0.05

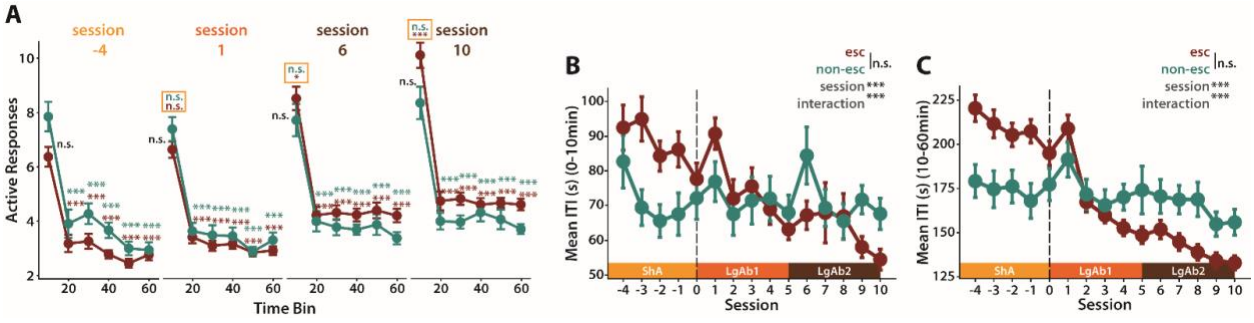


Figure 6. Load up and Maintenance of Cocaine Self-Administration Separated by Escalation Status

(A) Number of operant responses on the active hole during cocaine SA broken down into ten-minute bins for the first hour. The first day of ShA (session -4), first day of LgAb1 (session 1), first day of LgAb2 (session 6), and the last day of LgAb2 (session 10) are shown. A repeated-measures ANOVA revealed a significant main effect of time bin (p-value < 0.001), a significant main effect of session (p-value < 0.001), no main effect of escalation status (p-value = 0.90), a significant interaction between time bin and session (p-value < 0.01), no interaction between escalation status and time bin (p-value = 0.94), a significant interaction between escalation status and session (p-value < 0.001), and no interaction between escalation status, time bin, and session (p-value = 0.31). Statistics shown on time bins 20-60 of the plot are post-hoc Tukey of the given time bin compared to time bin = 10 for the given sex/session. Statistics shown in the boxes above time bin = 10 on the plot are post-hoc Tukey of the time bin = 10 for the given session compared to time bin = 10 for session -4 for the given sex. Statistics shown in black to the side of time bin = 10 for each session is post-hoc Tukey comparing female versus male for time bin = 10 of the given session. **(B)** Mean inter-trial interval (ITI) during the first ten minutes of cocaine SA across behavioral sessions. A repeated-measures ANOVA revealed a significant main effect of session (p-value < 0.001), no main effect of escalation status (p-value = 0.51), and a significant interaction (p-value < 0.001). **(C)** Mean inter-trial interval (ITI) during the 10-60 minute period of each cocaine SA session. A repeated-measures ANOVA revealed a significant main effect of session (p-value

< 0.001), no main effect of escalation status (p-value = 0.75), and a significant interaction (p-value < 0.001). **(all)** *** = p-value < 0.001 ; ** = p-value < 0.01 ; * = p-value < 0.05

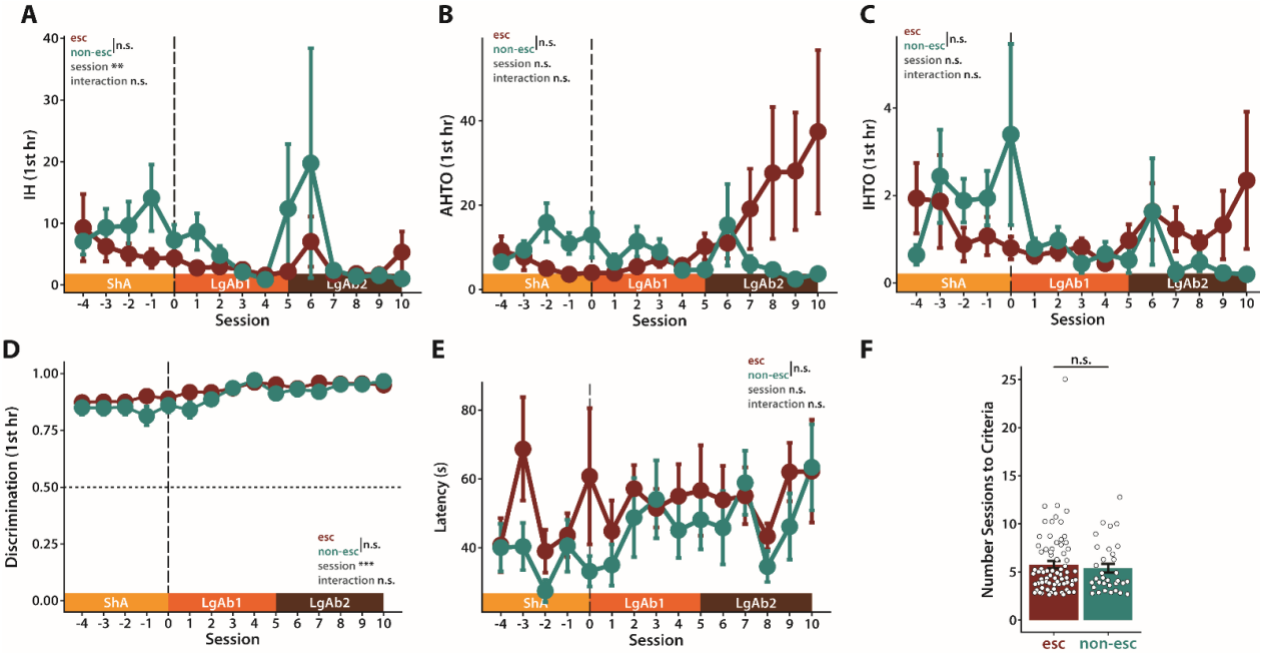


Figure 7. Other Operant Responses during Cocaine Self-Administration Separated by Escalation status

(A) Operant responses in the inactive hole (IH) during the first hour of cocaine SA across behavioral sessions. A mixed effects model revealed a significant main effect of session ($F(14,1427) = 2.1$, p-value < 0.01), no main effect of escalation status ($F(1,105) = 1.7$, p-value = 0.20), and no interaction ($F(14,1427) = 1.0$, p-value = 0.41). **(B)** Operant responses in the active hole during the time-out period (AHTO) during the first hour of cocaine SA across behavioral sessions. A mixed effects model revealed no main effect of session ($F(14,1426) = 0.7$, p-value = 0.79), no main effect of escalation status ($F(1,104) = 0.45$, p-value = 0.50), and no interaction ($F(14,1426) = 1.7$, p-value = 0.06). **(C)** Operant responses in the inactive hole during the time-out period (IHTO) during the first hour of cocaine SA across behavioral sessions. A mixed effects model revealed no main effect of session ($F(14,1425) = 1.2$, p-value = 0.30), no main effect of escalation status ($F(1,103) = 0.04$, p-value = 0.85), and no interaction ($F(14,1425) = 1.4$, p-value = 0.16). **(D)** Discrimination during the first hour of cocaine SA across behavioral sessions. Discrimination was calculated as the number of operant responses on the active hole divided by the number of operant responses on either active or inactive hole (note: only responses during

cocaine available periods). A mixed effects model revealed a significant main effect of session ($F(14,1426) = 11.5$, p-value < 0.01), no main effect of escalation status ($F(1,104) = 1.9$, p-value = 0.17), and no interaction ($F(14,1426) = 1.5$, p-value = 0.09). **(E)** Latency to first operant response in the active hole across behavioral sessions. A mixed effects model revealed no main effect of session ($F(14,1424) = 1.1$, p-value = 0.34), no main effect of escalation status ($F(1,104) = 1.2$, p-value = 0.28), and no interaction ($F(14,1424) = 0.4$, p-value = 0.98). (F) Number of days to meet criteria for escalators and non-escalators. Minimum number of days to meet criteria necessary is 3 days. A t-test revealed no significant difference in number of days to meet criteria (p-value = 0.79). **(all)** *** = p-value < 0.001 ; ** = p-value < 0.01 ; * = p-value < 0.05

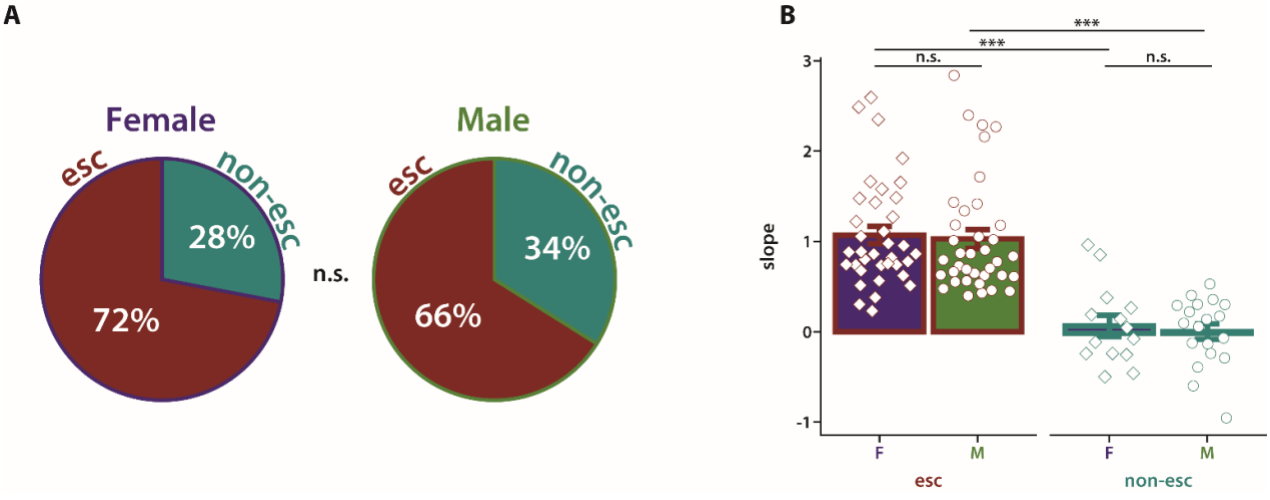


Figure 8. Escalator and Non-Escalator Populations between Sexes

(A) Percent of escalators versus non-escalators in females and males. A chi-squared goodness of fit test demonstrated no significance. **(B)** Slope of the regression separated by escalator and non-escalator females and males. An ANOVA revealed a significant main effect of escalation status (p-value < 0.001), no main effect of sex (p-value = 0.66), and no interaction (p-value = 0.94). **(all)** *** = p-value < 0.001 ; ** = p-value < 0.01 ; * = p-value < 0.05

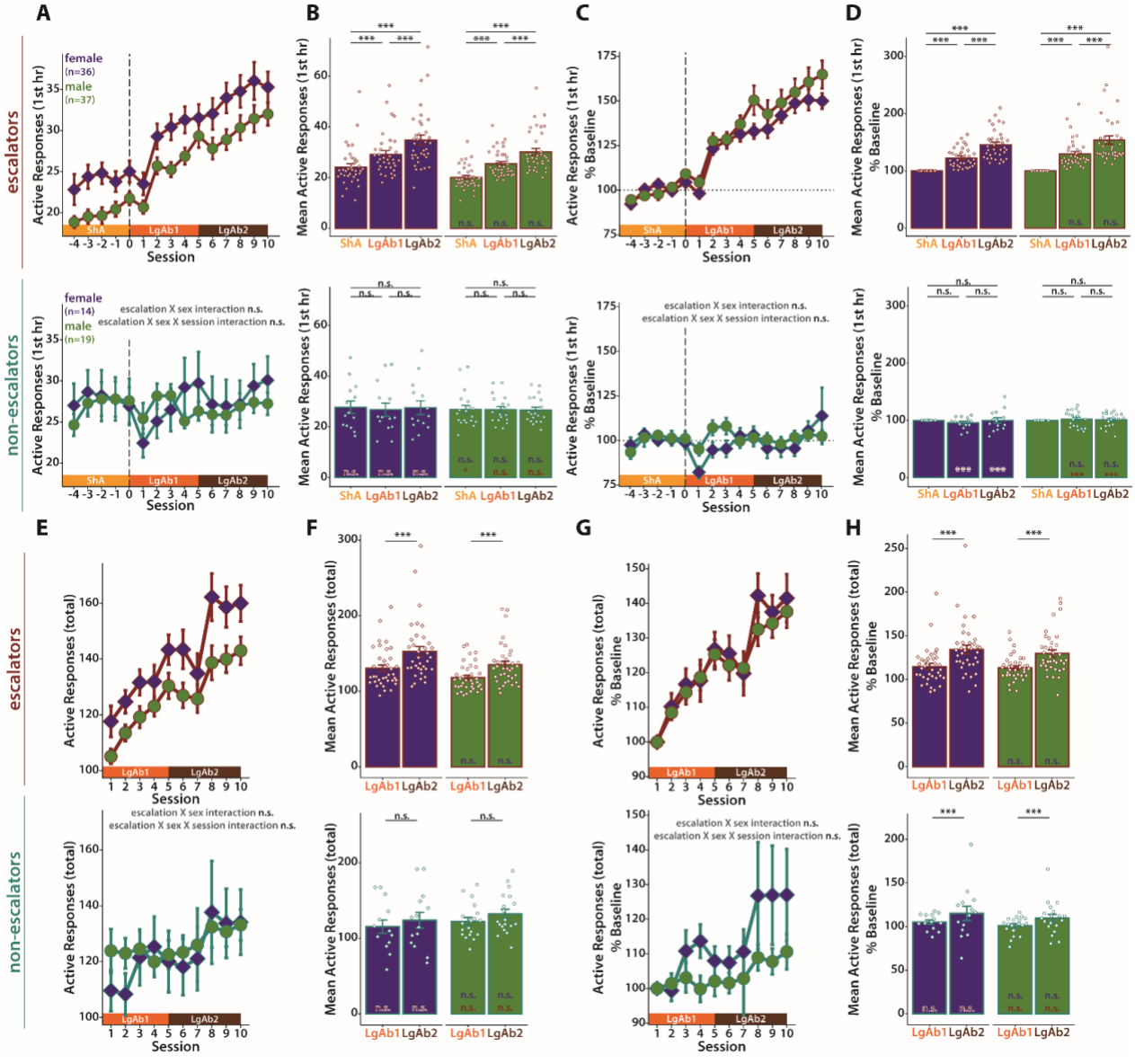


Figure 9. Cocaine Self-Administration across Behavioral Sessions Separated by Sex and Escalation status

(A) Number of operant responses in the active hole during the first hour of cocaine SA across behavioral sessions. A mixed effects model revealed a no interaction between escalation status and sex ($F(1,102) = 1.2$, p-value = 0.27) and no interaction between escalation status, sex, and session ($F(14,1397) = 1.2$, p-value = 0.25). **(B)** Mean number of responses in active hole during the first hour of cocaine SA across behavioral blocks. A repeated-measures ANOVA revealed no interaction between escalation status and sex (p-value = 0.26) and no interaction between

escalation status, sex, and block (p-value = 0.95). **(C)** Number of operant responses in the active hole during the first hour of cocaine SA computed as a percent baseline (ShA sessions used as baseline) across behavioral sessions. A mixed effects model revealed no interaction between escalation status and sex (p-value = 0.65) and no interaction between escalation status, sex, and session (p-value = 0.28). **(D)** Mean number of responses in active hole during the first hour of cocaine SA computed as a percent baseline (ShA sessions used as baseline) across behavioral blocks. A repeated-measures ANOVA revealed no interaction between escalation status and sex (p-value = 0.71) and no interaction between escalation status, sex, and block (p-value = 0.78). **(E)** Total number of operant responses in the active hole during cocaine SA across long-access behavioral sessions. A mixed effects model revealed a no interaction between escalation status and sex ($F(1,102) = 3.4$, p-value = 0.07) and no interaction between escalation status, sex, and session ($F(9,884) = 1.4$, p-value = 0.20). **(F)** Mean total number of responses in active hole during cocaine SA across long-access behavioral blocks. A repeated-measures ANOVA revealed no interaction between escalation status and sex (p-value = 0.06) and no interaction between escalation status, sex, and block (p-value = 0.38). **(G)** Total number of operant responses in the active hole during cocaine SA computed as a percent baseline (long-access session 1 used as baseline) across long-access behavioral sessions. A mixed effects model revealed no interaction between escalation status and sex (p-value = 0.48) and no interaction between escalation status, sex, and session (p-value = 0.88). **(H)** Mean total number of responses in active hole during cocaine SA computed as a percent baseline (long-access session 1 used as baseline) across long-access behavioral blocks. A repeated-measures ANOVA revealed no interaction between escalation status and sex (p-value = 0.83) and no interaction between escalation status, sex, and block (p-value = 0.80). **(all)** top row is escalators and bottom row is non-escalators. *** = p-value < 0.001 ; ** = p-value < 0.01 ; * = p-value < 0.05

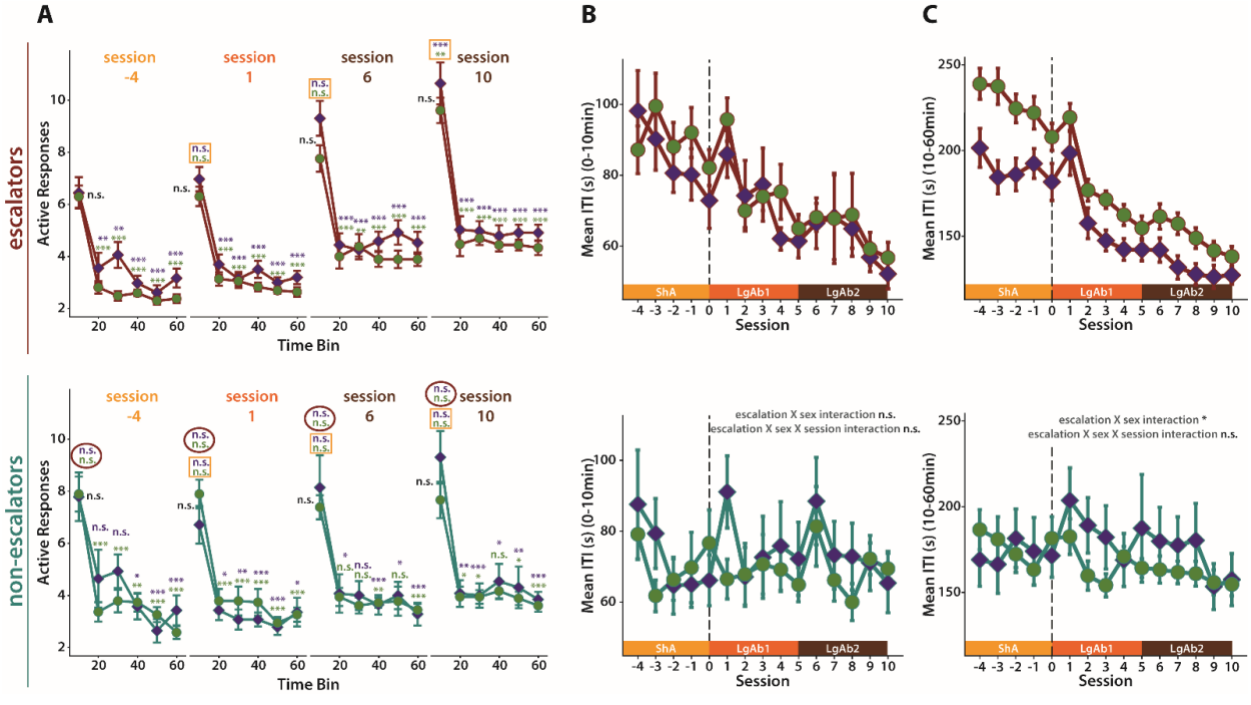


Fig 10. Load up and Maintenance of Cocaine Self-Administration Separated by Sex and Escalation status

(A) Number of operant responses on the active hole during cocaine SA broken down into ten-minute bins for the first hour. The first day of ShA (session -4), first day of LgAb1 (session 1), first day of LgAb2 (session 6), and the last day of LgAb2 (session 10) are shown. A repeated-measures ANOVA no interaction between escalation status and sex (p-value = 0.65), no interaction between escalation status, sex, and time bin (p-value = 0.96), no interaction between escalation status, sex, and session (p-value = 0.38), and no interaction between escalation status, sex, time bin, and session (p-value = 0.96). Statistics shown on time bins 20-60 of the plot are post-hoc Tukey of the given time bin compared to time bin = 10 for the given sex/session. Statistics shown in the boxes above time bin = 10 on the plot are post-hoc Tukey of the time bin = 10 for the given session compared to time bin = 10 for session -4 for the given sex. Statistics shown in black to the side of time bin = 10 for each session is post-hoc Tukey comparing female versus male for time bin = 10 of the given session. Statistics shown in the circle above time bin = 10 for each session is post-hoc Tukey comparing escalators versus non-escalators for a given

sex at that time bin and session. **(B)** Mean inter-trial interval (ITI) during the first ten minutes of cocaine SA across behavioral sessions. A repeated-measures ANOVA revealed no interaction between escalation status and sex (p-value =0.55) and no interaction between escalation status, sex, and session (p-value = 0.72). **(C)** Mean inter-trial interval (ITI) during the 10-60 minute period of each cocaine SA session. A repeated-measures ANOVA revealed a significant interaction between escalation status and sex (p-value < 0.05) and no interaction between escalation status, sex, and session (p-value = 0.66). **(all)** top row is escalators and bottom row is non-escalators. *** = p-value < 0.001 ; ** = p-value < 0.01 ; * = p-value < 0.05

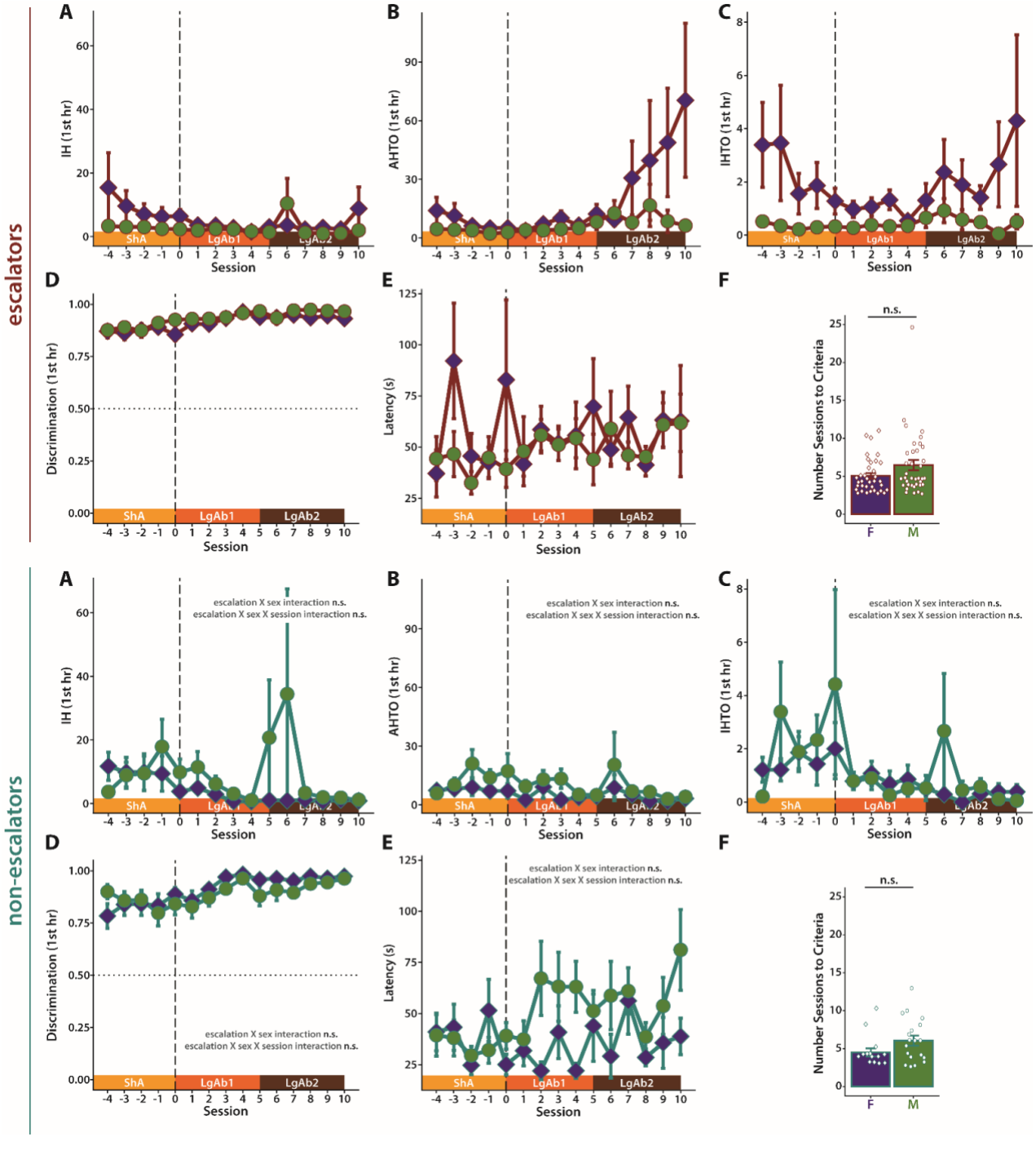
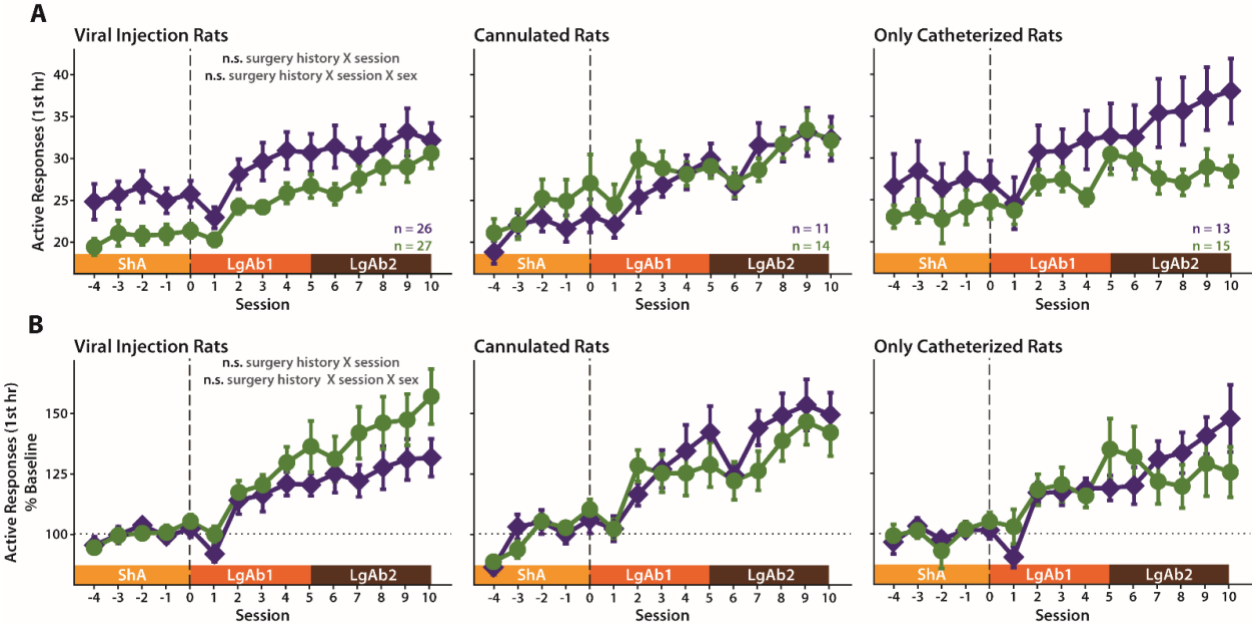


Figure 11. Other Operant Responses during Cocaine Self-Administration Separated by Sex and Escalation status

(A) Operant responses in the inactive hole (IH) during the first hour of cocaine SA across behavioral sessions. A mixed effects model revealed no interaction between escalation status and sex (p-value = 0.09) and no interaction between escalation status, sex, and session (p-value

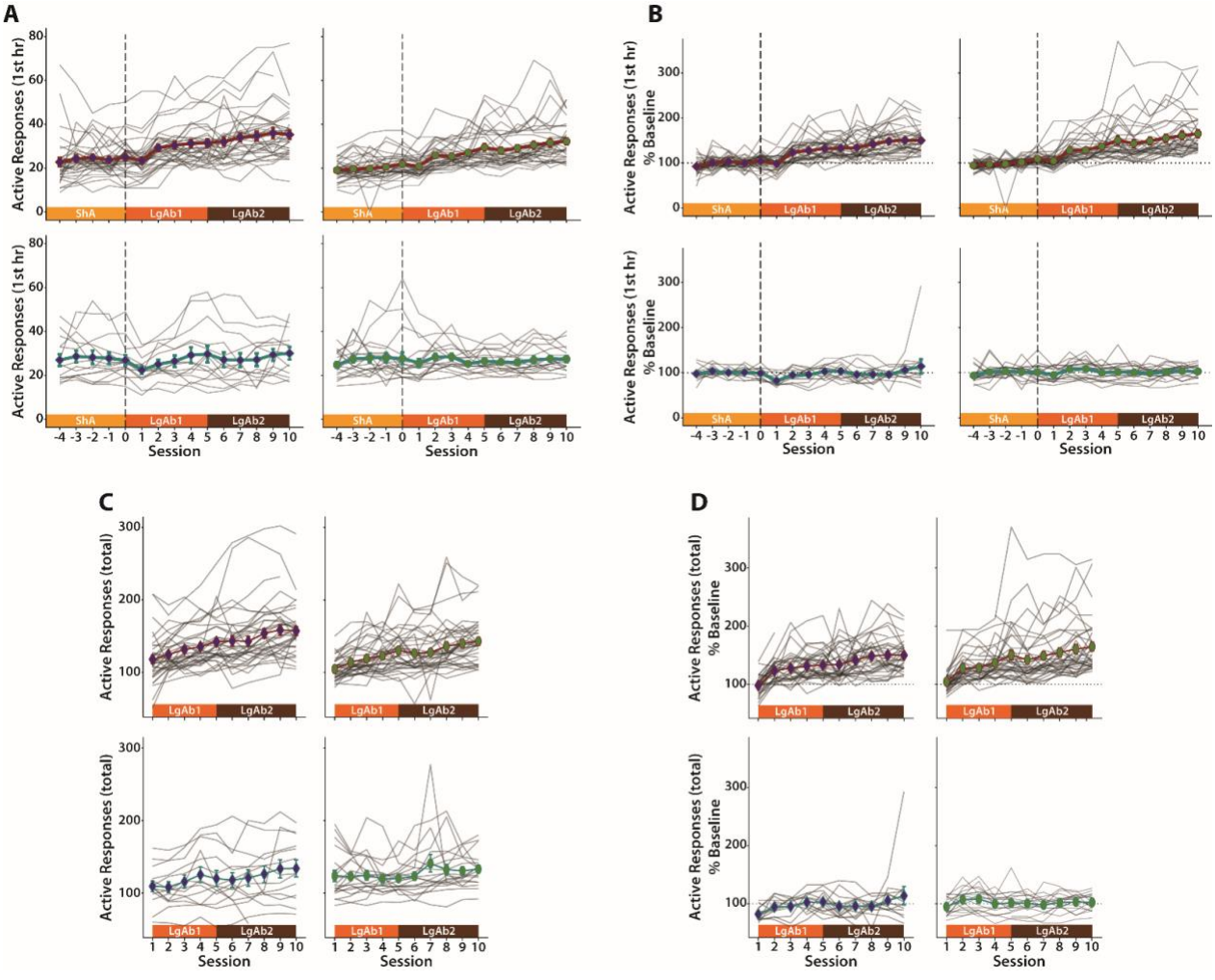
= 0.88). **(B)** Operant responses in the active hole during the time-out period (AHTO) during the first hour of cocaine SA across behavioral sessions. A mixed effects model revealed no interaction between escalation status and sex (p-value = 0.17) and no interaction between escalation status, sex, and session (p-value = 0.86). **(C)** Operant responses in the inactive hole during the time-out period (IHTO) during the first hour of cocaine SA across behavioral sessions. A mixed effects model revealed no interaction between escalation status and sex (p-value = 0.06) and no interaction between escalation status, sex, and session (p-value = 0.89). **(D)** Discrimination during the first hour of cocaine SA across behavioral sessions. Discrimination was calculated as the number of operant responses on the active hole divided by the number of operant responses on either active or inactive hole (note: only responses during cocaine available periods). A mixed effects model revealed no interaction between escalation status and sex (p-value = 0.20) and no interaction between escalation status, sex, and session (p-value = 0.18). **(E)** Latency to first operant response in the active hole across behavioral sessions. A mixed effects model revealed no interaction between escalation status and sex (p-value = 0.18) and no interaction between escalation status, sex, and session (p-value = 0.92). **(F)** Number of days to meet criteria for escalators and non-escalators. Minimum number of days to meet criteria necessary is 3 days. An ANOVA revealed no main effect of escalation status (p-value = 0.46), a significant main effect of sex (p-value < 0.05), and no interaction (p-value = 0.91). **(all)** top two rows are escalators and bottom two rows are non-escalators. *** = p-value < 0.001 ; ** = p-value < 0.01 ; * = p-value < 0.05

SUPPLEMENTAL FIGURES



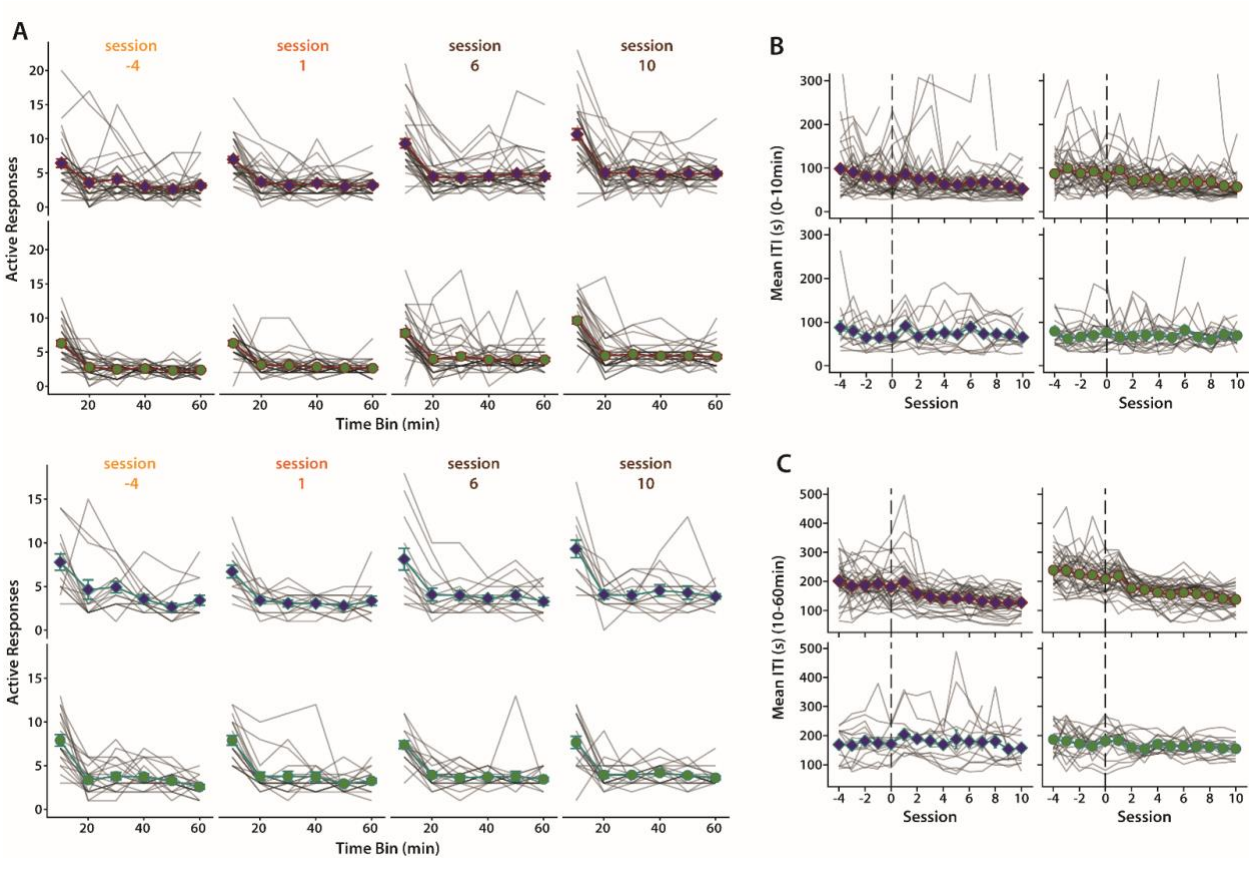
Supp Figure 1. Specific Experimental Design Does Not Impact Cocaine SA Behavior between Sexes

(A) Operant responses in the active hole during the first hour of cocaine SA separated by specific experimental design. A mixed effects model revealed no main effect of specific experimental design (p-value = 0.13), no interaction between sex and specific experimental design (p-value = 0.27), and no interaction between sex, session, and specific experimental design (p-value = 0.25). **(B)** Operant responses in the active hole during the first hour computed as a percent baseline. A mixed effects model revealed no main effect of specific experimental design (p-value = 0.26), no interaction between sex and specific experimental design (p-value = 0.65), and no interaction between sex, session, and specific experimental design (p-value = 0.28).



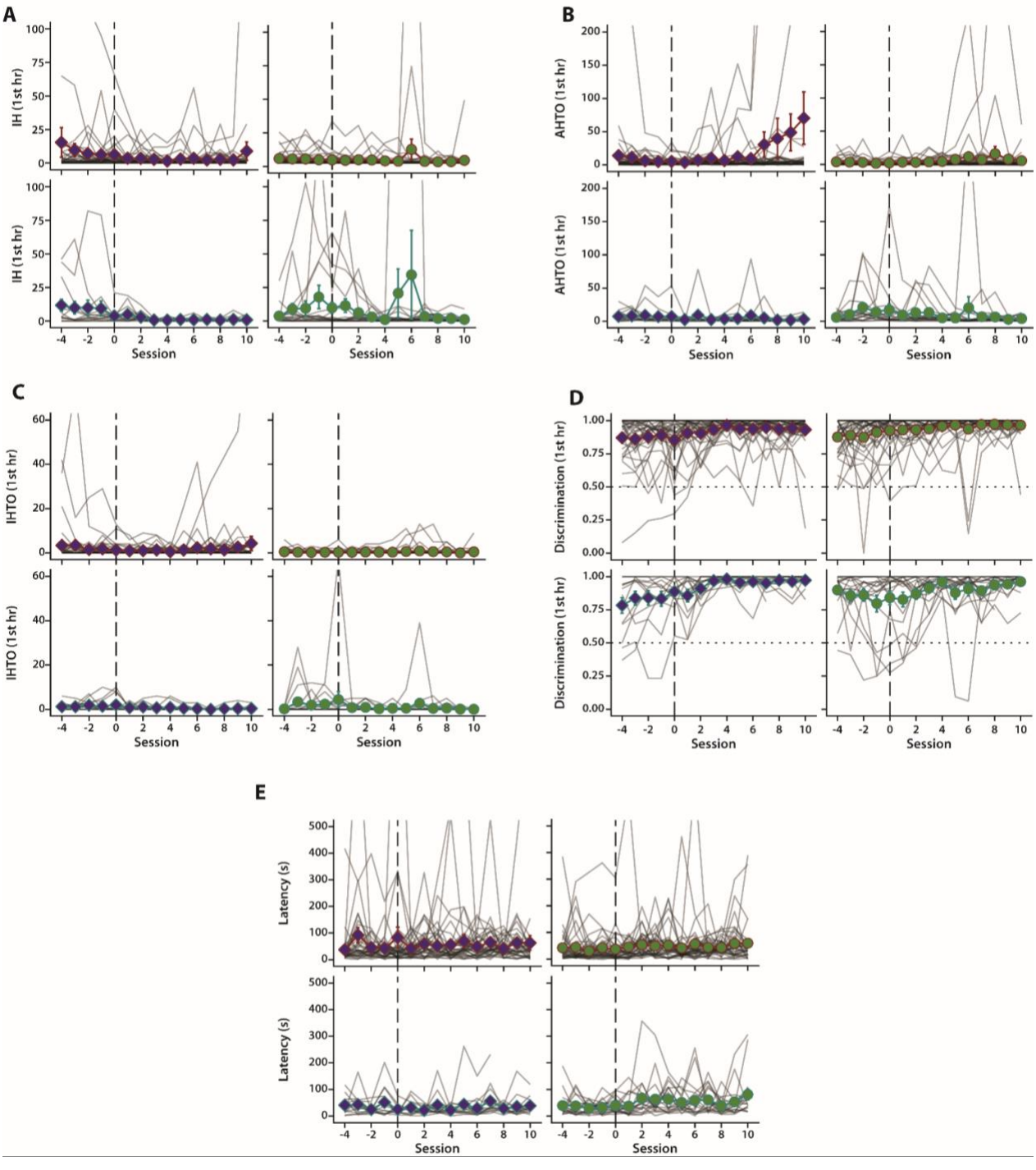
Supp Figure 2. Individual Traces for Active Responses

(A) Number of operant responses in the active hole during the first hour of cocaine SA across behavioral sessions. **(B)** Number of operant responses in the active hole during the first hour of cocaine SA computed as a percent baseline (ShA sessions used as baseline) across behavioral sessions. **(C)** Total number of operant responses in the active hole during cocaine SA across long-access behavioral sessions. **(D)** Total number of operant responses in the active hole during cocaine SA computed as a percent baseline (long-access session 1 used as baseline) across long-access behavioral sessions. **(all)** Colored line represents the mean and sem. Grey lines represent individual animals. For each panel, the top graphs are escalators (left: female, right: male) and the bottom graphs are non-escalators (left: female, right: male).



Supp Figure 3. Individual Traces for Load Up and Maintenance

(A) Number of operant responses on the active hole during cocaine SA broken down into ten-minute bins for the first hour. The first day of ShA (session -4), first day of LgAb1 (session 1), first day of LgAb2 (session 6), and the last day of LgAb2 (session 10) are shown. Top graph is escalators (upper: female, lower: male) and bottom graph is non-escalators (upper: female, lower: male). **(B)** Mean inter-trial interval (ITI) during the first ten minutes of cocaine SA across behavioral sessions. **(C)** Mean inter-trial interval (ITI) during the 10-60 minute period of each cocaine SA session. **(B & C)** top graphs are escalators (left: female, right: male) and the bottom graphs are non-escalators (left: female, right: male). **(all)** Colored line represents the mean and sem. Grey lines represent individual animals.



Supp Figure 4. Individual Traces for Other Operant Behaviors

(A) Operant responses in the inactive hole (IH) during the first hour of cocaine SA across behavioral sessions. **(B)** Operant responses in the active hole during the time-out period (AHTO) during the first hour of cocaine SA across behavioral sessions. **(C)** Operant responses in the

inactive hole during the time-out period (IHTO) during the first hour of cocaine SA across behavioral sessions. **(D)** Discrimination during the first hour of cocaine SA across behavioral sessions. Discrimination was calculated as the number of operant responses on the active hole divided by the number of operant responses on either active or inactive hole (note: only responses during cocaine available periods. **(E)** Latency to first operant response in the active hole across behavioral sessions. A mixed effects model revealed no interaction between escalation status and sex (p-value = 0.18) and no interaction between escalation status, sex, and session (p-value = 0.92). **(all)** Colored line represents the mean and sem. Grey lines represent individual animals. For each panel, the top graphs are escalators (left: female, right: male) and the bottom graphs are non-escalators (left: female, right: male).

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Chapter 5:

Discussion

The work presented in this dissertation furthers our understanding of the neurobiology behind escalation of drug use. This work will inform therapeutic interventions to reduce harm by preventing individuals from escalating their drug intake and reversing escalation in individuals already presenting this SUD-like phenotype. This will lead to reduced likelihood of infections, overdose, death, and loss (economic and personal) for individuals suffering from SUD and society as a whole.

SUMMARY OF FINDINGS

Chapter 2 investigates the role of the KOR system in escalation of cocaine consumption. Through pharmacology and gene editing technology, I demonstrated that functionally active KOR on VTA neurons (particularly VTA terminals in the NAc), but not NAc neurons themselves, is necessary for the development of escalation. Chapter 3 utilized similar techniques to investigate the role of the CRF system in escalation of cocaine consumption. I validated the ability of systemic CRF-R1 antagonism to slightly decrease overall levels of cocaine consumption under long access self-administration conditions and sought to uncover the specific CRF-R1 population underlying this effect. I demonstrated that functionally active CRF-R1 in the NAc is not necessary for the development nor sustainment of escalation. Chapter 4 details sex differences in cocaine self-administration and escalation behavior observed across several experiments in our lab. While there is often verbal discussion of observed sex differences in drug use working with rodent models, this type of data and meta-analyses are currently rarely published. The goal of this chapter is to provide sex difference data for consideration when generally interpreting experimental results and designing experiments with sex as a biological factor. Overall, this

dissertation provides evidence for the dynorphin/KOR system in the canonical reward pathway, irrespective of the CRF system in the NAc, to play a role in the SUD-like phenotype of escalation.

DYNORPHIN / KAPPA OPIOID RECEPTOR SYSTEM

There is still a lot unknown about the dynorphin/KOR system, particularly regarding its release patterns and dynamics. The overall temporal dynamics of dynorphin is poorly understood due to historically a lack of tools necessary for such measurements. Some studies have utilized microdialysis, but this approach can be challenging and the time scale of microdialysis typically analyzed is quite long (minutes to tens of minutes) (You et al., 1994; Marinelli et al., 2006; Lam et al., 2008). However, with the recent invention of in vivo fluorescence imaging coupled with a biosensor based on the KOR protein (klight), we can now measure sub-second resolution of dynorphin dynamics during freely moving behavior (Dong et al., 2024). This technology will allow us to uncover the signaling pattern of dynorphin (and other systems) like never before. Additionally, utilizing a KOR sensor (klight) and a red-shifted dopamine sensor (dlight) in the same brain region simultaneously during cocaine self-administration will allow us to determine the relationship between dynorphin and dopamine during single sessions of cocaine self-administration as well as across escalation. To implicate dopamine in the KOR's downstream pathway in modulating escalation, you could perform a unilateral knock out of KOR in VTA neurons with bilateral dopamine recording in the NAc during cocaine self-administration and examine within-subject differences in dopamine release across sessions. Alternatively, to investigate KOR activity more broadly, you could utilize a KOR sensor with a CREB or calcium sensor to investigate neuronal activity of populations in relation to dynorphin release, one such population of interest being dynorphin-containing medium spiny neurons in the NAc. These technologies can also be combined with pharmacology, systemic or local, to investigate causality and further implicate the relationship between these systems. Further understanding the dynamics and relationships of dynorphin in this system will inform the more 'psychological' role

dynorphin may be playing in the escalation of drug use – in other words, which SUD theory it is best described under and if it is truly acting as a β-process to the opponent process theory. Fitting dynorphin into its temporal dynamics and 'psychological' role of SUD will inform future pharmacological and behavioral therapeutics for SUD centered on the dynorphin/KOR system.

CORTICOTROPIN RELEASING FACTOR SYSTEM

The work presented in Chapter 3 centers mainly on the necessity of CRF-R1 activity in the NAc for the development and sustainment of cocaine escalation. Previous studies, confirmed here (Chapter 3, Fig 1), demonstrate the necessity of CRF-R1 globally to maintain high levels of cocaine consumption (Goeders and Guerin, 2000; Specio et al., 2008). This effect is small in magnitude, and we hypothesized that dialing in on SUD-like phenotypic rats and brain region specific manipulations would allow us to unmask a larger effect. Based on prior literature implicating CRF and the NAc in drug-related behaviors (Chapter 1), interactions between CRF and the dynorphin/KOR system, and our data demonstrating the necessity of KOR activation in the NAc for escalation (Chapter 2), we decided to target CRH-R1 in NAc neurons. Ultimately, our data suggests that CRH-R1 activity in the NAc does not seem necessary for the development nor sustainment of cocaine escalation regardless of SUD-like phenotype classification. Considering the small effect size from systemic manipulation, it could be feasible that with larger sample sizes a similar small effect could be achieved. The decision to focus on CRH-R1 stems from previous systemic antagonism and CRF-R mRNA localization studies (Goeders and Guerin, 2000; Pett et al., 2000; Specio et al., 2008). However, CRH-R2 has also been demonstrated to mediate behavioral effects driven by stress through activation of KORs, including KOR in the NAc (Land et al., 2008). Therefore, a logical next step would be to investigate the necessity of NAc CRH-R2 in the development and sustainment of cocaine escalation. However, it is also plausible that the CRF system is not working upstream of the KOR system to mediate escalation of cocaine consumption. While both systems have been implicated in similar behavioral outputs and

withdrawal symptoms related to SUD, there are key distinctions in their current literature. The CRF system has majorly been implicated in changes in drug taking/seeking behavior prompted by external stressors with a large focus on the reinstatement of drug taking while the KOR system has more been implicated in overall negative affect and dysphoria. The majority of work relating stress to drug taking, mediated by CRF, utilizes experimenter-imposed stressors. While there are some studies that examine drug-induced stress, they have typically utilized involuntary drug administration which is known to have differential effects on neural systems (Sarnyai et al., 1992, 1995; Jacobs et al., 2003). An unaddressed assumption is that drug-taking behavior itself is stressful under voluntary conditions and causes increases in drug taking in a similar manner to experimenter-imposed stressors. Under this assumption, the CRF system fits nicely in the β-process of the opponent process theory and as an upstream modulator of the KOR system in the SUD-like phenotype of escalation. There is still currently a lack of evidence to demonstrate the relationship between drug-induced stress, CRF activity, and increases in drug-related behaviors (particularly outside reinstatement paradigms). Potentially this identifies a divergence between the CRF and KOR systems in relation to drug-taking behaviors.

SEX AS A BIOLOGICAL FACTOR

Chapter 4 utilizes control animals across various experiments performed throughout this dissertation (and some experiments not included) to investigate potential sex differences in cocaine consumption and escalation. The main findings were that females overall consume more cocaine than males, females learn cocaine SA faster than males, the pattern of cocaine SA behavior over sessions is similar between females and males, and similar proportions of males and females demonstrate the SUD-like phenotype of escalation to similar degrees. Overall, the data presented in chapter 4 provides a validation that our cocaine SA paradigm is capable of producing a similar SUD-like phenotype regardless of sex. Importantly, this does not validate that the neurobiological underpinnings of escalation is the same across sex. As SUD research

continues to implement sex as a biological factor as a design and analysis consideration, it is imperative that we validate that assays developed using male-only cohorts are also effective in and informative for females. Chapter 4 provides strong evidence that this paradigm is appropriate to study sex differences in cocaine SA / escalation and to study this behavior in mixed-sex cohorts. Additionally, chapter 4 provides a resource of various measures of cocaine SA behavior divided by sex and escalation status that could be utilized in future meta-analyses and for consideration within the field.

CONCLUSION

This dissertation provides evidence for the role of KOR on VTA neurons in escalation of cocaine consumption utilizing a long-access cocaine SA paradigm that is shown to be effective in both female and male rats. While there is a strong demand for studying cocaine consumption specifically, due to a lack of pharmacotherapies for psychostimulant SUD, the hypotheses presented within this dissertation are under an assumption that SUD of all addictive drugs are mediated by the same neural adaptations. In support of this assumption, the literature on the KOR system (as well as CRF) is not restricted only to cocaine related behaviors. The ultimate goal is to identify processes shared across drug classes to identify common therapeutic strategies under a harm reductionist approach. The work presented here furthers our progress toward that goal.

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