Mechanisms and treatments of drug consumption: A preclinical exploration in harm reducing pharmacotherapies for substance use disorders

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Abstract

Mechanisms and treatment of drug consumption: A preclinical exploration in harm reducing pharmacotherapies for substance use disorders

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The loss of control in amounts of drug consumed is a common trait experienced by persons living with drug addiction. The motivation of this dissertation is to further our understanding in the neural mechanisms that mediate consumption of drugs of abuse, to reveal targets for pharmacological intervention to decrease uncontrollable drug use, and ultimately promote harm-reduction.

Most contemporary theories of drug abuse posit that adaptations in mesolimbic dopamine signaling are a fundamental mediator in the development, and maintenance of drug addiction. However, the manner and direction of alterations in dopaminergic signaling during drug addiction are heavily debated. Within Chapter 2 of this dissertation we demonstrate that dopaminergic signaling to drug paired stimuli has diametric changes across the addiction cycle that depends on the context in which the cues are presented. We explore how drug-taking history, drug-taking patterns, and abstinence influence alterations observed in nucleus accumbens (NAc) dopamine signaling to stimuli associated with drug-delivery. We further demonstrate causal evidence that differential changes in phasic dopamine transmission can drive drug-taking and drug-seeking behaviors, ultimately revealing that treating people living with addiction will heavily depend on what stage of recovery that person is currently within.

Harm reduction is an approach often used to treat drug addiction. One way to accomplish this is to help people decrease drug consumption to more moderate levels. In Chapter 2 we found that replacement of diminished phasic dopamine release within the NAc during a drug-paired cue decreases escalated drug consumption. In Chapter 3 I explore the mechanisms mediating the observed decrement cue evoked dopamine release within the NAc cues. Overall, we find that the endogenous signaling of the kappa opioid receptor ligand, dynorphin is a likely candidate responsible for attenuated dopamine signaling. Altering the ability for dynorphin to act on kappa, with pharmacologic or genetic approaches, prevents the development of escalated cocaine intake and decreases drug consumption.

It was previously found that administration of L-DOPA, a precursor of dopamine, replenishes the attenuated cue evoked dopamine in the NAc, and decreases cocaine consumption (Willuhn et al., 2014). In Chapter 4 I present our investigations in the effect L-DOPA has on ethanol and fentanyl consumption. We observed significant decreases in drug consumption of both ethanol and fentanyl following L-DOPA treatment. These results demonstrate that L-DOPA, and FDA approved drug taken by humans, has harm-reducing properties in psychostimulant, alcohol, and opioid use.

Another FDA approved drug, finasteride, has shown promise in decreasing gambling disorders in people living with Parkinson's and decreases ticks in people living with Tourette's. Because it is known that both of these disease have heavily disrupted dopamine signaling we were interested in how this drug would affect drug-taking behaviors. We found that administering animals with finasteride decreased their intake of hydrocodone, fentanyl (Chapter 5), as well as cocaine (Chapter 6). Interestingly, we also found that finasteride decreases drug-seeking behaviors that are observed after animals experience prolonged abstinence and are shown the stimuli that are paired with imminent drug delivery.

Collectively, the results within this dissertation reveal neurobiological mechanisms promoting excessive drug use. Additionally, the data within demonstrate two promising pharmacological therapies to aid in decreasing drug consumption, and such reduce harm in people struggling with drug addiction.

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Chapter 1: Introduction

Parts of this introduction were included in a published book chapter that was written by RDF and PEMP. (Farero & Phillips, 2019)

Drug addiction is a chronic brain disease. Although this point is sometimes still debated, empirical data collected from preclinical and clinical studies has allowed for great advancement in our knowledge of the biological mechanisms involved with substance use disorders. As such, drug addiction is now known to be comparable to other chronic relapsing diseases such as cardiovascular disease, or diabetes. Despite our advancement in the biology associated with substance use disorders, identifying successful treatments has proven to be difficult.

This difficulty is evident by the increasing and overwhelming amount of drug related deaths in the United States, which totaled more than 63,000 from drug overdose, and another 88,000 deaths due to excessive alcohol use (NIDA, 2018; NIDA et al., 2018). In addition, drug addiction also carries a severe economic burden. In 2018 National Institute of Drug Abuse (NIDA) estimated that costs associated with substance use disorders within the United States exceed $740 billion annually.

Defining Drug Addiction

Drug addiction is a relapsing brain disease characterized by irrepressible drug use, and compulsive drug seeking (APA, 2013; Koob & Volkow, 2016). Diagnosis of drug addiction by clinicians is based on the *Diagnostic and Statistical Manual of Mental Disorders 5* (DSM-5), which lists diagnostic criteria for given substance use disorders (SUD). Although each class of drug has its own diagnostic criteria, most of these characteristics are shared across all drugs of abuse. A majority of SUDs have 11 criteria listed (Table 1), and people matching > 2 or 3 of these criteria could be diagnosed with an SUD (APA, 2013).

Treating Substance Use Disorders:

Treatment options of drug addiction typically fall into two categories: abstinence or harm reduction. Both of these philosophies have merit and have successfully helped many people. No treatment will work for everyone and the intervention used should be matched to the individual (NIDA, 2018). As a quick disclaimer a large portion of the scientific work within this text is dedicated to preclinical studies on harm reducing pharmacological therapies. As such, I will provide more in-depth background knowledge in harm reduction approaches and pharmacological therapies. Pharmacotherapies are more successful in treating SUDs when they are paired with behavioral therapy (Ray et al., 2020), however, in-depth behavioral therapy treatment discussions are outside the scope of this dissertation. If you are interested in learning more about behavioral therapies for the treatment of SUDs, please see NIDA's *Principles of Drug Addiction Treatment: A Research-Based Guide (3rd edition)* (NIDA, 2018), and Carroll KM & Onken LS (2005) (Carroll & Onken, 2005).

Abstinence

Abstinence-based approaches focus on complete cessation of drug use. The success and failures of these treatments is measured by how many days a client has remained abstinent from drug use or how many days they continue to use drug (Musalek, 2013; Soyka et al., 2017). Historically this approach was the primary strategy for treating individuals suffering from drug addiction. One complication of the abstinence approach is convincing the person living with a SUD that abstinence is an attainable goal (Musalek, 2013). Because the reinforcement provided from not using drugs is often distant, and potentially convoluted, abstinence does not seem attainable. One strategy clinicians have used to combat this problem is incentivizing the cessation of drug use in shorter time-windows, so abstinence is reinforced regularly. This strategy, called contingency management, has been shown to be quite effective in treating SUDs (Prendergast et al., 2006; NIDA, 2018).

Harm Reduction

Harm reduction approaches focus on minimizing the amount of both direct and indirect consequences of drug addiction. These consequences include individual health, public health, society, and economic impacts. Harm reduction as a philosophy for the treatment of drug addiction has historically faced societal and governmental opposition, due to various reasons (Des Jarlais, 2017). A shift in this opposition came after the late 1980s when syringe exchange programs were being established to reduce HIV transmission (Des Jarlais, 2017). Currently, a large portion of harm reduction approaches involve creating safe environments and materials to use drug (injection sites) or provide resources to someone despite if they are actively using drugs (housing first).

Pharmacotherapies

Other harm reduction approaches focus on decreasing drug consumption to moderate amounts or treating physical dependence and withdrawal with medicine / pharmacotherapies. Each class of SUD has its own established pharmacotherapies, and besides naltrexone, these medicines are specific to a class of drug. Within this dissertation I present data that similar neurobiological mechanisms of drug addiction occur across multiple classes of drugs and targeting treatments for these mechanisms could reduce harm through a reduction in consumption of multiple classes of drugs. This may also be used to treat people struggling with addiction to multiple SUDs. Within this section I will present a brief summary of current pharmacotherapies being used. These FDA approved pharmacological treatments are categorized into the class of drugs the treatments are being used for.

Opioids:

Methadone is a synthetic opioid agonist that has prolonged actions (~24 hours), it is often taken chronically and daily to prevent withdrawal symptoms as well as drug craving, which are very

common after cessation of opioid use in people struggling with an opioid substance use disorder (Dole et al., 1966; Pasternak & Pan, 2013).

Buprenorphine is another synthetic opioid but acts as a partial mu opioid agonist. Because of its agonist actions at the mu opioid receptor it decreases withdrawal symptoms, and drug craving. Moreover, buprenorphine also has antagonistic activity on the kappa opioid receptor (Leander, 1987), which may aid in decreasing drug craving.

Naltrexone is a synthetic opioid antagonist; as such it prevents other opioids from binding to their receptors, and prevents the hedonic effects of opioids. Naltrexone is often used to prevent overdose as it directly competes with opioid agonists. Naltrexone should only be used for maintenance treatment after someone has detoxed, as it can precipitate withdrawal symptoms (Bart, 2012).

Alcohol:

Naltrexone discussed above is also used for the treatment of alcoholism, as it decreases the rewarding effects of alcohol as well as the craving to use alcohol (Thomas et al., 2003).

Acamprosate is a medication used to prolong abstinence from alcohol, because of this it is thought to decrease craving for alcohol. The drug interacts with the NMDA glutamate receptors as well as with GABA(A) receptors (Kalk & Lingford-Hughes, 2014).

Disulfiram is an aversive agent and attempts to create a conditioned aversion to alcohol consumption. In short, disulfiram inhibits aldehyde dehydrogenase, which is a key component needed for metabolism of alcohol. This causes nausea, sweating, and increased heart rate when alcohol is consumed (Mutschler et al., 2016)

Psychostimulants: Currently there are no FDA approved pharmacotherapies to treat psychostimulant substance use disorders.

Tobacco:

<u>Nicotine replacement therapy</u> is a commonly used therapy in which persons can obtain nicotine through less hazardous routes of administration, such as gum, or a transdermal patch.

<u>Bupropion</u> blocks the reuptake of the catecholamines, dopamine and norepinephrine and facilitates a decrease in tobacco use (NIDA, 2018). Although the neurobiological mechanisms of how bupropion decrease tobacco use are not fully understood (Warner & Shoaib, 2005).

<u>Varenicline</u> interacts with nicotinic receptors and is thought to block the reinforcing effects of nicotine, by inhibiting its ability to stimulate dopamine release (NIDA, 2018).

All of the treatments listed above are recommended to be used in combination with cognitive or behavioral therapy treatment.

Animal Models of Addiction:

In order to understand the complex neurobiological mechanisms that occur through the development of drug addiction, animal models have been developed. The most common animal models utilize rodents, such as rats and mice; however, other organisms including non-human primates are used (Nader et al., 2002). No single model is able to capture the complex pathology of humans living with SUDs, so individual behavioral paradigms have been designed to probe different stages of the addiction cycle (Koob & Le Moal, 2008; Koob & Volkow, 2016). The goal of these behavioral paradigms is to model one or more of the diagnostic criteria associated with addiction. As such, these models provide an avenue for preclinical research to explore the neurobiological mechanisms that are involved in phenotypes associated with any given criteria. Many behavioral paradigms have been used to explore drug addiction, and the neural circuits involved. Below I have provided brief overviews of some the models used that relate to the primary research content within this dissertation. This list is in no way exhaustive and novel paradigms are often created to further our understanding of this complex chronic

disease. For further exploration into animal models of drug addiction please see (Spanagel, 2017)

Conditioned Place Preference

Conditioned place preference (CPP) behavioral paradigms investigate associative learning mechanisms from administered rewards such as a substance of abuse. Typically animals are placed in two separate contexts for a discrete amount of time, and just prior to being placed in one of the contexts animals are administered a substance of abuse. This method will cause animals to develop an association between the context and the drug they received. Following the conditioning period, animals are placed between the two contexts and their time spent in each of the contexts is recorded. This time measurement is compared to a baseline time measurement that is obtained prior to any conditioning (Bardo & Bevins, 2000). This method allows for experimenters to probe both the reinforcing properties of drugs of abuse and the neurobiological mechanisms of associative learning.

Drug Self-administration

Another paradigm often used within animal models to study substance abuse is drug self-administration. This behavioral procedure is an operant conditioning task, in which animals perform an action to receive drug delivery. These sessions are repeatedly provided, allowing the animal to learn the action-outcome contingency. After acquisition of this task animals respond consistently to maintain a desired threshold of drug (Pickens & Thompson, 1968; Zimmer et al., 2011). These sessions often only require a single response for drug delivery, known as a fixed-ration 1 (FR1) (Spealman & Goldberg, 1978), however other schedules of reinforcement are used and have been shown to effect self-administration behavior (Oliva & Wanat, 2019). One schedule of reinforcement, that is particular useful in studying motivation to obtain drug, has increases in the effort needed to obtain drug with each successive trial, this is known as progressive ratio (Hodos, 1961). During daily 1-hour self-administration sessions utilizing an FR1 schedule of reinforcement animal's drug consumption remains stable across days and

weeks. However, if animals are provided longer daily drug access periods, drug intake will escalate over sessions (Ahmed & Koob, 1998). The length of the extended session can differ depending on the class of drug (Ahmed & Koob, 2005; Wade et al., 2015). In addition to escalating intake, protracted sessions also increase an animal's motivation to obtain drug (Ahmed & Koob, 1998; Edwards & Koob, 2013).

Modeling Drug Relapse

One characteristic of drug addiction is the high rates of relapse following periods of abstinence (APA, 2013). Those who are living with substance use disorders experience drug craving and often relapse following exposure to stress (Sinha et al., 1999, 2000, 2011), or environmental stimuli that have previously been associated with the drug (O'Brien et al., 1992; O'Brien, 2005). Animal models of relapse typically incorporate a reinstatement session following extinction of operant responding. In this paradigm animals are provided access to the self-administration chamber but responses on the operandum are no longer reinforced. Following the extinction and a period of abstinence animals are tested in the previously extinguished responding following a manipulation, such as presentation of a drug-paired conditioned stimulus (CS), stress, or a small dose of the drug itself (Meil & See, 1996; Weiss et al., 2000; Crombag & Shaham, 2002). Another variation of reinstatement has also been used in which animals go through forced abstinence without having extinguished operant responding (Grimm et al., 2001; Shaham et al., 2003; Lu et al., 2004). Instead, they are provided probe conditioned reinforcement sessions, where responses on the operandum are not reinforced by drug but the cues previously associated with drug delivery are still presented. They found that the longer the animals are in forced abstinence the more enhanced their responding is during the extinction / conditioned reinforcement probe sessions, a phenomenon that has been termed the "incubation of craving" (Grimm et al., 2001).

Relevant Background

Initial drug use has a hedonic component, and provides positive reinforcement, i.e. an action is performed and a reward is provided. However, as drug use continues, the hedonic component becomes masked due do homeostatic biological processes. Following this homeostasis it is hypothesized that drug is used to alleviate aversive negative states, i.e. an action is performed to remove the aversion being experienced such as withdrawal (Koob & Le Moal, 2008; Wise & Koob, 2014). Understanding the neurobiological mechanisms associated with these phases of drug addiction and the intermediate causes for the switch between positive reinforcement and negative reinforcement will help guide the development of future treatments for addiction. Within this section I present neuromodulators that I hypothesize are involved with these phases.

Although different classes of substances of abuse have diverse pharmacological properties, they have one common denominator, they increase extracellular dopamine in the brain (Koob & Volkow, 2016; NIDA, 2018; Wise & Robble, 2020). As such, a large amount of research and theories of drug addiction focus on dopaminergic signaling within the brain. The primary brain regions of dopamine synthesis are ventral tegmental area (VTA) and the substantia nigra, which have dense dopaminergic projections to the ventral and dorsal striatum (Swanson, 1982). Alterations of dopamine signaling within striatal regions are heavily implicated in multiple behavioral characteristics observed in addiction (Wise, 1987; Robinson & Berridge, 1993; White, 1996; Robbins & Everitt, 1999; Berke & Hyman, 2000; Dackis & O'Brien, 2001). The nucleus accumbens, a neuronal substructure in the ventral striatum, receives dense dopaminergic projections from the VTA and is a vital component for drug reinforcement (Wise & Bozarth, 1987; Ritz et al., 1987; Kalivas & McFarland, 2003). It is hypothesized that dopaminergic transmission in the nucleus accumbens (NAc) mediates drug satiety and thus regulates drug intake (Wise, Newton, et al., 1995; Wise, Leone, et al., 1995; Suto & Wise, 2011). Dopamine release in this region of the brain has also been ascribed to encoding

incentive salience to conditioned stimuli associated with drugs, as well as the augmentation of these stimuli (incentive sensitization), following continued drug use (Robinson & Berridge, 1993). Other investigations concluded that dopamine transmission in the dorsal-lateral striatum, a region linked to the formation of habitual behavior (Yin et al., 2007; Smith & Graybiel, 2016), subsumed control of drug seeking following chronic drug use (Everitt & Robbins, 2016). All of these accounts predict alterations in dopamine signaling during the development of addiction.

The neuromodulator and endogenous opioid dynorphin has also been attributed to characteristics associated with substance use disorders. Dynorphin is an endogenous ligand for the kappa opioid receptor (KOR) (Chavkin et al., 1982). KOR is a $G\alpha_i$ coupled receptor meaning activation inhibits cyclic AMP, an effect that essentially decreases neuronal activity (Brust et al., 2015). Kappa opioid receptors are expressed on dopaminergic terminals (Mulder et al., 1984; Schlosser et al., 1995; Lemos et al., 2012; A. Karkhanis et al., 2017), and chronic drug administration elevates dynorphin levels within the striatum (Hurd & Herkenham, 1993; Daunais & McGinty, 1994). Administration of KOR agonists decreases dopamine release within the NAc (Ehrich et al., 2014; A. N. Karkhanis et al., 2016; Escobar et al., 2017). It has also been shown that long-lasting KOR antagonists infused directly into the NAc, decrease consumption of methamphetamine, heroin, and alcohol (Walker et al., 2011; Schlosburg et al., 2013; Whitfield et al., 2015). Stress potentiated cocaine CPP is modulated by KOR activation within the NAc (McLaughlin et al., 2006; Schindler et al., 2010, 2012; Fontaine et al., 2021). Additionally, although somewhat seemingly contrary, treatment with KOR agonist also reduces relapse rats (Heinsbroek et al., 2018).

Corticotrophin releasing factor (CRF) also known as corticotrophin releasing hormone (CRH), is also known to be involved in mediating both drug consumption as well as relapse (Logrip et al., 2011; Zorrilla et al., 2014). CRF has two known receptors Crhr1 and Crhr2, both of which are G-protein coupled but are promiscuous in that they couple to both Gs and Gq secondary messengers, and in high concentrations have shown to couple to Gi/o (Milan-Lobo et

al., 2009). Crhr1 more readily binds CRF at low concentrations than its counterpart, Crhr2 (Bale & Vale, 2004). CRF and its receptors have been shown to be expressed through-out the mesolimbic dopaminergic pathway (Van Pett et al., 2000). Systemically administered Crhr1-receptor antagonists prevents escalation of heroin, and reverses escalated cocaine consumption (Specio et al., 2008; Greenwell et al., 2009; Roberto et al., 2010). Crhr1 antagonists also reduced reinstatement of drug seeking for heroin, cocaine (Shaham et al., 1998), and alcohol (Lê et al., 2000). Interestingly, Crhr1 antagonism also diminished reinstatement of methamphetamine following both cue and drug-prime challenges (Moffett & Goeders, 2007).

Specific Aims of Thesis

The majority of scientific works presented and discussed through-out this text are aimed to further our understanding of the neurobiological mechanisms contributing to drug consumption and pharmacotherapies that aid in reduction of consumption to moderate levels. In addition, I present data demonstrating a causal effect of dopamine transmission in the NAc and drug seeking behaviors, as well as preliminary data of a medicinal drug that may help alleviate augmented drug-seeking that is observed in prolonged periods of abstinence.

The underlying hypothesis being tested within this dissertation is, that cue evoked phasic dopamine transmission during drug taking sessions is attenuated from activation of kappa opioid receptors on dopaminergic terminals within the ventral striatum. Furthermore, elevated dynorphin, which is responsible for kappa activation, is downstream from CRF activity on Crhr1 receptors. Overall this hypothesis can be simplified into serial model (Figure 1), in which CRF elevates dynorphin, thus diminishing dopamine release and causing an increase is drug consumption behaviors.

The results presented in this body of work furthers our understanding of the neurobiological mechanisms mediating excessive drug consumption behavior, and tests FDA approved treatments to decrease drug consumption to moderate levels.

Figures

Hazardous use
Social/ interpersonal problems related to use
Neglected major roles to use
Withdrawal
Tolerance
Using larger amount for longer
Failed attempts to quit using
Much time spent using
Physical/ psychological problems related to us
Given of activities to use
Craving

Table 1. Diagnostic Criteria for substance use disorders listed in the DSM-5 (APA, 2013).

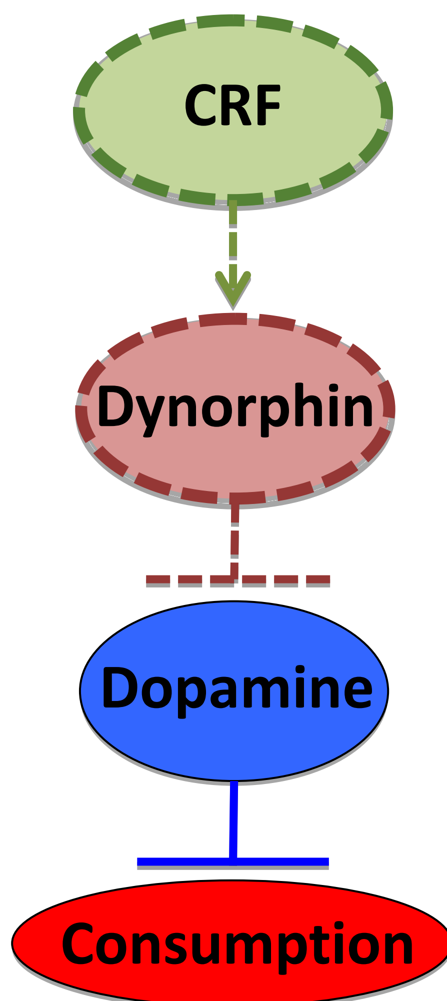

Figure 1. Serial model of neurobiological mechanisms mediating drug consumption in the NAc. Arrow indicated enhancement, the parallel line indicates reduction.

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Chapter 2: Diametric Changes in Dopamine Signals Regulate Drug Taking and Drug Seeking

This work is currently in preparation for submission as a manuscript by Lauren M. Burgeno, Ryan D. Farero*, Nicole L. Murray, Jennifer S. Steger, Marta E. Soden, Scott B. Evans, Ingo Willuhn, Larry Zweifel, Paul E.M. Phillips.*

Introduction

Drug addiction is a disorder characterized by compulsive drug seeking, irrepressible drug use, and high propensity to relapse (APA, 2013; Koob & Volkow, 2016). Despite great advances in our understanding of the biological mechanisms of addiction, treatment for substance use disorders has proven difficult. This difficulty resides in high relapse rates even well after pharmacological withdrawal or dependence (Gawin & Kleber, 1988; APA, 2013). Most contemporary theories of drug abuse implicate altered mesolimbic dopamine (DA) transmission as a fundamental mediator in the development, and maintenance, of drug addiction (Wise & Bozarth, 1987; Robinson & Berridge, 1993; Dackis & O'Brien, 2001; Everitt & Robbins, 2005; Volkow et al., 2012). However, the direction, timing, and context in which these alterations of dopamine signaling occur remain a matter of debate.

The nucleus accumbens (NAc), a substructure of the ventral striatum, receives dense dopaminergic input from the ventral tegmental area (VTA), and is responsible for learning associations between drugs of abuse and stimuli that predict their availability (Phillips et al., 2003; Day et al., 2007; Flagel et al., 2011). As drug use progresses, the drug associated conditioned stimuli (CS) acquires the ability to evoke NAc dopamine transmission, even when presented without drug reinforcement (Phillips et al., 2003; Willuhn et al., 2014). A presentation of the CS independent of an animals behavior (non-contingent drug-associated cue) promotes motivation to seek drug and drug-craving in both humans and in rodents (Meil & See, 1996; Di Ciano & Everitt, 2003; Hollander & Carelli, 2007; Volkow et al., 2012), and is prone to induce

relapse (Stewart et al., 1984; Meil & See, 1996; Shalev et al., 2002; Di Ciano & Everitt, 2003; Shaham et al., 2003; Hollander & Carelli, 2007). Furthermore, administration of dopamine antagonists to the NAc attenuates drug-seeking behaviors elicited by the drug-associated cues (Saunders et al., 2013; Xi et al., 2013; Ostlund et al., 2014).

In contrast, dopamine transmission in the NAc core also has an important role in regulating drug-satiety, and thus mediates drug-intake (Wise, 1987; Wise et al., 1995; Suto & Wise, 2011; Willuhn et al., 2014). Administering dopamine agonists into the NAc decreases drug consumption and increases the time between each successive infusion (Suto & Wise, 2011). During rodent cocaine self-administration phasic dopamine release is observed during the presentation of a drug-related cue following a successful response to obtain drug (Phillips et al., 2003; Stuber, Roitman, et al., 2005; Stuber, Wightman, et al., 2005; Owesson-White et al., 2009; Willuhn et al., 2012, 2014). Furthermore, as animal's transition into excessive drug consumption, measured by their escalation in daily drug-intake, there is an attenuation of NAc phasic dopamine transmission to response-contingent drug cues (Willuhn et al., 2014). Increasing dopamine through pharmacological intervention with L-DOPA, a dopamine precursor, both prevents and reverses escalation of drug-intake (Willuhn et al., 2014).

There is a large database of empirical evidence documenting bidirectional changes in phasic dopamine transmission following protracted drug use. The work within this chapter aims to test our hypothesis that diametric changes in phasic dopamine are contextually and temporally specific in animals that experience protracted cocaine access. To test this hypothesis, we employed fast-scan cyclic voltammetry (FSCV), and optogenetics within different rodent behavioral paradigms used to model drug addiction.

Methods

Animals

A total of 213 adult male Wistar rats (Charles River, Raleigh NC) weighing 300-350g were used in these studies. Rats were housed individually and kept on a 12-h light/12-h dark

cycle (lights on at 0700) with controlled temperature and humidity, and food and water available *ad libitum*. All animal use was approved by the University of Washington Institutional Animal Care and Use Committee, and surgical procedures were performed under aseptic conditions. Thirty rats completed the short-access voltammetry studies, and 55 completed long-access voltammetry studies. Thirty rats completed the optogenetics studies. Approximately 20% of the attrition was a result of head cap loss or catheter failure during the post-surgery recovery period prior to beginning experimentation. The remainder of subjects dropped out due to head cap loss, electrode failure, catheter failure /rejection, lack of viral expression, or in rare instances, failure to acquire self-administration once training began.

Stereotaxic and Catheter Surgeries

For dopamine recording experiments, chronically implantable carbon fiber microelectrodes, were constructed as described in Clark et al., 2009, and lowered unilaterally or bilaterally in the ventral striatum (nucleus accumbens core (Paxinos & Watson, 2007), AP: +1.3mm, ML: ±1.3mm, DV: -7.2mm) and secured with dental acrylic (Paxinos & Watson, 2007). Following two weeks of recovery from stereotaxic surgery, rats were outfitted with indwelling intravenous catheters and allowed to recover for at least one week prior to beginning cocaine self-administration training.

For optogenetics studies rats first underwent catheter implantation surgery, then following one week of recovery they were injected with AAV1-CAMKIIα-ChR2-mCherry or AAV1-CAMKIIα-mCherry for controls (viral vectors were provided by Dr. Larry Zweifel, Univ. of Washington) bilaterally into the VTA (AP: -6.35mm, ML: ±0.5mm, DV: -8.5mm) and 1.25mm optical fiber stub implants (Plexon Inc.) were implanted bilaterally in the ventral striatum (AP: -6.35, ML: ±0.5, DV: -7.0mm). After an additional week of recovery, rats began cocaine self-administration training. All optogenetic manipulations were done at least 3 weeks post virus injection. Before starting cocaine self-administration, and prior to days off from self-administration, catheters were backfilled with a viscous mixture of polyvinylpyrrolidone,

gentamicin, and heparin to prevent formation of blood clots. Otherwise, catheters were flushed daily with saline, and with heparin as needed, to maintain catheter patency throughout experimentation.

Cocaine Self-Administration

Rats were trained to self-administer cocaine during daily one hour (short-access, ShA) sessions in an operant chamber outfitted with a liquid swivel and containing two nose-poke ports. During self-administration sessions, the illumination of a house light paired with white noise signaled the availability of drug. A nose poke into the active port elicited a 0.5 mg/kg cocaine infusion (fixed-ratio one schedule), accompanied by a 20-second presentation of an audiovisual conditioned stimulus (nose-poke light + tone), during which any additional nose-poke was without consequence (time out). Nose pokes into the inactive port at any time were without consequence. After meeting the acquisition criterion of three sequential sessions earning 10 or more infusions, animals received daily one hour sessions for five additional days to establish baseline intake and were then divided into two drug access groups, each receiving the same number of sessions but with sessions differing with respect to the number of hours the animals had access to self-administer cocaine. The short-access (ShA) cohort continued with one hour sessions daily for 10 more days, while the long access (LgA) group received daily six hour sessions for 10 days. A subset of LgA animals continued six hour access for an additional five sessions (mirroring the duration of LgA used in previous studies. Using our previously validated method for separating escalators and non-escalators (further detailed in Willuhn *et al.*, 2014), a linear regression analysis of each animal's first hour drug intake over sessions (beginning the day after acquisition) was used to assess the relationship between session and drug intake. Rats having a significant, positive non-zero slope were considered escalators.

Assessment of Drug-Seeking behavior

Animal's behavioral responses during probe sessions in which, a non-contingent CS (20s, audiovisual stimulus) was presented were recorded and hand scored. Behavioral scores

ranged from 0-5 using the following criteria: 0 = no response; 1= startled action to cue onset; 2 = animal's head directs towards nose-poke port; 3 = animal orients body towards nose-poke port; 4 = animal orients body and approaches nose-poke port; 5= animal orients, approaches, and interacts with the nose-poke port. In some cases the animal's head was outside of the camera view, causing an inability to score the animal's behavioral response the CS presentation. In these cases, the data for this particular CS presentation was not included in statistical analysis.

In addition to scoring approach behavior, animals also underwent extinction sessions to assess incubation of craving. The day after the CS probe sessions provided in abstinence (Figure 2), animals underwent a 30-minute extinction sessions. These sessions were identical to self-administration sessions, except that responses on the active nose-pokes elicited the CS without the reinforcement of drug infusion. Responses for the CS were then used to compare conditioned reinforcement after 1 or 30 day(s) of abstinence.

Fast-Scan Cyclic Voltammetry

Behaviorally relevant stimuli elicit rapid changes in dopaminergic neuron firing, resulting in transient changes in dopamine release over the course of seconds, and requiring the use of a detection method with high temporal resolution. In the studies phasic dopamine release events were measured at carbon fiber microelectrodes in the ventral striatum using fast-scan cyclic voltammetry (FSCV) (Clark et al., 2010). Briefly, chronically implanted carbon-fiber microelectrodes were connected to a head-mounted voltammetric amplifier, interfaced with a PC- driven data-acquisition and analysis system (National Instruments, TX, USA) through a commutator (Med Associates, VT, USA) that was mounted above the test chamber. A potential was applied to the electrode as a triangular waveform such that it was linearly ramped from the initial holding potential (-0.4 V vs Ag/AgCl) to a maximum voltage (1.3 V vs Ag/AgCl, anodic sweep), then returned to the holding potential (cathodic sweep). Each voltage scan lasted 8.5 ms, yielding a scan rate of 400 V/s. The holding potential was maintained between voltage

scans. Scans were applied every 100 ms (10 Hz sampling). When dopamine was present at the surface of the electrode, it was oxidized during the anodic sweep to form dopamine-*o*-quinone (peak reaction detected at approximately +0.7 V), which was reduced back to dopamine in the cathodic sweep (peak reaction detected at approximately −0.3 V). The ensuing flux of electrons was measured as current and was directly proportional to the number of molecules that underwent electrolysis. Voltammetric data was band-pass filtered at 0.025–2,000 Hz. The background-subtracted, time-resolved current obtained from each scan provided a chemical signature characteristic of the analyte, allowing resolution of dopamine from other substances (Rodeberg et al., 2017)}. Dopamine was isolated from the voltammetric signal by chemometric analysis using a standard training set (Clark et al., 2010) based on electrically stimulated dopamine release detected by chronically implanted electrodes, and dopamine concentrations estimated on the basis of the average post-implantation sensitivity of electrodes. Before analysis of average concentration, all data were smoothed with a five-point within-trial running average. The concentration of dopamine was averaged over seven seconds (approximate duration of the observed phasic signal) following the non-contingent presentation of the CS or operant response to obtain drug. Data collected on recording days was included if there was a detectable dopamine response at any point within the session. Data was excluded from sessions where electrical noise exceeded 0.2 nA, animals became disconnected from the drug delivery tubing, or when tethering for recordings altered the animal's regular behavior patterns (some animals won't move when tethered). In cases where more than one electrode was functioning in an animal during a given session, the average of the signals obtained from both electrodes was used for analysis. In cases where we only obtained voltammetry data from a single session from an animal we excluded the animal.

Optogenetics

In order to validate virus expression and function, and to determine what the minimum optogenetic stimulation parameters required to evoke physiologically relevant dopamine

responses having similar amplitude and temporal profile to cue-elicited responses observed during behavior, we initially performed immunohistochemistry and measurements of optically evoked dopamine release in acute NAc rat brain slices, three weeks after virus injection. Optical stimulation of NAc dopamine terminals elicited dopamine responses comparable to standard electrical stimulation parameters used in slices, and we found that optical stimulation of 30 Hz, 6 pulses, at 10mW reliably evoked dopamine responses with the desired amplitude and temporal profile. This stimulation parameter was used for all stimulations during behavior.

In order to assess the impact of behavior-contingent CS-elicited dopamine release on drug-intake, we trained animals to self-administer cocaine in ShA sessions, then switched them to LgA for two weeks before augmenting CS elicited dopamine release by pairing optogenetic stimulation with the behavior-contingent CS presentations that occurred during drug self-administration on 0%, 50% or 100% of trials on different days within the same subject. We then assessed the impact of non-contingent CS-elicited dopamine release on drug-seeking behavior by measuring the CS-elicited conditioned approach behavior evoked by 5 x unexpected CS presentations delivered prior to self-administration in the second week of LgA, with or without optogenetic stimulation paired with CS. For all studies, all animals received all stimulation conditions in a counterbalanced fashion, allowing for within subject comparisons to be made. All experiments were also performed in mCherry controls with the same light stimulation patterns.

Histology

Upon completion of experimentation, animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg). Recording sites in animals implanted with electrodes were marked with an electrolytic lesion, and animals were transcardially perfused with saline followed by 4% paraformaldehyde. Brains were removed and postfixed in paraformaldehyde for at least 24 h before being frozen, and sliced on a cryostat (50μm coronal sections, −25°C). Brains from voltammetry studies were stained with cresyl violet to aid visualization of anatomical structures and the electrolytic lesions. In some cases, placements

could not be obtained because lesions were not clearly visible or animals lost head caps before perfusion. Immunohistochemistry was performed on brain slices from optogenetic experiment subjects to validate viral expression patterns and selectivity for dopamine neurons.

Immunohistochemistry

Following completion of optogenetic experiments, rats were transcardially perfused with saline and 4% PFA. Brains were extracted and fixed in 4% PFA, then serially transferred into 15% and 30% sucrose solutions. Brains were frozen and cut in coronal sections (40μm) on a cryostat (Leica CM1850). All sections were treated with a blocking solution (PBS containing 3% normal donkey serum and 0.3% Triton) for 1 hour and incubated overnight at 4°C with two primary antibodies: anti-tyrosine hydroxylase (monoclonal, 1:1000 Millipore, MAB318) and anti-dsRed (polyclonal, 1:1000; Clontech, 632496). Sections were then washed for three times for ten minutes each, then incubated for ~2 hours with at room temperature with secondary antibodies conjugated to AlexaFluor 488 and CY3 (donkey anti-mouse and donkey anti-rabbit, 1:250; Jackson Immunoresearch). Sections were washed three times for ten minutes each and cover slipped with a mounting medium. Fluorescent images were taken to examine protein expression, and fiber placement.

Results

Drug-use history impacts non-contingent cue elicited phasic dopamine transmission

Animals surgically implanted with carbon fiber microelectrodes and a jugular catheter, were trained to nose-poke an active port to receive intravenous cocaine infusions (0.5 mg/kg/delivery; Figure 1A) in daily one hour sessions (short-access; ShA). Every drug infusion was paired with the presentation of an audiovisual conditioned stimulus (CS; Figure 1B). Once animals reached acquisition criteria of ten or more active nose-pokes for three consecutive sessions they received an additional five baseline ShA sessions. Prior to the start of the last day of cocaine access animals were given a probe session where no cocaine was available and presented with the drug paired CS in a non-contingent manner. Dopamine transients were

recorded with FSCV during this probe session. A group of animal's naïve to cocaine self-administration also received these probe sessions (Figure 1C). It was previously shown that, by this stage of training, the CS had acquired the capacity to elicit phasic dopamine release in the NAc when presented non-contingently (Phillips et al, 2003). Accordingly, when rats were tested in a probe session where previously drug-paired cues were presented non-contingently in the absence of drug (Figure 1c); we also observed cue evoked dopamine release (Figure 1d, e). However, no dopamine release was evoked by the cue in rats naive to drug exposure and training (Figure 1d, e) confirming that these dopamine signals are a learned, rather than innate, response to the cue. We next examined the impact of drug-use history on cue-elicited dopamine release in the NAc. Following training and five 1-hour baseline sessions, animals were split into two groups. Both groups received ten additional self-administration sessions, but one group's daily sessions were six hours (long-access; LgA) while the other group continued to receive one-hour (ShA) sessions (Figure 1f). As shown previously, animals provided LgA escalated their drug-intake over time whereas their ShA counterparts did not (Figure 1g; Ahmed & Koob, 1998). We measured non-contingent cue elicited dopamine responses during drug-free probe sessions after the 9 to 10 sessions of either ShA or LgA drug use and compared these signals with signals from post-acquisition baseline sessions (Figure 1f). While we observed no significant change in cue-evoked dopamine release to non-contingent cues presented following prolonged ShA training, animals that undergo LgA exhibit a robust increase in non-contingent cue evoked dopamine release (Figure 1h,i). These data demonstrate that the magnitude of cue-evoked dopamine responses change with drug-use history.

Drug Abstinence further increase non-contingent cue elicited dopamine transmission

In order to gain insight into how non-contingent cue elicited dopamine release influences drug-seeking and thus contributes to relapse, we also investigated the dynamic changes in cue evoked dopamine release occurring over the course of prolonged abstinence. It has been well established that animals will more frequently perform an action to earn the presentation of a

previously drug-paired cue following periods of abstinence of increasing duration (Grimm et al., 2001), a phenomenon termed 'incubation of craving'. This phenomenon was originally demonstrated by comparing different time points between subjects, however, more recently, it has been observed using repeated measurements within subjects (Theberge et al., 2013). We replicated these data to confirm that we were able to observe this incubation of craving within subjects (Figure S1) and that the repeated-measure design did not distort the result (Figure S1). To test the hypothesis that changes in phasic dopamine evoked by drug-paired cues correspond to incentive sensitization of those cues, we measured non-contingent cue evoked dopamine release in probe sessions given one day and one month since the last day of LgA cocaine self-administration (Figure 2a). In order to assess drug-seeking behavior elicited by CS presentations, conditioned approach behavior was scored during probe sessions. During each probe sessions the animals were presented the cue a total of 5 times to examine the robustness of dopaminergic signaling. Responding for the CS within extinction sessions was measured one-day after the probe sessions (Figure 2a). We observed a robust increase in non-contingent cue evoked dopamine release following one month of abstinence (Figure 2d & e), an effect which paralleled increases in both cue-elicited approach behavior (Figure 2c) and extinction responding (Figure 2b). We then tested to see if the increase in dopamine release to the non-contingent CS is causally related to the observed increases in conditioned approach behavior. Animals received bilateral microinjections of a viral vector that contained Channelrhodopsin-2 behind a CaMKIIα promoter into the VTA, which allowed us to elicit dopamine release with temporal precision (Figure 2j). Animals were equipped with bilateral optic fibers into the NAc and trained to self-administer cocaine. After 10-15 LgA sessions animals were provided back to back drug free probe sessions, where they were presented with five non-contingent CS events. During one of these sessions all 5 of the CS presentations were paired with photostimulations of DA (Figure 2k). We observed that stimulating dopamine in the NAc during the presentation of the CS increased the animals approach scores (Figure 2l), thus showing a causal relationship between dopamine release and drug-seeking behaviors.

Individual drug-taking patterns influence changes in dopamine transmission to non-contingent drug cues

It was previously demonstrated, that animals provided with LgA cocaine self-administration can be classified as escalators and non-escalators by linear regression of their daily (first-hour) intake (Willuhn et al., 2014). We replicated these findings and show two populations of drug-taking patterns (Figure 3a).

We aimed to identify if individual differences in drug-taking had differential dopaminergic signaling across weeks of cocaine self-administration to non-contingent cue presentations. As such animals that were within the LgA group in Figure 1 were split into two groups based on their drug-taking patterns across sessions of cocaine self-administration (Figure 3a). Animals that escalated their daily cocaine intake across weeks showed a significant increase in their phasic dopamine release to non-contingent CS presentations, whereas animals that maintained stable intake also showed stable dopamine transmission (Figure 3b, c). Escalators also had an increase in their CS elicited approach behavior across self-administration weeks. Non-escalators approach behavior remained stable across self-administration weeks (Figure 3d). Additionally, we found, through a simple linear regression, that larger non-contingent cue evoked dopamine signals predicted a larger increase in approach behavior (Figure 3e).

Individual drug-taking patterns influences changes in dopamine transients during response-contingent drug paired cues

Previous work from our lab demonstrated that phasic dopamine release during response-contingent drug paired cues during self-administration sessions is negatively correlated with increases in cocaine consumption (Willuhn et al., 2014). It was also found that pre-session administration of L-DOPA decreases the amount of cocaine consumed by animals that have previously escalated their cocaine intake. Within the current work we replicated these findings showing that animals that escalate their cocaine intake have a decrement in response-contingent cue evoked dopamine (Figure 4a-d).

Animals classified as escalators demonstrated an increase in drug-taking across weeks of cocaine self-administration, whereas non-escalators did not (Figure 4a). A decrease in dopamine release to the drug-paired CS decreased significantly only in escalators, but this was not observed in non-escalators (Figure 4b&c). We additionally found that the animal's escalation slope was negatively correlated to cue evoked dopamine release observed in the LgA self-administration task (Figure 4d). Our previous work utilized L-DOPA to replace the decrement of dopamine observed during the response-contingent CS (Willuhn et al., 2014). However, L-DOPA is likely to increase tonic levels of dopamine and is not temporally specific to the presentation of the cue. Thus, to further examine if the phasic cue evoked dopamine response is responsible for an increase in drug-consumption we employed optogenetics during cocaine self-administration allowing for temporally precise dopamine stimulation during response-contingent cues (Figure 4e). We found that escalators decreased drug-taking during stimulated sessions compared to unstimulated sessions (Figure 4f & g). Interestingly, there was an observed increase in drug-taking in non-escalators during these stimulated sessions (Figure 4f & g). With further analysis we discovered that non-escalators have an increase in their drug-taking towards the end of the 1-hour stimulated sessions (Figure 4f; top left panel).

Discussion

Collectively these data demonstrate diametric changes in drug-paired cue evoked phasic dopamine transmission through-out the addiction cycle. We establish a causal relationship with the observed diametric changes and two distinct behaviors associated with addiction; drug-seeking and drug-consumption. These data further support two contemporary theories of addiction, one attributing increased dopamine release being responsible for increased motivation to seek drug (Robinson & Berridge, 1993). The other theory posits that dopamine modulates drug satiety and decreased dopamine release increases amount of drug consumed to reach satiation (Wise & Bozarth, 1987).

We used an established rodent model of addiction, in which animals are giving extended daily access to self-administer drug. The longer daily sessions produces the loss of control of drug consumption, measured by increasing amounts of drug consumed, and increased rates of drug-seeking behavior (Ahmed & Koob, 1998). Thus, we were interested if there were differences in non-contingent cue evoked dopamine based on drug history. We observed that animals provided with 6-hour daily drug access had increased cue evoked dopamine release compared to animals with only 1-hour of daily drug access (Figure 1).

Given our interest in dopamine transmissions role in mediating drug-seeking behaviors we studied it within periods of abstinence, which has been found to have heightened amounts of drug-seeking behaviors (Grimm et al., 2001). Animals that experienced extended access of drug were provided with non-contingent probe sessions to measure cue evoked dopamine after 24 hours of abstinence and compared to 30 days of abstinence. In addition we examined how non-contingent cues elicited drug-seeking behaviors. After 30 days of abstinence we observed that animals cue evoked dopamine release was amplified compared to 1 day of abstinence, which correlated with an increase in their drug-seeking behavior during the probe sessions. In a second experiment we utilized a viral vector to express ChR2 in dopamine terminals in the NAc, allowing for artificial enhancement of dopamine signaling during cue presentation, and measure approach behaviors. We found a causal effect, in which stimulating dopamine release during the non-contingent cue increased animal's drug-seeking behaviors.

It was previously found that not all animals that are provided protracted drug-access escalate their intake over the course of the experiment (Willuhn et al., 2014), and that animals who escalate their intake show higher break-points in a progressive ratio tasks. Individual animals drug-taking patterns were assessed and grouped based on if they significantly escalated their intake across sessions (escalators), or did not (non-escalators). We then investigated if non-contingent cue evoked dopamine and drug-seeking behavior differed between the two groups. We observed that across weeks of long-access drug availability

animals that non-escalators had no change in non-contingent cue evoked dopamine, and decreased approach behaviors during the cue presentation. Escalators showed an enhancement of non-contingent cue evoked dopamine compared to short-access baseline sessions, and showed enhanced approach behavior as well (Figure 3). Utilizing many of the same animals we also examined cue evoked dopamine transients during drug-taking sessions. We replicated previous findings (Willuhn et al., 2014), in which escalators have diminished dopamine signaling to response-contingent cue presentations, which negatively correlates with their escalation slope. Willuhn and crew demonstrated that administration of L-DOPA, a precursor of dopamine, rescues the diminished dopamine transients and decreased drug consumption back to pre-escalated levels (Willuhn et al., 2014). One caveat of their findings is that L-DOPA may increase tonic levels of dopamine, and is not temporally specific. Thus we expanded on their findings and utilized optogenetics to stimulate dopamine release from terminals in the NAc following an active nose-poke and paired with the cue onset. Similar to the effects of L-DOPA we found photostimulations decreased cocaine consumption in animals that escalated their intake. Interestingly we also observed an increase in non-escalators intake during these stimulation effects, although interestingly this increase of active nose-pokes did not occur until later in the 1 hour sessions provided during this test.

Given the current data we conclude that bidirectional changes in cue evoked dopamine release develop through-out the addiction cycle, and that the direction of changes in dopamine depends on the context and the state the animal is in during the presentation of the drug paired cue. During drug taking the cue is predicted and dopamine release to the cue will inhibit further responding for drug, the attenuation of this signal in animals who escalate lose their feedback and continue to respond for drug more frequently. In contrast, when a cue is presented unexpectedly, it acquires attention and promotes drug-seeking behaviors.

Figures

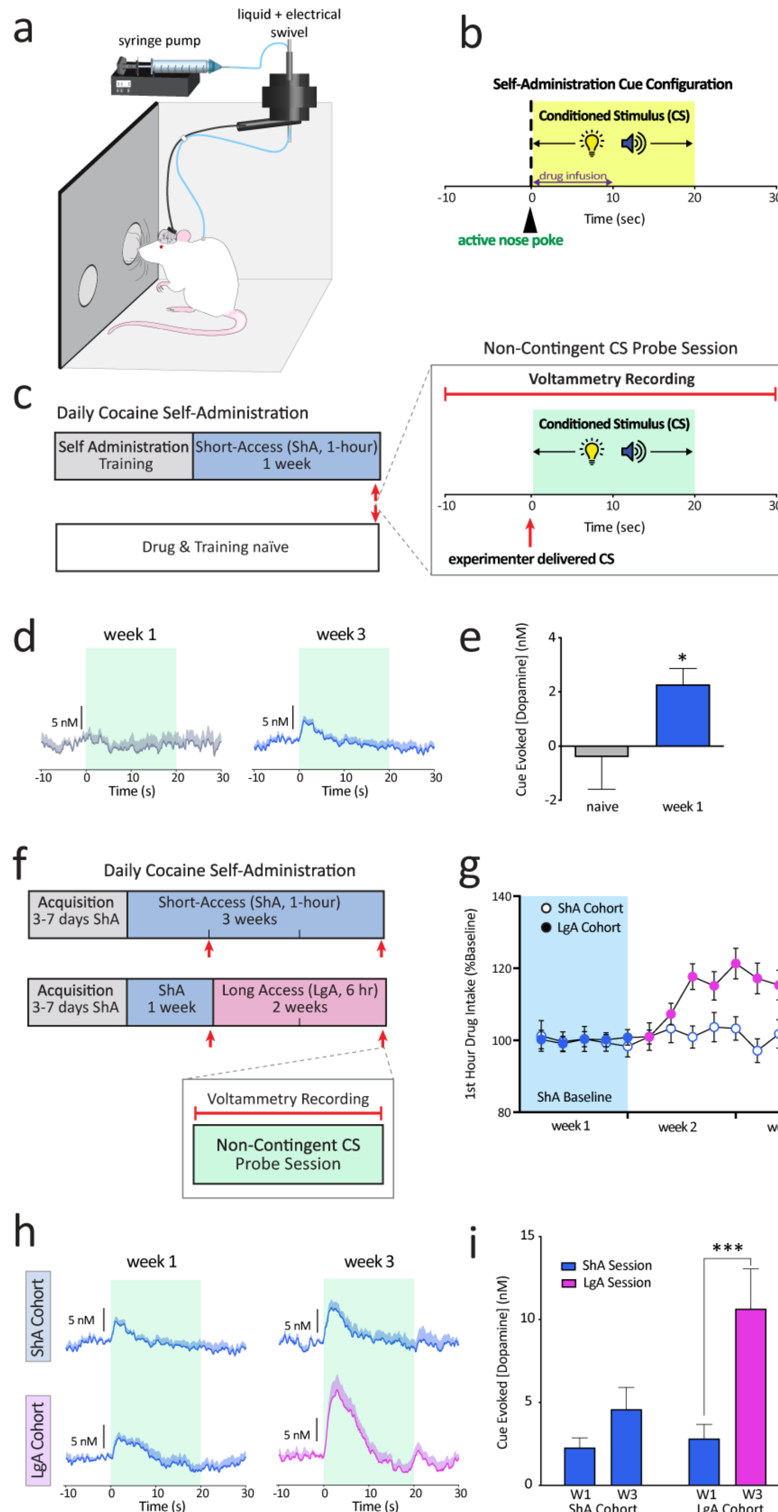

Figure 1. Drug use history impact non-contingent cue elicited phasic dopamine transmission. a) Cartoon of operant chamber used for drug access sessions. **b)** Schematic of cue parameters demonstrating that a 10 s audiovisual stimulus is played (yellow box) following a nose-poke. **c)** Paradigm

used to present the non-contingent conditioned stimulus within a probe session. The probe session occurred following 5 1-hour cocaine self-administration sessions, indicated by the red arrows. **d & e)** Following one week of drug self-administration a non-contingent delivered CS gained the ability to elicit dopamine transients (blue trace), whereas drug-naïve animals show no such dopamine release (gray trace). A non-parametric Mann-Whitney found a significant difference of cue evoked dopamine between naïve animals (gray) and animals with one week self-administration (blue) ($U = 26$, $p=0.0282$). **f)** Experimental design to compare non-contingent cue evoked dopamine in animals with different drug access histories. Red arrows indicate probe sessions. **g)** Animals given six hour cocaine access escalate their intake over time, to directly compare to the 1 hour access animals only the 1 hour drug-consumption is shown. **h & i)** Animals who are allotted with more drug access per session have a significant increase in non-contingent cue evoked dopamine release. A main effect of drug-access group was observed (mix-effects analysis (REML) ($F(1,26)=4.297$, $p<0.05$). A post-hoc Tukey's test completed and demonstrated a significant difference between the LgA cohort's week 1 and week 3 ($p<0.01$). The ShA cohort had no significant difference between weeks ($p>0.05$).

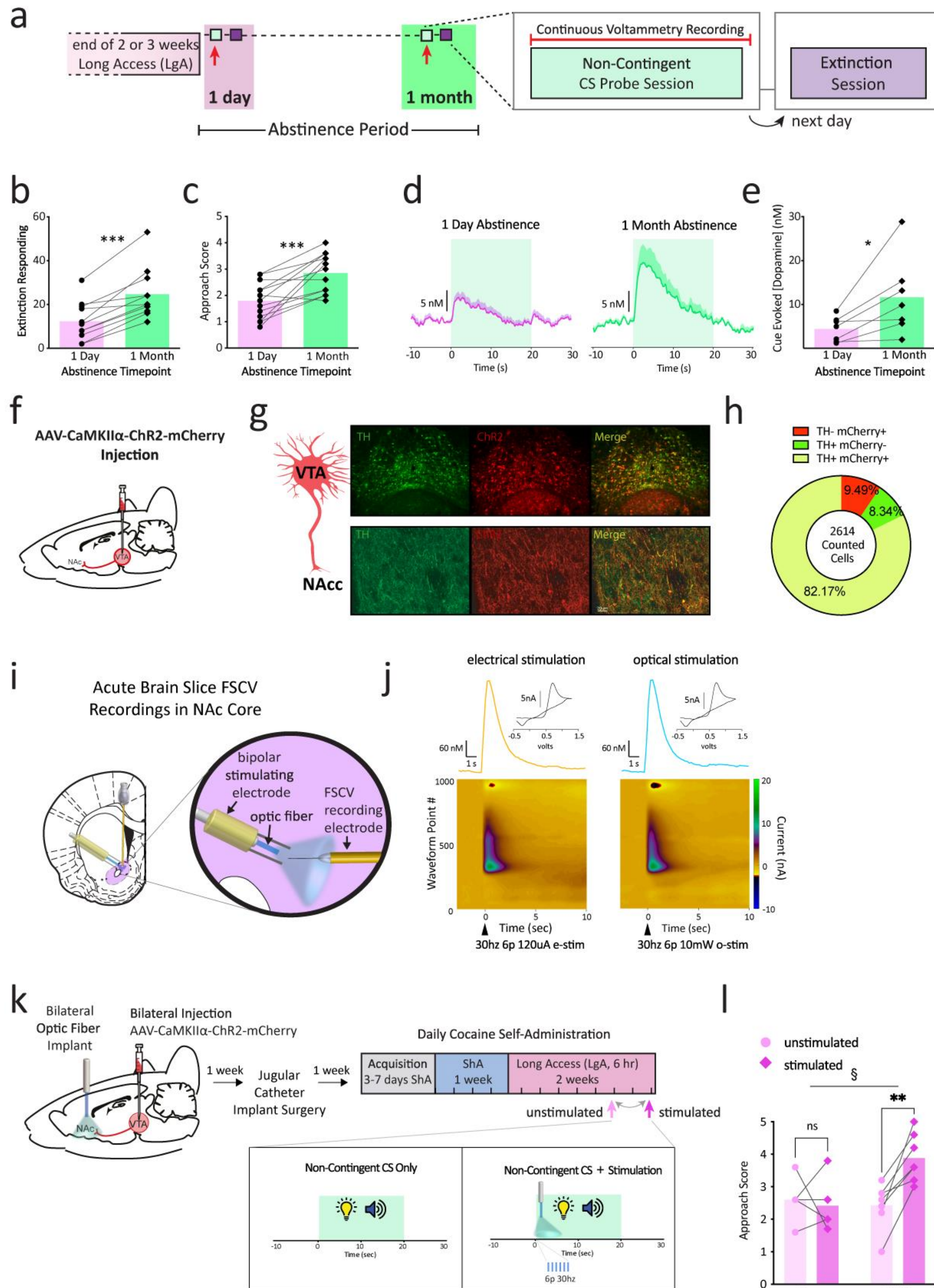

Figure 2. Drug abstinence increases non-contingent cue elicited dopamine transmission.

a) Experimental paradigm, outlining that animals received a 30 day drug abstinence period in which probe sessions were provided at day 1 and day 30 of abstinence. The day after both probe sessions animals had an extinction session. **b)** Following 1 month of abstinence animals had a significant increase in their extinction responding (Wilcoxon signed rank test, n=12 W= 78, p<0.001). **c)** During probe

sessions animals approach behavior was scored. We found that after 30 days of abstinence animals had a significant increase in their approach score (Wilcoxon signed rank test, n=13 W=86, p=0.001). **d & e)** non-contingent cue evoked dopamine significantly increases after 1 month of abstinence (paired t-test; t(6)=2.816, p<0.05). **f)** Animals were injected with a viral vector to express ChR2 in dopamine terminals in the NAc. **g)** Fluorescent IHC images were captured to in demonstrating ChR2 expression in tyrosine hydroxylase containing cell bodies (top panel) and their terminals (NAc). **h)** TH positive and mCherry positive neurons were counted in 7 VTA slices from 3 separate animals to estimate ChR2 expression in putative dopamine neurons. **i & j)** To functionally test ChR2 expression in dopamine terminals, live brain tissue containing the NAc underwent photostimulation and electrical stimulation while recording with FSCV. Similar amounts of dopamine release were observed using our parameters. **k)** Animals were bilaterally injected with ChR2 into the VTA and bilaterally implanted with optic fibers in the NAc, then equipped with intravenous catheters and trained to self-administer cocaine (top left panel). Animals were given two non-contingent probe sessions, one of which each CS paired with ChR2 photostimuation of dopamine terminals (bottom middle panel), and approach behavior was scored. A two-way ANOVA revealed an interaction of group by stim in approach scores to the cue(F(1,10)=8.778, p<0.05). We performed a Sidak post-hoc analysis and found that the stimulated session had a significant increase in approach scores during the probe session (p<0.01).

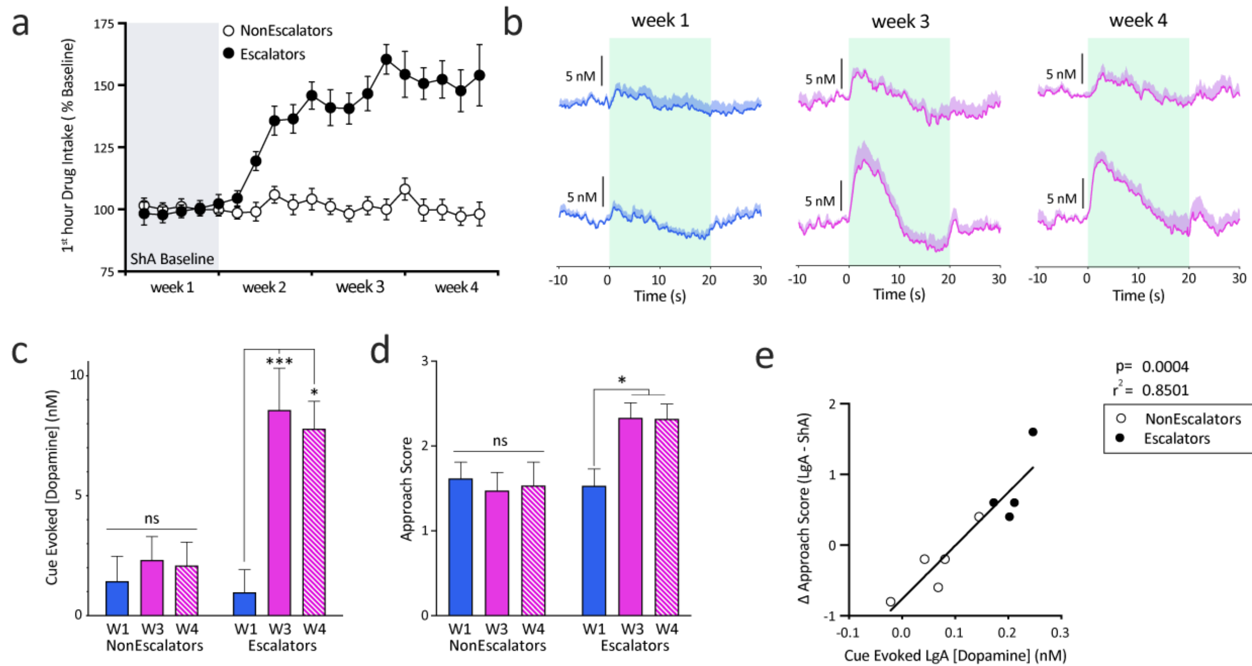

Figure 3. Individual drug-taking patterns influence changes in dopamine transmission to non-contingent drug cues. a) Animals in the LgA group in Figure 1 were grouped based on drug-taking patterns. Animals that had a significant positive regression between session and cocaine consumption were classified as escalators (filled circles). The other animals were classified as non-escalators (open circles). **b)** Cue evoked dopamine traces to non-contingent CS. At the end of each 5-session week of cocaine self-administration animals received a probe session where they were non-contingently presented the 20-s CS (lime green box). Cue evoked dopamine was recorded with FSCV in each of these sessions. **c)** Peak cue evoked dopamine was calculated and a mix-effects analysis with REML revealed an interaction between group and week ($F(2,17)=6.546$, $p<0.01$). A Sidak post-hoc analysis was completed and found a significant difference in Escalators Week 1 to Week 3 ($p<0.001$), and Week 1 to Week 4 ($p<0.05$). Additionally a main effect of escalator group was also observed ($F(1,20)=7.328$, $p<0.05$). **d)** Approach behavior was scored and compared based on escalation group. An interaction of Approach Scores was found between escalation group and week ($F(2,18) =4.853$, $p<0.05$). A post-hoc Dunnet test was completed and found a significant differences in Escalators Week 1 vs Week 3 ($p<0.05$) and Week 1 vs Week 4 ($p<0.05$). **e)** A regression between cue evoked dopamine in LgA, and the change in Approach score from ShA to LgA was performed. A significant positive regression was found ($r^2=0.8501$, $p<0.001$), so larger Cue Evoked DA predicated a large change in Approach Score.

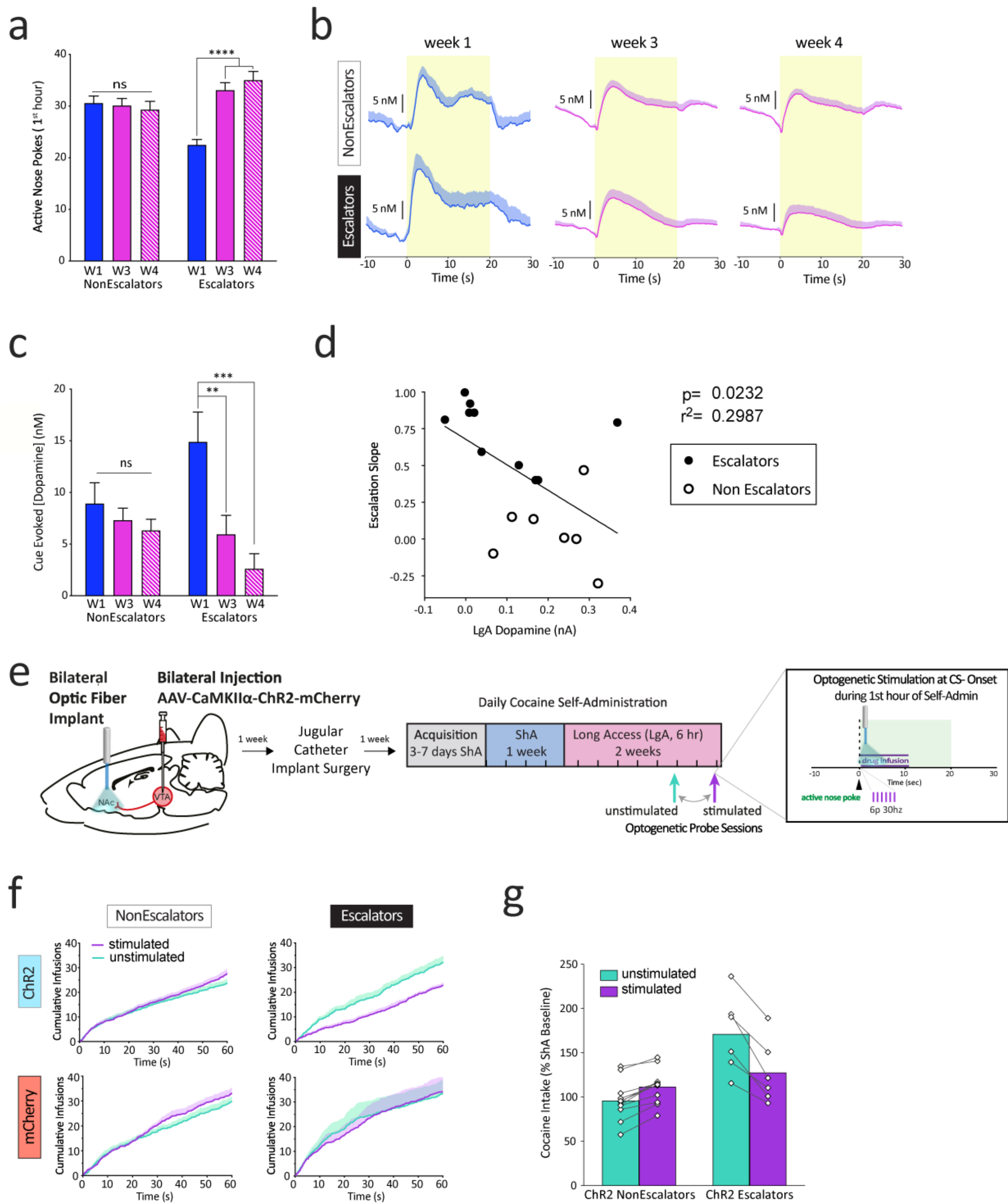

Figure 4. Photostimulated phasic dopamine release during response-contingent drug paired cues decrease escalated cocaine consumption. a) Animals classified as escalators have a significant increase in their cocaine intake across weeks. An interaction of drug-taking was observed between escalation group and week (mixed-effects analysis, $F(2,95)=63.51$. $p<0.0001$). A post-hoc Tukey's test

revealed that Escalators had significant change in their drug intake comparing Week 1 (ShA) to Week 3 ($p<0.0001$), and comparing Week 1 to Week 4 ($p<0.0001$). **b)** Response contingent cue (yellow box) evoked dopamine was recorded in animals during cocaine self-administration sessions across the 5-session weeks of self-administration. Blue traces represent ShA sessions whereas, pink indicates LgA sessions. **c)** A mixed-effect s analysis on the peak cue evoked dopamine revealed an interaction between Escalation group and Week ($F(2,23)=3.655$, $p<0.05$). A Tukey's post-hoc analysis revealed that escalators cue evoked dopamine in Week 1 was significantly higher than Week 3 ($p<0.01$) and Week 4 ($p<0.001$). **d)** Given animals had an inverse relationship of their drug-taking and cue evoked dopamine we ran a regression between their LgA cue evoked dopamine and the slope of their drug-taking over sessions. Cue evoked dopamine release in LgA predicts the animals escalation slope ($r^2 = 0.2987$, $p<0.05$). **e)** To test if there is a direct casual effect of cue evoked dopamine release and drug consumption, we utilized ChR2 to stimulate dopamine release from NAc terminals when an animal completed an active NP and elicited a CS. **f)** Cumulative nose-poke plots allow for visualization of drug-taking patterns during the 1-hr session. **g)** Using the total nose-pokes completed within the 1-hour sessions with photostimulation, we found a significant interaction of drug-taking between Escalation group and Stimulation (Two-way ANOVA, $F(1,15) = 104.8$, $p <0.0001$). A Sidak post-hoc analysis revealed that stimulation increased drug-taking in non-escalators ($p<0.001$), but decreased drug-taking in escalators ($p<0.0001$).

Supplemental Figures

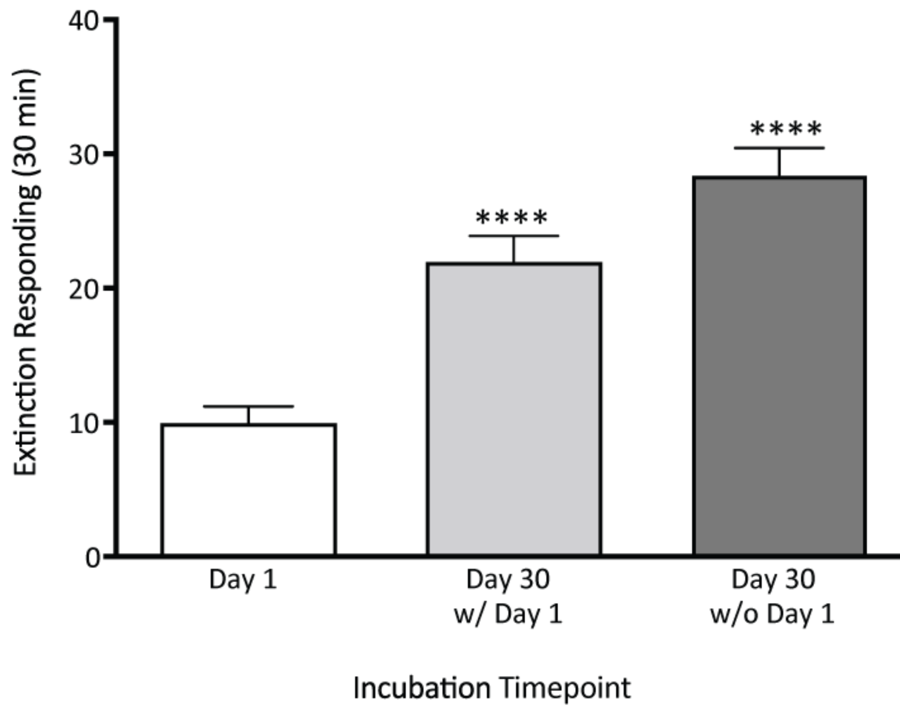

Figure S1. Repeated measures of extinction responding does not block incubation of craving. A one-way ANOVA revealed a significant difference of incubation time-point ($F(2,85) = 28.60$, $p<0.0001$). A Tukey's post-hoc analysis further revealed a significant difference between Day 1 and both Day 30 with a Day 1 (middle bar; $p<0.0001$), and Day 30 without a Day 1 (right bar; $p<0.0001$).

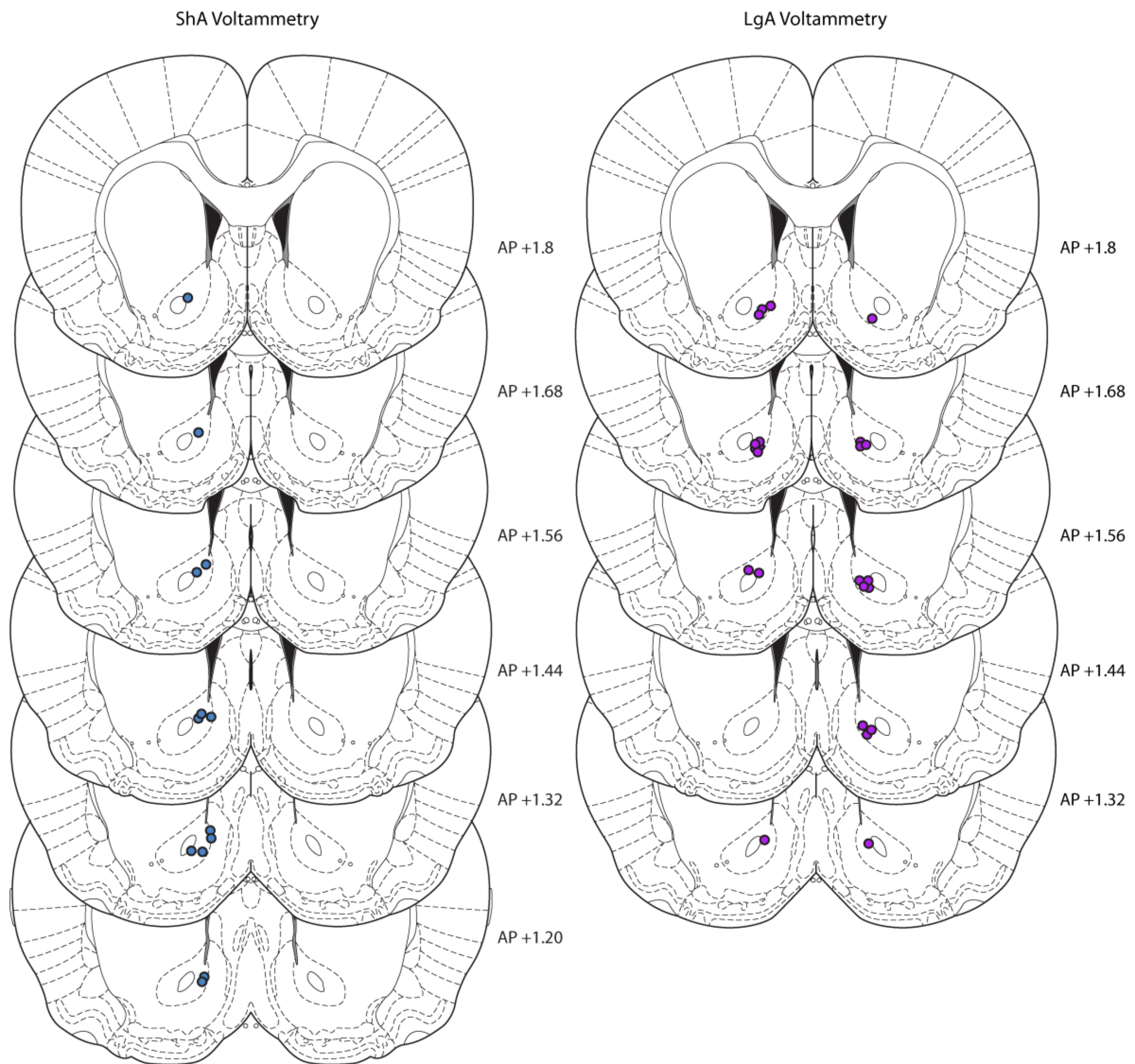

Figure S2. Electrode placement for all FSCV animals.

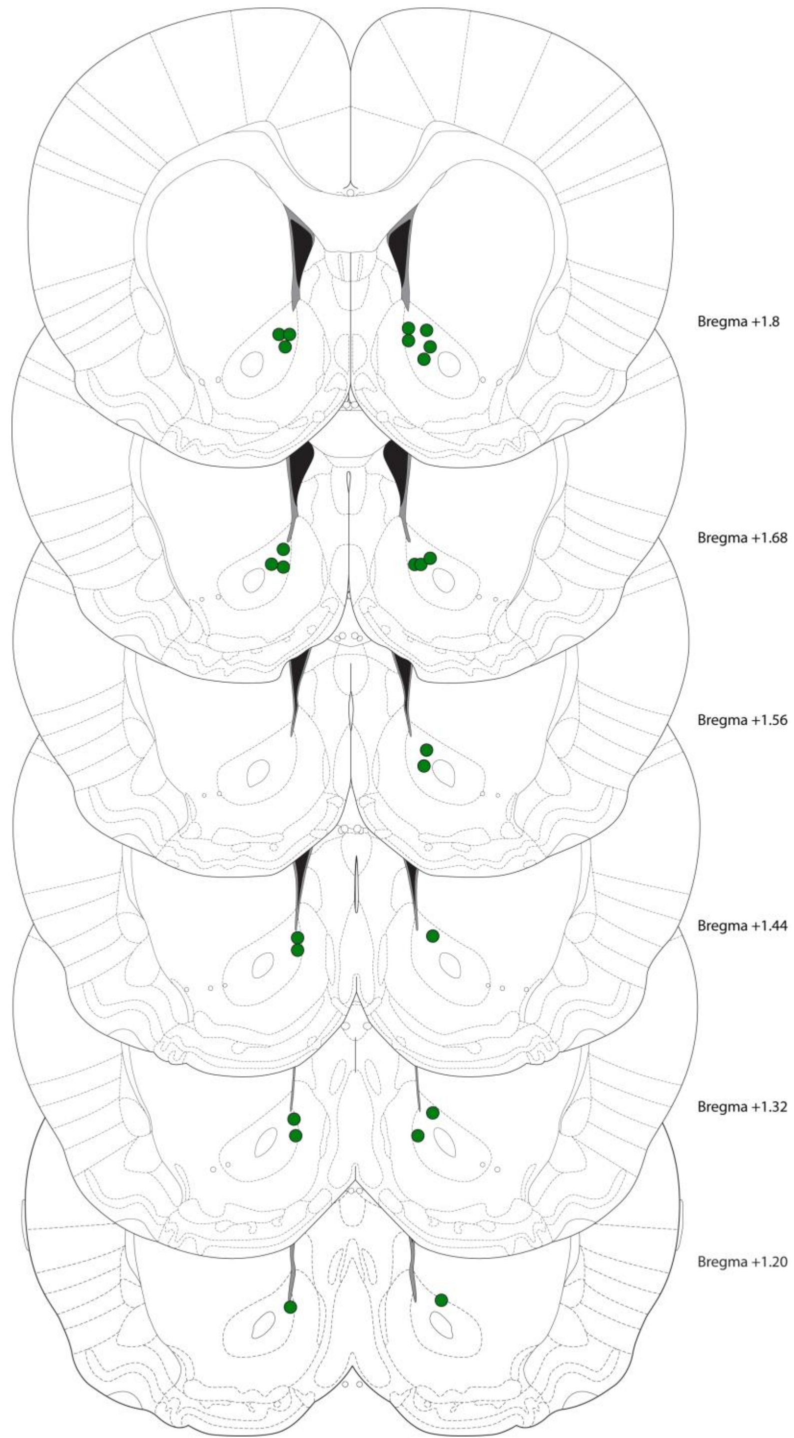

Figure S3. Optic fiber placements in animals that had visible fiber tracts in optogenetic experiments.

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Chapter 3: Kappa Opioid Receptors on VTA terminals in the Nucleus Accumbens Promote Excessive Cocaine Consumption

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Introduction

Through the use of human brain imaging and animal model studies, several contemporary theories of drug addiction implicate alterations in dopaminergic signaling during the progression of addiction (Wise & Bozarth, 1987; Robinson & Berridge, 1993; Dackis & O'Brien, 2001; Everitt & Robbins, 2005; Volkow et al., 2012). Importantly, the transition from moderate to excessive drug-consumption has been shown to occur in animal models when protracted access to self-administer drugs of abuse is provided (Ahmed & Koob, 1998). This protracted self-administration access model allows for investigation of escalated drug-consumption. One anatomical region within the brain that is heavily associated with the progression of drug abuse behaviors is the nucleus accumbens core (NAc), a substructure of the ventral striatum. The NAc has dense dopaminergic input from the ventral tegmental area (VTA), and has been shown to mediate associative learning between drugs of abuse and stimuli that predict their availability (Phillips et al., 2003; Day et al., 2007; Flagel et al., 2011). As drug use progresses, drug-associated stimuli become conditioned, and acquire the ability to evoke NAc dopamine (DA) transmission (Phillips et al., 2003; Willuhn et al., 2014). One theory of drug addiction posits that DA signaling within the NAc is responsible for the loss-of-control in drug-consumption, and mediates drug satiety (Wise & Bozarth, 1987). Consistent with this theory, recent work from our lab and others have shown that NAc DA transmission mediates drug satiety, and thus regulates drug intake (Wise et al., 1995; Suto & Wise, 2011; Willuhn et al.,

2014). It was demonstrated that an attenuation of phasic DA transmission elicited by response contingent drug-associated cues in rodents correlated with escalated daily drug-intake of cocaine (Willuhn et al., 2014). Furthermore, increasing DA concentration through pharmacological intervention with L-DOPA, the DA precursor, prevented and reversed escalation of drug-intake: an effect absent in non-escalators (Willuhn et al., 2014). Additionally, stimulating phasic DA transmission during the presentation of a response-contingent drug-associated cue resulted in a decrease of drug-consumption in animals that escalated their daily drug-intake (Chapter 2, Figure 3). These data indicate that an attenuation of phasic DA within the nucleus accumbens core is necessary to produce escalation of drug-consumption. However, the mechanisms promoting attenuation of DA signaling within the NAc are unknown. Exploration into these mechanisms will reveal potential targets for future pharmacotherapies in the treatment of substance use disorders. Several lines of evidence implicate kappa opioid receptor (KOR) activation as a potential mechanism for the attenuation of DA in the ventral striatum during chronic drug taking. KORs are located on dopaminergic terminals (Mulder et al., 1984; Schlosser et al., 1995; Lemos et al., 2012; A. Karkhanis et al., 2017) and bind the neuropeptide dynorphin (Schlosser et al., 1995). Following repeated drug exposure, dynorphin levels are elevated in the striatum (Hurd & Herkenham, 1993; Daunais & McGinty, 1994). KOR activation, accomplished through administration of KOR agonists, decreases DA release in the NAc (Ehrich et al., 2015; A. N. Karkhanis et al., 2016; A. P. Escobar et al., 2017). D1-type medium spiny neurons (MSNs) contain dynorphin (Al-Hasani et al., 2015) and have collaterals onto dopaminergic terminals (Schlosser et al., 1995). Moreover, KOR inactivation by administration of the long-lasting kappa antagonist, norbinaltorphimine (norBNI), has been shown to block escalation of both methamphetamine, heroin, and alcohol intake (Walker et al., 2011; Schlosburg et al., 2013; Whitfield et al., 2015).

Recent research has also implicated corticotrophin releasing factor (CRF), also known as CRH, to be involved in the escalation of drug-consumption (Specio et al., 2008; Greenwell et

al., 2009; Roberto et al., 2010). CRF is a 41 amino-acid neuropeptide (Vale et al., 1981) that was initially discovered for its role in initiating the HPA axis response. Within the last 20 years CRF has also been found to have physiological actions beyond the hypothalamus (Roberto et al., 2017). CRF has two known receptors Crhr1 and Crhr2, both of which are G-protein coupled but are promiscuous in that they couple to both Gs and Gq receptors, and in high concentrations have shown to couple to Gi/o (Milan-Lobo et al., 2009). Crhr1 more readily binds CRF at low concentrations than its counterpart, Crhr2 (Bale & Vale, 2004). CRF and its receptors have been shown to be expressed through-out the mesolimbic dopaminergic pathway (Van Pett et al., 2000). Although CRF has mainly been implicated in relapse and withdrawal of drugs of abuse, there is recent evidence showing that Crhr1 antagonists, when given systemically, block increased alcohol consumption, prevent the escalation of heroin intake, and reverse escalated cocaine consumption (Specio et al., 2008; Greenwell et al., 2009; Roberto et al., 2010). However, the role Crhr1 has in escalated drug use is not fully understood. Crhr1 antagonists decrease heightened current thresholds induced by nicotine and alcohol withdrawal during intracranial self-stimulation (ICSS), a behavioral assay in which animals press a level for electrical stimulation to the major dopaminergic axons from the VTA to the NAc (Bruijnzeel et al., 2007, 2009). Interestingly, there is an observed increase in ICSS threshold currents when kappa agonists are infused directly into the NAc (Todtenkopf et al., 2004). This is intriguing as we know that dopamine attenuation in the NAc is necessary for the development of escalated drug consumption. However, investigation of CRFs effects within the nucleus accumbens is very sparse, but has been shown to increase cue responding (Pecina et al., 2006), and in mouse brain slices to potentiate DA prior to stress but loses this effect following repeated forced swim exposure (Lemos et al., 2012). There is no clear indication to the actions of CRF within the NAc during escalation of drug intake. CRF administered intracerebroventricular (I.C.V.) has been shown to produce KOR activation within the nucleus accumbens, and Crhr1 agonists I.C.V. elicit anxiety like behaviors that are blocked by a KOR antagonist administration to the basolateral amygdala (Land et al., 2008; Bruchas et al., 2009). Additionally, CRF increases CREB

phosphorylation in cultured MSN (Stern et al., 2011), a transcription factor that is known to regulate dynorphin (Cole et al., 1995; Carlezon Jr. et al., 1998).

The work completed within this chapter examines two mechanisms which we hypothesize are responsible for promoting escalated drug consumption. First we show that KOR antagonism in the NAc blocks the development of escalated cocaine consumption, and examine which population of KOR within the NAc is driving these effects. Secondly, it's been previously shown that Crhr1 antagonists can reverse escalated cocaine consumption when administered systemically (Goeders & Guerin, 2000). Given that we now know the necessity of NAc DA attenuation in the development of escalated consumption (Willuhn et al., 2014 & Chapter 2, Figure 3); we tested if Crhr1 antagonists infused directly into the NAc have a similar effect on volunteer cocaine intake.

Methods

Animals

A total of 96 Wistar rats were included in the studies. Thirty-one of the animals were Female Wistar rats, meaning the remaining 65 were Male Wistar rats. Following histological verification one male rodent from the viral vector studies was removed. All animals were delivered from Charles Rivers, and weighed between 200-250g (females) and 275-325g (males) at the start of the first surgery. Animals were individually housed in ventilated cage racks following the completion of the first surgery. All animals experienced a 12-hour light/dark cycle. All food and water was provided *ad libitum*.

Stereotaxic and Catheter Surgeries

Rats in experiments requiring intracranial microinjections of drug were equipped with bilateral intracranial cannula in the NAc core (Figure 1A & 4A, AP: +1.3mm, ML: ±1.3mm, DV: -6.2mm for guide cannula and injector extends to -7.2mm). For experiments utilizing viral vectors, animals received microinjections into either the NAc (Figure 3A, AP: +1.3mm, ML:

±1.3mm, DV: -7.0mm) or into the VTA (Figure 3A, AP: -6.35mm, ML: ±0.5mm, DV: -8.5mm). All viral vectors were provided by Dr. Larry Zweifel, University of Washington and prepared as described previously (Gore et al., 2013). CRISPR/SaCas9 viruses were made using serotype AAV1 packaging vector with AAV2 ITRs (Hunker et al., 2020). All experiments involving viral vectors had a 4 week incubation period following viral injection to allow for sufficient transfection.

Following 1-3 weeks of recovery from intracranial surgery animals were outfitted with indwelling intravenous jugular catheters and given at least one week to recover before beginning behavioral training. Prior to behavioral training and any extended days off (>2 days) from self-administration catheters were backfilled with a viscous mixture of polyvinylpyrrolidone, gentamicin, and heparin to prevent blood clots. Otherwise catheters were flushed daily with saline and heparin as needed.

Cocaine Self-administration

Rats were trained to self-administer cocaine in daily one hour (short-access, ShA) sessions within an operant chamber outfitted with a liquid swivel and containing two nose-poke ports. During self-administration sessions, the illumination of a house light paired with white noise signaled the availability of drug. A nose poke into the active port elicited a 0.5 mg/kg cocaine infusion (fixed-ratio one schedule), accompanied by a 20-second presentation of an audiovisual conditioned stimulus (nose-poke light + tone), during which any additional nose-poke was without consequence (time out). Nose pokes into the inactive port at any time were without consequence. After meeting the acquisition criterion of three sequential sessions earning 10 or more infusions, animals received daily one hour sessions for five additional days to establish baseline intake. Animals then received daily six hour sessions (long-access, LgA) for 10 additional days. A subset of animals within this chapter received up to 13 days of LgA in order to test the effects of a Crhr1 antagonist after escalation of cocaine intake was established. Animals were identified as escalators or non-escalators by performing a linear regression

analysis of an animal's individual first hour drug-intake over the session number. Animal's that had a significant, positive slope were considered escalators (see Willuhn et al, 2014 for more details).

Drugs

Norbinaltorphimine (norBNI) was suspended in ACSF and injected at 5µg/0.3µL bilaterally into the NAc. The KOR agonist, U69,593 (Sigma Aldrich) used for slice FSCV, was dissolved in solvent 0.01% DMSO and aCSF for a final concentration of 1µM. The Crhr1 antagonist CP154,526 (Sigma Aldrich), was suspended in 1% DMSO, 5% Cremophor, and 94% ACSF, and bilaterally infused into the NAc at 1µg/0.5µL 20 minutes before the behavioral session start.

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 300 µm coronal slices containing the NAc were prepared in an oxygenated sucrose solution. Slices were held in a recovery chamber containing oxygenated aCSF for at least 45 minutes 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 ~175 µm. Transients were evoked by a single pulse of a blue LED through the microscope objective. 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 10 minutes. Evoked transients during baseline and experimental recordings took place every 2 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 10

recordings were performed. Waveform generation, data acquisition, and analysis were carried out using 2 PCI cards and software written in LabVIEW 7.1 (National Instruments, Austin, TX).

Histology and immunohistochemistry

Animals equipped with cannula (Figure 1 & Figure 4) were injected with a sky-blue dye; animal's brains were then fixed via intracardiac perfusion with 4% paraformaldehyde. Brains were then submerged in sucrose before being frozen and sectioned at 40μm experiment end to identify cannula / injector placement. Following completion of CRISPR/SaCas9 experiments, rats were transcardially perfused with 4% PFA, then serially stored in 15% and 30% sucrose solutions. Brains were frozen and cut into 40μm coronal sections on a cryostat (Leica CM 1850). All sections were treated with a blocking solution (PBS containing 3% normal donkey serum and 0.3% Triton x-100) for 1 hour and incubated overnight at 4°C with primary antibodies. Sections were then washed 3 times for 10 mins each in 1x phosphate buffer solution (PBS), incubated in secondary antibodies for ~2 hours at room temperature, and then went through 3 more 10 min washes. Sections were mounted and cover slopped with Fluromount. Fluorescent images were taken on a ZEISS Apotome Fluorescence Microscope (Jena, Germany) to examine if viral vector injections were correctly targeted and transfected.

Results

Long-term KOR antagonism blocks the development of escalated cocaine consumption

Animals acquired cocaine self-administration behavior within 3-7 one-hour sessions, and provided an additional 5 days of ShA to collect baseline post-acquisition drug consumption. Two days prior to starting the ten LgA sessions animals were microinjected with norBNI or its vehicle, ACSF (Figure 1A). Plotting animal's intake within the first hour of LgA, allowed us to compare the change of intake including baseline ShA sessions, and is an established way to detect escalation of cocaine intake (Willuhn et al., 2014). Here we observe that animals treated with norBNI as a group do not have a positive significant regression between intake and session,

whereas vehicle treated animals do (Figure 1B). Additionally, we saw an interaction between drug treatment and session, and a main effect of drug, indicating that norBNI was effective in decreasing the amount of drug consumed when compared to vehicle treated animals. We then split the drug-taking sessions into 3 bins, with 5 sessions in each bin, and averaged across these 5 sessions (Figure 1C). By doing this we can see there is a lack of escalation in the norBNI treated animals, whereas ACSF treated animals have significant increases in their drug intake compared to the ShA baseline bin (Figure 3C). Interestingly, when plotting the cumulative active nose-pokes within each bin there is a visual difference at the point in which the norBNI treated group has a change in rate of drug consumption for LgA Block 1 and LgA Block 2 (Figure 1D). This is further confirmed by comparing the number of nose-pokes animals complete within the first 10 minute of the session (Figure S1). It was previously shown that animals trained to self-administer fall within a bimodal distribution in which, animals either escalate their intake (escalators) or maintain stable intake across sessions (non-escalators). This was accomplished by running individual regressions for an animal's intake across session number and animals with positive significant regressions were labeled as escalators. Willuhn and colleagues (Willuhn et al., 2014) performed this analysis they also found there was a clear visual bimodal split when plotting individual animal's r^2 values. Within the current experiment we observed, ~50% of vehicle treated animals were escalators, whereas only ~12% of norBNI animals were classified as escalators (Figure 1E). We additionally demonstrate through a binomial test, that the distribution of escalators and non-escalators was significantly different in the norBNI group than the expected Vehicle treated group (Figure 1E, inset). We also found there were differences in animal's cocaine intake across the entire 6 hour sessions (Figure S2).

Identifying the NAc KOR population mediating escalated cocaine consumption

KOR in the NAc is expressed on multiple neuronal populations (Tejeda et al., 2017), and microinjection of norBNI into the NAc will cause long-term antagonism of all those populations. Our hypothesis is that the effect of norBNI on drug-taking is through its blockade of KOR on

VTA DA terminals in the NAc. To test this hypothesis we utilized CRISPR/SaCas9 to target and disrupt gene regulation of KOR. Genetic disruption of KOR signaling was done previously utilizing Cre mouse lines (Ehrich et al., 2015), thus we first sought to replicate this effect in non-transgenic rats. Male and female rats were injected with viral vector constructs in the VTA (Figure 2A), which permitted the expression of SaCas9, and ChR2 in VTA neurons. The SaCas9 single-guide RNA was targeted exon 2 in the Oprk1 gene of the rat (Figure 2A, right panel). After injection of viral vectors animals were given 4 weeks to allow for ample viral expression and KOR turn over in DA terminals. Live brain tissue sections of the NAc were then used for *ex vivo* FSCV recordings. First, we demonstrate that ChR2 expression in DA terminals was accomplished and that blue light stimulation (1pulse) elicits a dopamine transient (Figure 2B). It has been well documented that KOR agonists in the NAc decrease stimulated DA release; as such we tested our approach to knock-out KOR by stimulating dopamine release while a KOR agonist is present. We found that SaCas9 mediated genetic knock-out of KOR significantly decreased the ability of the KOR agonist, U69,593, to inhibit DA release (Figure 2 C, D, & E). Representative traces from a control viral vector and a KOR knock-out animal are shown in Figure 2C. Stimulated DA release remains relatively stable across 30 minutes of recording without the presence of a KOR agonist (Figure 2D). The summary data in Figure 2E is the average decrease of DA release across the five stimulated epochs before U69,593 application (0-10 mins in Figure 2D) versus the last five stimulated epochs during the 20 minute U69,593 application window (20-30 mins in Figure 2D).

Following characterization and functional confirmation that the CRISPR/SaCas9 approach sufficiently knocked-out KOR (Figure 2), we examined which KOR population in the NAc is responsible for the blockade of escalated cocaine consumption. We injected the viral vector into either the VTA, to knock-out KOR from VTA neurons and thus their terminals that reside in the NAc, or injected the viral vector into the NAc to remove KOR from medium spiny neurons (MSNs) and interneurons that express KOR. Animals that received the control viral

vector had injections in either the VTA or NAc, these animals were combined into one control group as no statistical differences were found between these groups (Figure S3). A 4-week period between viral injections and behavior allowed for sufficient expression of SaCas9 and turn-over of KOR. Animals were then equipped with intravenous jugular catheters and trained to self-administer cocaine. Following the acquisition period animals received an additional 5 days of ShA and then 10-sessions of LgA (Figure 3A). We first examined if there were significant regressions within the treatment groups first hour drug-taking and session number (Figure 3A). We found that both the control group and the group receiving NAc viral vector injections had a significant positive regression between first hour drug-intake and session number, indicating they escalated their intake over session, whereas the VTA injected viral vector animals failed to escalate their intake and no significant regression was observed (Figure 3B). We also observed there was an interaction between the groups and a main effect of viral vector (Figure 3B). When averaging across the 5 sessions within each block, we found that control animals had a significant increase from LgA Block 2 and ShA, and that the NAc injected group had significant increases in both LgA Block 1 and LgA Block 2 compared to its ShA Block (Figure 3C). Animals in which KOR was removed from VTA neurons and its terminals had no significant change across the self-administration blocks. The average cumulative active NP across each self-administration block reveals a separation of drug-taking in the VTA KO group when compared to the control and NAc KO groups (Figure 3D). A bimodal distribution was observed in both the control and NAc injected animals and a binomial test was run in comparison to controls. This test revealed that there was no difference in the distribution of escalators and non-escalators in the NAc KO animals but the VTA KO animals had a significant shift in the distribution of escalators and non-escalators (Figure 3E).

One difference between the pharmacological approach with norBNI and the genetic approach with CRISPR/SaCas9 is that normal KOR function was present during acquisition and baseline drug-taking time points. We also investigated if disrupted KOR signaling had an effect

of cocaine self-administration acquisition and baseline consumption. There were no statistically observed differences in animals baseline drug taking (Figure 3F), nor was there any significant difference in the number of sessions it took for animals to reach criteria (Figure 3G).

All genetic manipulation studies with male and female rodents matched to age. Both males and females within the control viral vector group escalate their cocaine intake over sessions (Figure S4A, left panel). As such the main figures combined male and females during the investigations of how KOR signaling may contribute to escalation of drug intake. Although the direction and slope between males and females is not different, we did observe that females take more cocaine than males (Figure S4A, left panel). Males and females that had KOR knocked-out of VTA cell bodies showed no escalation of their cocaine intake, and again a main effect was observed demonstrating the females take more overall cocaine (Figure S4A, right panel). In both sexes there was an observed escalation of intake when KOR was removed from cells that had cell bodies residing in the NAc, but interestingly males and females did not have differences in total drug consumed (Figure S4, middle). If compiling all the treatment groups by sex we observe similar effects on escalation slope (Figure S4B), as the Control Vector and NAc injected KOR KO's had a positive regression between drug-taking and session number, whereas in the VTA injected KOR KO no significant regression was observed. It should be noted that Figure 3 was all completed using only the first hour of the LgA sessions, we observed similar results using the full 6-hour sessions (Figure S5).

Crhr1 antagonist microinjected into the NAc decrease escalated drug-consumption

CRF signaling has been implicated in multiple aspects and stages of addiction (Zorrilla et al., 2014; Roberto et al., 2017). Interestingly, antagonists for the CRF receptor, Crhr1, have been used to decrease escalated cocaine consumption when giving systemically (Goeders & Guerin, 2000). I further explored Crhr1's role in promoting increased cocaine intake by testing if a Crhr1 antagonist confined to the NAc is sufficient to decrease escalated cocaine consumption. To do this I implanted bilateral cannula into the NAc, and trained animals to self-administer

cocaine. After animals were given sufficient time to observe escalated cocaine intake (~9 – 10 sessions of LgA), we treated animals with CP154,526 and its vehicle (Figure 4A). The order in which CP154,526 or vehicle was administered was counterbalanced across animals. We found there was a statistical interaction between the animal's drug-taking classification and the treatment given (Figure 4B). Post-hoc analysis revealed that there was a statistical trend in non-escalators increasing intake during CP154,526 administration, and a statistical trend in the decrease of drug-taking observed in escalated animals when treated with the Crhr1 antagonist. These data are currently underpowered and we will be increasing the sample size in this experiment to allow for proper statistical power in our analyses (Figure 4B). Interestingly, just as it was previously shown in chapter two non-escalators treated with CP154,526 started to increase their drug-taking towards the end of the 60 minute sessions (Figure 4C, left panel), whereas escalators had a noticeable dip in their drug-taking during the first 10 minutes of the session (Figure 4C, right panel). More experiments will need to be completed to elucidate the role Crhr1 in the NAc has in the maintenance, and possibly the development of escalated cocaine intake. For instance, one question we have is what population of Crhr1-receptors is mediating this effect we see if Figure 4B? In the near future we plan on utilizing a CRISPR/SaCas9 mediated knock-out of Crhr1 in cocaine self-administration assays. I was able to characterize and show functional success in our ability to knock-out Crhr1-receptors from MSNs and interneurons within the NAc. I unilaterally injected the viral vector containing the SaCas9 targeted for Crhr1 into the NAc, and implanted bilateral cannula into the NAc and after 4 weeks of incubation time. Animals were bilaterally injected with 1µg of CRF in 0.5µL of ACSF into the NAc (Figure 4D). Crhr1 is a Gs-coupled GPCR and its activation will cause early gene factors such as C-Fos to be upregulated. Ninety minutes after the CRF injection animals were euthanized and brain tissue fixed through intracardial perfusion with 4% paraformaldehyde. We used fluorescence immunohistochemistry to stain for the HA tag on SaCas9, and for C-Fos (Figure 4E). Cells that were marked from the C-Fos antibody were counted in both hemispheres

and we found that the hemisphere(s) that were injected with our CRISPR/SaCas9 viral vector had significantly less C-Fos activation from a CRF injection (Figure 4F).

Discussion

The work within this chapter demonstrates that Kappa opioid receptors on dopamine terminals within the ventral striatum mediate escalated cocaine consumption. Additionally, we present novel data that Crhr1-receptors within the NAc also play a role in maintenance of escalated cocaine consumption.

It has been well documented that long-term antagonism of KOR within the NAc prevents excessive drug consumption (Wee et al., 2009; Walker et al., 2011; Schlosburg et al., 2013; Whitfield et al., 2015). We replicated these findings and reveal a microinjection of norBNI into the NAc blocks the development of escalated cocaine consumption (Figure 1). KOR is expressed on both D1 and D2 MSNs in the NAc,(Tejeda et al., 2017), and on dopamine terminals (see reviews: A. del P. Escobar et al., 2020; Estave et al., 2020). Given that micoinjections of KOR antagonists in the NAc will inactive all local KOR, we sought to elucidate which population of KOR was mediating escalated drug consumption. We utilized a viral delivered CRISPR/SaCas9 strategy (Hunker et al., 2020) to target and genetically knock-out the Oprk1 gene out of VTA neruons, and thus their terminals, or NAc neurons (i.e. MSNs and interneurons). We found that this strategy effectively removed KOR from dopamine terminals in the NAc (Figure 2), and blocked the development of escalated cocaine consumption (Figure 3). We found there was no effect on drug-consumption when we genetically removed KOR from MSNs and NAc interneurons (Figure 3). One caveat of these data is we are unaware if inactivation of KOR on VTA cell bodies and dendrites, or on terminals in other regions such as the PFC or the Hippocampus contributes to the overall behavioral effect we observed. However, we do show that microinjections of norBNI into NAc have similar results (Figure 1).

Similar to the observations made by Willuhn and gang (Willuhn et al., 2014), we found a bimodal distribution in animals' r^2 values, calculated from a linear regression of their drug-taking across sessions (Figures 1E, & 2E). We demonstrate that microinjection of norBNI or genetically inactivating VTA neuron's KORs skews this bimodal distribution. In both manipulations, it appears that animals with high r^2 values, which would likely be classified as escalators, are being skewed towards lower r^2 values. As such we hypothesize that there are differences in KOR signaling between escalators and non-escalators.

Previous studies (Wee et al., 2009; Walker et al., 2011; Schlosburg et al., 2013; Whitfield et al., 2015) and our own data (Figure 1) that used KOR antagonism to prevent or decreasing consumption of addictive substances exclusively used male rodents. Given that female rats have shown a greater amount of overall cocaine consumption (Swalve et al., 2016) , and have an enhanced escalation of cocaine intake (Roth & Carroll, 2004), we thought it necessary to include female rodents in our genetic knock-out studies (Figure 2 & 3). Our *a priori* motivation was to examine if KOR knock-outs had an effect on drug consumption in both male and female rodents, rather than study sex differences in KOR signaling or overall drug consumption. Genetically knocking-out KOR from VTA neurons blocked the development of escalation in both male and female rodents. We did find that females consumed more drug than males in both the control and VTA knock-out groups (Figure S4A), similar to observations previously found (Roth & Carroll, 2004; Swalve et al., 2016). Interestingly, we observed that males and females had near identical levels of drug consumption when we removed KOR from cell bodies that reside within the NAc (Figure S4A). Further analyses revealed that in male rats removing KOR from NAc neurons increased their drug-consumption above control animals (Figure S4B). Further experiments will need to be run to understand this mechanism, but it has been reported that a KOR antagonist injected into the NAc core increased heroin intake in male rats (Schlosburg et al., 2013). However, we saw the opposite effect when injecting norBNI into the NAc core of male rats (Figure 1). We did not observe an increase in female's drug-intake

when KOR was removed from NAc neurons (Figure S4B). Interestingly, there are known sex differences in KOR signaling (Abraham et al., 2018; Reichard et al., 2020), and these data suggest there are sex differences in KOR signaling within NAc neurons.

It has previously been found that antagonists for the CRF receptor Crhr1, decrease cocaine consumption (Goeders & Guerin, 2000; Specio et al., 2008), even after the animals escalate their intake. However, these studies administered Crhr1-receptor antagonists systemically; as such the brain region that was mediating the behavioral effects observed is unknown. Our current data reveal that Crhr1-receptors in the NAc are important for the maintenance of escalated drug consumption. Given, that CRF transmission recruits dynorphin signaling within the NAc (Bruchas et al., 2009), our current hypothesis is that CRF actions in the NAc recruit dynorphin signaling, which promotes escalated drug consumption. Furthermore, CRF increases CREB (Stern et al., 2011), which has been shown to increase dynorphin synthesis (Carlezon Jr. et al., 1998; Muschamp & Carlezon, 2013). Currently we hypothesize that Crhr1-receptors on MSNs mediate this effect. Although I do not directly test this hypothesis in the current work, in Figure 4D-F, I present experiments where we functionally confirm CRIPSR mediated Crhr1 receptor knock-outs in MSNs and interneurons in the NAc. We will utilize this technique in future experiments to reveal more information about NAc Crhr1's role in cocaine consumption.

In conclusion these data demonstrate that both KOR and Crhr1 within the NAc promote escalated drug consumption in both male and female rats. Further experiments should asses if terminal or somatodendritic Crhr1-receptors promote escalated drug consumption.

Figures

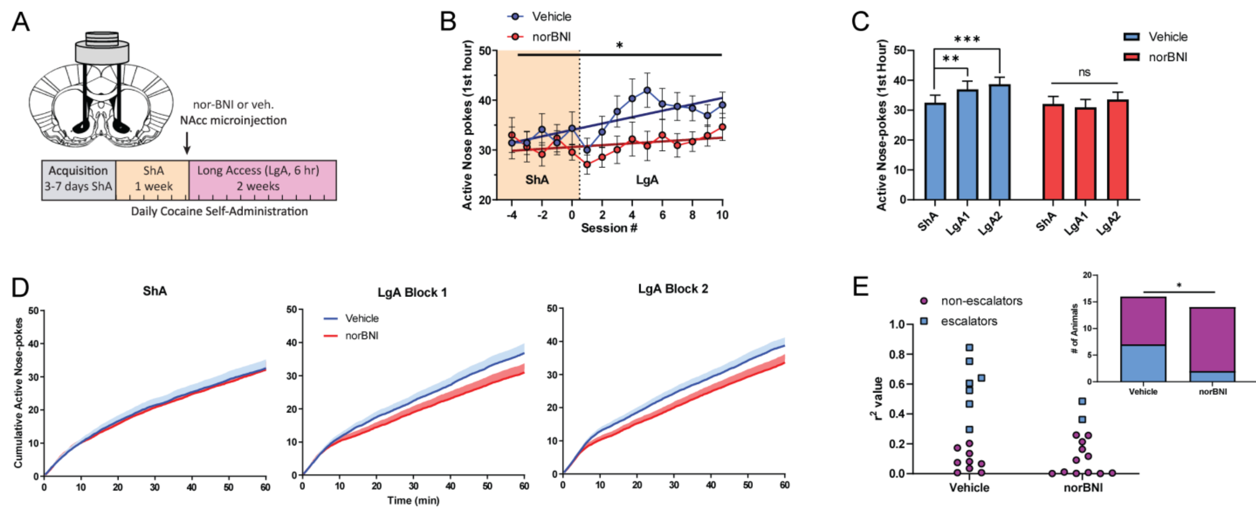

Figure 1. norBNI prevents escalation of cocaine consumption. **A)** Schematic of experimental timeline and location of microinjections. **B)** Animals treated with vehicle (blue) escalated their daily drug intake indicated by a significant positive regression (blue line, r^2 = 0.057, F(1,229)=13.91, p<0.001). norBNI treated animals (red) did not escalate their cocaine intake across sessions (red line, r^2 = 0.007, F(1,199) = 1.465, p > 0.05). A direct comparison between animals was completed with a mix-effects model and an interaction between session and treatment was observed (F(14,374) = 2.02, p>0.05). **C)** Animals drug-consumption was averaged for each 5 session block. A two-way ANOVA revealed an interaction between session blocks and drug treatment (F(2,56) = 4.36, p<0.05), and post-hoc Tukey test showed a significant difference between vehicle treated ShA block to LgA Block 1 (p<0.01) and LgA Block 2 (p<0.001), no significant differences were found in the norBNI treated animals. **D)** Cumulative cocaine intake plots for the first hour of each session reveals a separation between the group's consumption patterns in starting in LgA Block 1, after norBNI was administered. This data visualization shows that norBNI animals appear to have a lower load-up phase in LgA Block 2 (right panel; see Figure S1). **E)** r^2 values that were calculated from a linear regression for each animals intake vs session number. Animals that had a significant positive correlation between drug-consumption and session were classified as escalators. A bimodal distribution is observed within the vehicle treated animals r^2 values that matches their escalation classification. In animals treated with norBNI this bimodal distribution appears to flatten. **E inset)** To test if there is a shift in the number of escalators present in the norBNI treated animals we ran a binomial test defining the expected number of escalators and non-escalators to match the vehicle group. The binomial

test indicated that the proportion of escalators of 0.1429 was lower than the expected 0.4375 (p<0.05; two-tailed).

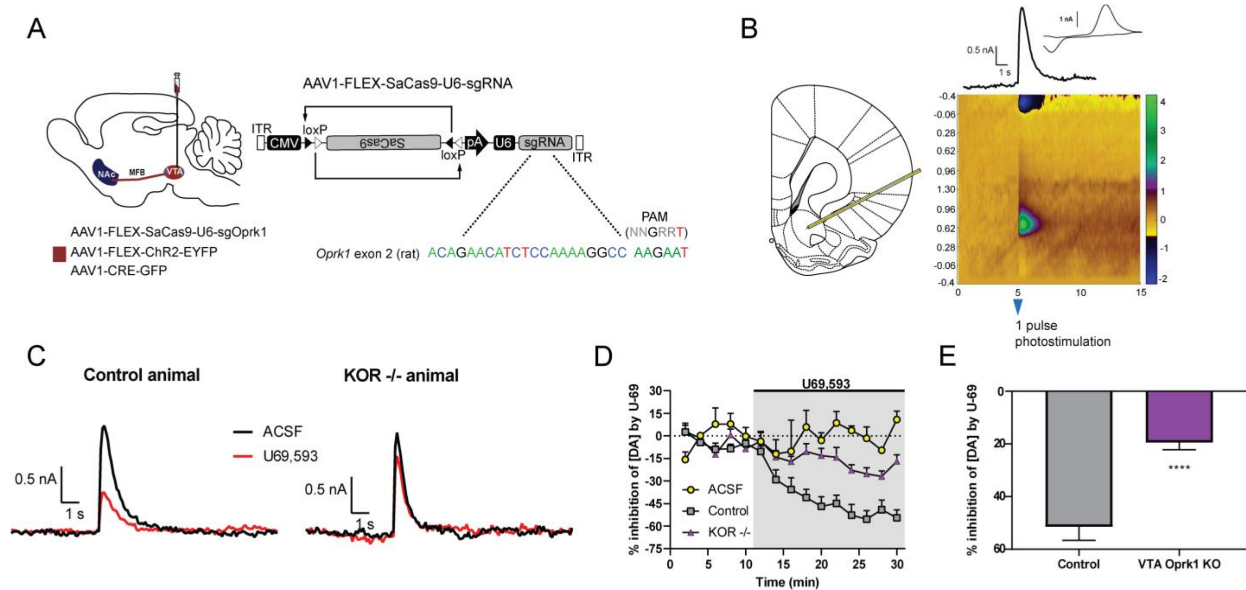

Figure 2. CRISPR/SaCas9 mediated knock-out of Oprk1 in VTA neurons disrupts KOR agonists inhibitory effect on stimulated dopamine release in NAc terminals. A) Schematic of experimental approach. Left panel shows injection site of the viral vectors used to knock-out KOR from dopamine terminals as well as achieve expression with ChR2. The right panel shows the sgRNA used to target exon 2 in the rat Oprk1 gene. **B)** Expression of ChR2 was sufficient in stimulating dopamine transients following a pulse of blue light onto a coronal slice. The left panel shows the recording site within slices. The right panel shows a current vs time (right panel; top left), the corresponding CV (right panel; 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 both a control animal (left panel) and an animal with SaCas9 mediated KOR knock-out (right panel). **D)** Time series of stimulated dopamine release under various conditions. A subset of slices underwent 15 stimulations without the U69,593 being washed on (yellow circles, n=3). The other two groups, contro (gray squares n=7) or KOR knock-outs (purple triangles n=9), had 5 stimulations (10 mins), before U69,593 was applied to the ACSF bath (grey box). A mixed-effects model revealed there was a main effect of group (F(2,16) = 12.81, p<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 the KOR knock-outs and control

(p<0.0001), and a difference between the KOR knock-outs and ACSF (p<0.0001). **E)** To test the normalized change in dopamine release after U69,593 application, the 5 stimulations just prior to U69,593 wash on were used as a baseline, 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).

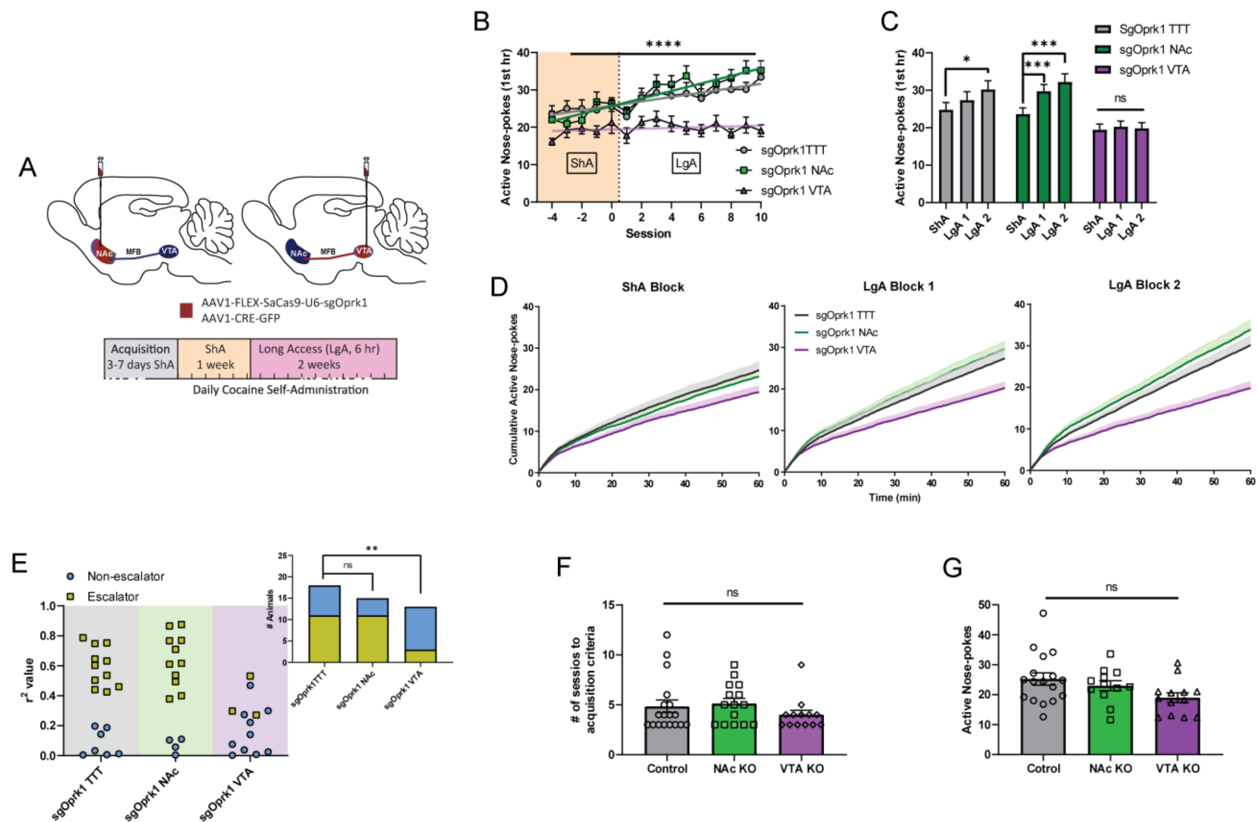

Figure 3. Knocking out Oprk1 from VTA neurons prevents the development of escalated cocaine intake. **A)** Schematic showing viral vector injection sites (top panel), the time-line of cocaine sefl-administration (middle panel). **B)** Animals who had KOR knocked-out of VTA neurons did not have a positive significant regression of drug-intake across session (purple line; r^2= 0.004, p>0.05). Both control animals (gray line; r^2=0.061, p<0.0001) and animals that had KOR knocked of the NAc (green line; r^2 = 0.1989, p<0.0001) escalated their daily cocaine consumption. A mixed-effects model revealed a main effect of group (F(2,42) = 6.632, p<0.01), and an interaction of time by group (F(28,571)=2.491, p<0.0001). A post-hoc Tukey's test showed a significant difference of sgOprk1TTT and the sgOprk1 VTA (p<0.0001), and a significant difference between sgOprk1 NAc and sgOprk1 VTA was observed (p<0.0001). **C)** Drug-taking in the first hour of each session was collapsed into 3 blocks with 5 sessions in

each block. A two-way ANOVA was ran and revealed a main effect of group (F(2,42)=6.544, p<0.01), and a significant interaction of session block by group (F(4,84)=3,945, p<0.01). A post-hoc Tukey test was completed to compare within group differences across weeks. **D)** Cumulative plots within the first hour of drug-taking. This data visualization reveals how drug-taking can change within session. **E)** Animals individual r^2 values calculated from individual regressions of drug intake across sessions. Both the control group (sgOprk1 TTT) and the Oprk1 NAc knock-outs had a visible bimodal distribution. A binomial test indicated that the proportion of escalators in the VTA Oprk1 knock-outs (0.23) was statistically different than the expected (0.611) (E inset, p<0.01; two-tailed). **F)** Animals in all three groups did not differ significantly in the number of days it took them to reach acquisition criteria (F(2,43)=0.914, p>0.05), **G)** nor did they differ in their total amount of drug intake during the ShA baseline block of sessions (F(2,39)=0.7099, p>0.05).

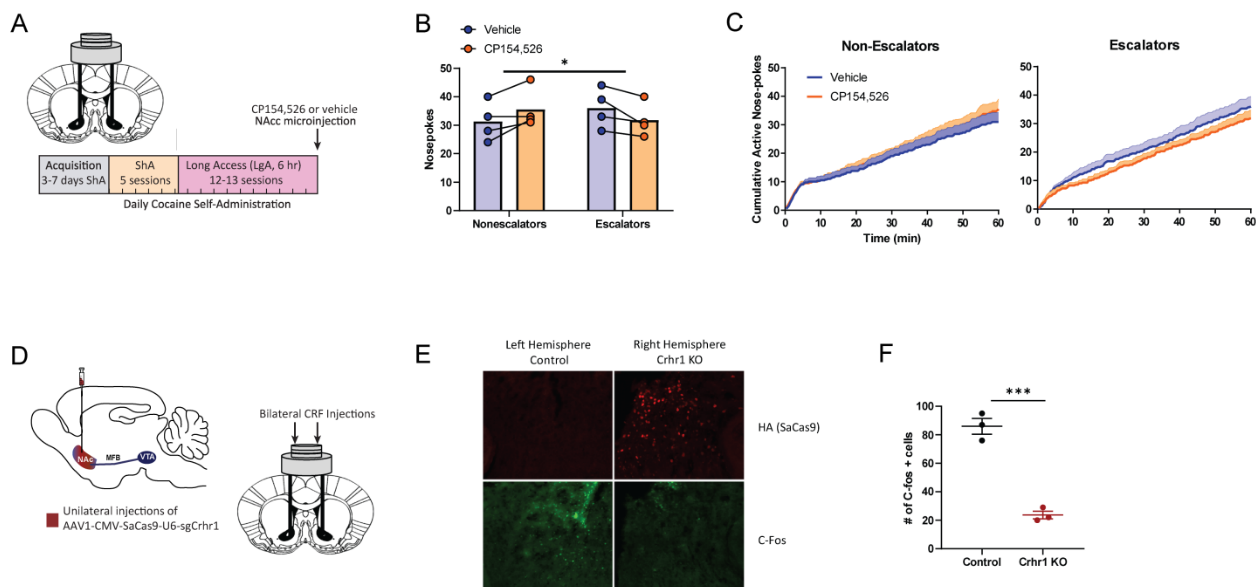

Figure 4. Antagonism of Crhr1-receptor in the NAc differentially affects cocaine intake in escalators and non-escalators. **A)** Schematic of experimental design. **B)** CP154,526 has differential effects on escalators and non-escalators. An interaction between drug-taking group and drug treatment was found by a two-way ANOVA (F(1,6)=12.47, p>0.05). **C)** Cumulative drug-intake during the 1 hour sessions following pre-treatment. **D)** Animals were unilaterally injected with viral vector containing SaCas9 targeted to knock-out Crhr1 in the NAc of the rat. Following viral injections an intracranial cannula was equipped to the animals. **E&F)** Following a 4 week incubation time animals were bilaterally

injected with CRF and perfused 90 minutes later to examine C-Fos staining. Representative IHC images are shown in **E.** We counted the number of C-Fos positive cells following the CRF injection and found that there were significantly fewer C-Fos positive cells in the hemisphere injected with the SaCas9 targeted to Crhr1 (unpaired t-test ($t(4)=10.14$ $p<0.001$).

Supplemental Figures

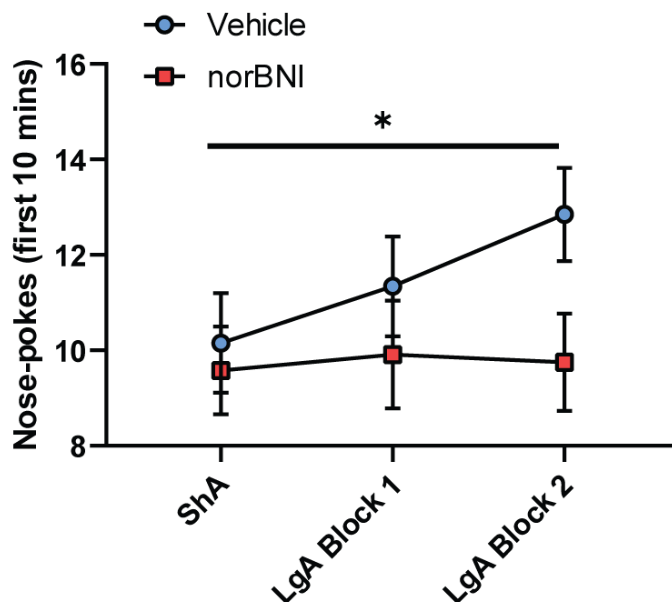

Figure S1. norBNI blocks escalated cocaine consumption observed in the load-up phase. During cocaine self-administration tasks animals take more drug in the beginning of the session. We observed that in vehicle treated animals there appears to be an increase in the amount of drug being taken in the first 10 minutes of the session. We also found that animals treated with norBNI did not have the same growth over the session's blocks, indicated by the interaction between Session Block and Drug Treatment in a two-way ANOVA ($F(2,56) = 3.506$, $p<0.05$).

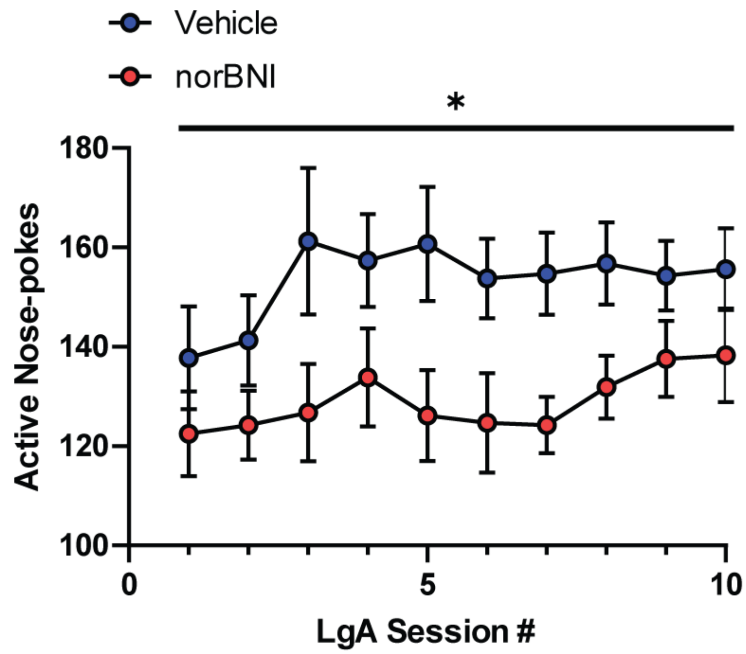

Figure S2. norBNI treated animals have decreased cocaine consumption during the across six-hour LgA sessions. Similar effects of norBNI on animals drug-taking was observed when using all nose-pokes completed within the six hour long-access sessions. An interaction was observed between session and drug treatment when (mixed-effects model $F(1,28)=6.012$ $p< 0.05$).

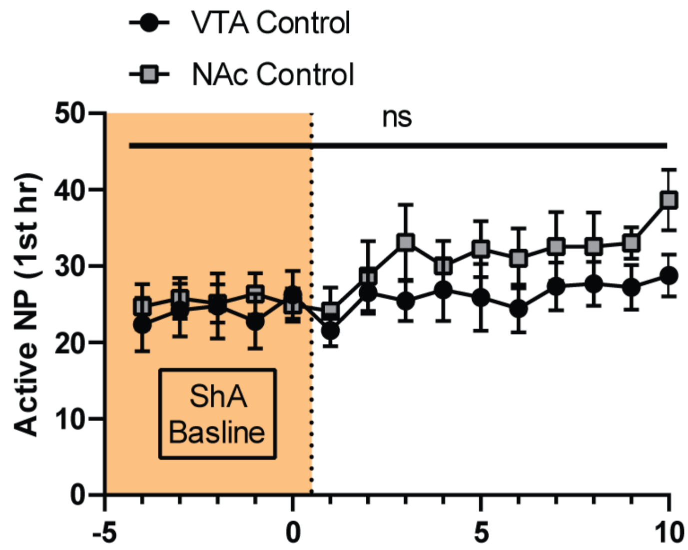

Figure S3. The location of the non-targeting CRISPR control sgOprk1TTT had no statistical effect on drug consumption. Animals in the control sgOprk1 TTT group were counter balanced on what brain location the vector was injected into. To confirm injection site was not responsible for the effects we observed we compared the two groups. A mixed-effects model was used and no main effect of vector injection site ($F(1,16) = 0.9803$, $p>0.05$) nor an interaction between time and injection site ($F(14,221)=1.173$, $p>0.05$) was observed.

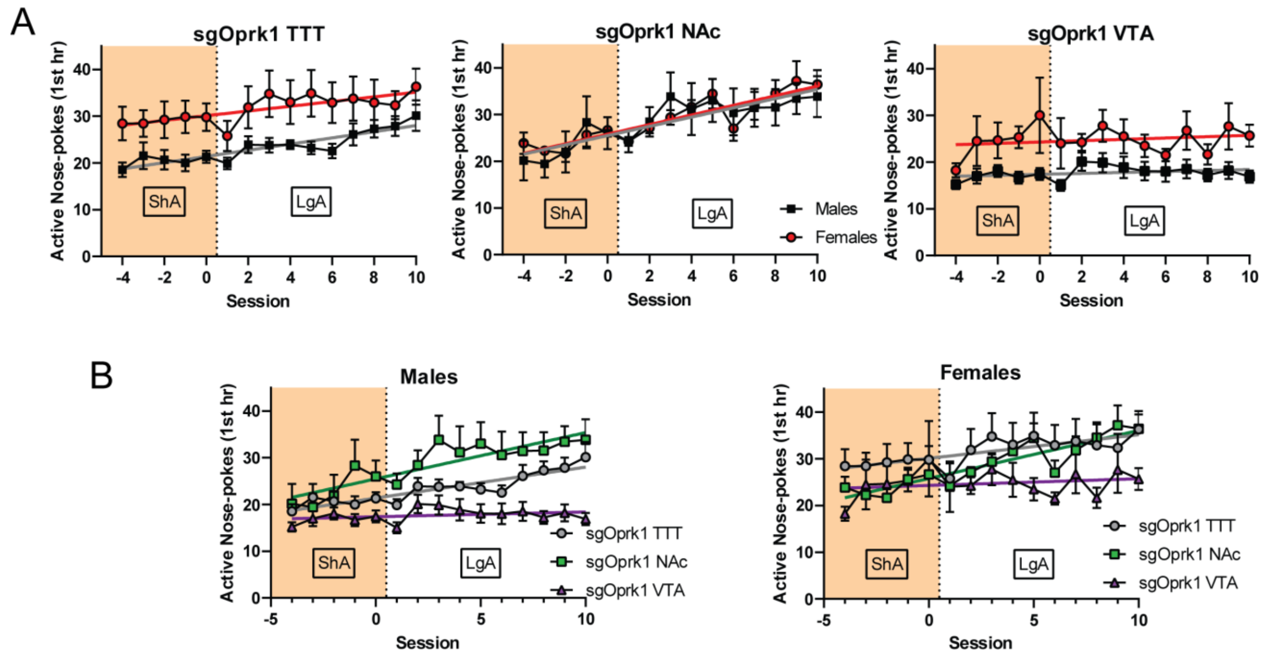

Figure S4. Sex-differences across the Oprk1 knock-out groups. A) A mixture of males and females were used in viral vector groups. There was a significant difference of overall drug consumption between males (black) and females (red) in both the control group (Left panel, F(1,16)=5.025, p<0.05) and the group with KOR KO in the VTA (right panel, F(1,11)=9.157), p<0.05). No interactions were found in any of the groups. There was no sex difference between males and females in the KOR NAc KO group (F(1,13) = 0.016, p>0.05). All analyses were run using a mixed-effects model with REML. **B)** In both males and females the group of animals that received the control viral vector (gray; males: r^2=0.2072, p<00001; females: r^2 = 0.034, p<0.05), and had the sgOprk1 injected into the NAc (green; males: r^2=0.118, p=0.001; females: r^2 = 0.3376, p<0.0001) had a significant positive regression of their intake over sessions. In both males and females animals that had KOR removed from VTA neurons had no significant regression in their intake across sessions (purple; males: r^2= 0.008, p>0.05; females: r^2=0.007, p>0.05).

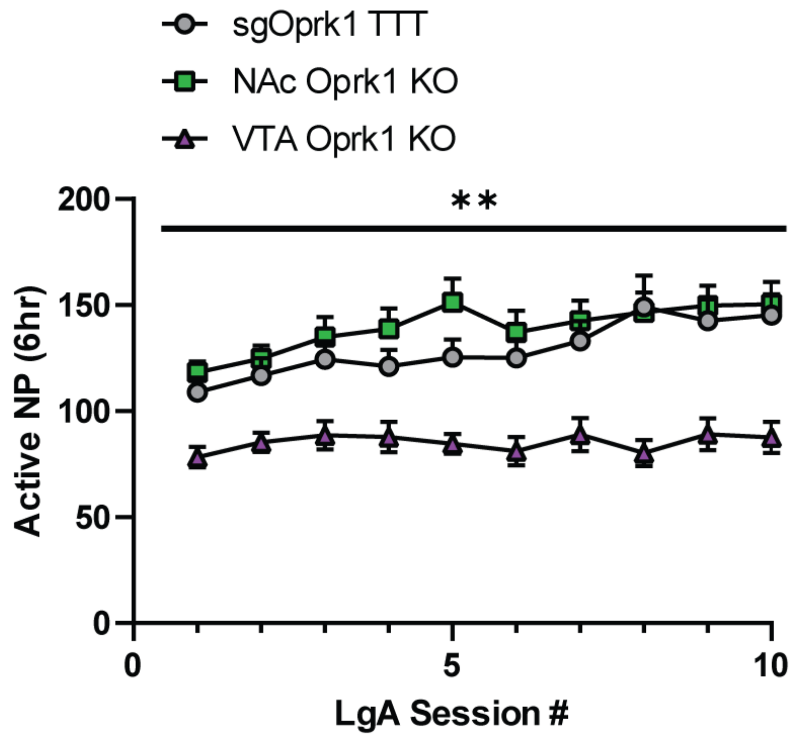

Figure S5. VTA Oprk1 knock-outs decrease total cocaine intake in six hour LgA sessions. A mixed-effects model was ran to test if there were statistical differences in the animals drug-intake within all six hours. An interaction was of sessions by group was found ($F(18,357)=2.509$, $p<0.001$). A Tukey's post-hoc analysis revealed that the VTA KOR KO (purple) group was significantly different from the control group (gray), $p<0.0001$) and from the NAc KOR KO (green, $p<0.0001$).

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Chapter 4: Levodopa as a pharmacotherapy for multiple classes of drugs

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Introduction

Drug addiction is a neuropsychiatric disorder distinguished by compulsive and continued drug seeking, and long-lasting changes to the brain (Koob & Volkow, 2016; NIDA, 2018). Substance use disorders (SUDs) are characterized by the class of drug being abused (APA, 2013), two classes of substances that have a heavy impact on society are alcohol and opioids. This is evident given that 63,000 drug overdose deaths occurred in 2016 and in the same year there were 88,000 deaths related to excessive alcohol use (NIDA, 2018; NIDA et al., 2018). All currently approved pharmacological based treatments for substance use disorders are specific to the class of drug being abused (e.g. alcohol, opioids, nicotine) (NIDA, 2018). As such, a treatment for multiple classes of SUDs could be beneficial especially for people living with addictions to multiple substances.

Alcohol and opioids increase dopamine activity in the mesolimbic dopamine system (Di Chiara & Imperato, 1988; Koob & Volkow, 2016), an effect linked to their reinforcing and rewarding properties (Perry et al., 2014). Given the opportunity animals will administer drugs of abuse to maintain a concentration of extracellular dopamine (Wise, Newton, et al., 1995; Wise, Leone, et al., 1995). Stimuli that are paired with drugs obtain the ability to increase extracellular dopamine signaling within the nucleus accumbens (NAc) (Katner & Weiss, 1999; Phillips et al., 2003; Stuber et al., 2005; Day et al., 2007; Flagel et al., 2011). Interestingly, in chronic cocaine

experiments animals that have escalated consumption show a decrement in phasic dopamine signaling to cocaine-paired cues across drug-taking sessions (Willuhn et al., 2014). Experimenters restored phasic dopamine release in the NAc through systemic L-DOPA administration, which also decreased animal's intake back to pre-escalated levels (Willuhn et al., 2014). Although pharmacological mechanisms between cocaine, alcohol, and fentanyl differ, we hypothesize that similar allostatic processes are responsible for the decrement of cue evoked phasic dopamine observed by Willuhn and colleagues (Willuhn et al., 2014), and can be generalized across other drugs of abuse. Therefore, we tested the effects of L-DOPA on rodent self-administration of alcohol and fentanyl.

Methods

Subjects:

A total of 64 Wister rats (18 female, 46 male) from Charles Rivers (Hollister, CA, USA) were used. Animals weighed between 145-165 g (female) and 275-300 g (male) at the beginning of all behavioral experiments. All animals were individually housed in ventilated cage racks and experienced a 12-hour light/dark cycle. All food and water was provided *ad libitum*. Twenty-eight of the 46 male rats underwent alcohol self-administration experiments, however 5 were not used for analysis as they failed to self-administer at least 0.6 g/kg of ethanol (~approximately 10 nose-pokes). A total of 12 animals underwent two-bottle choice self-administration of fentanyl, evenly split between males and females. An even sex split of 16 animals underwent operant self-administration of fentanyl. The remaining 8 animals (4 males, 4 females) underwent operant water self-administration.

Alcohol Self-administration:

In addition to the water bottle provided in animal's home cage, they were given 24 hour access to a second bottle containing an ethanol solution for 20 consecutive days. The first 7 days of this 20 day period the ethanol bottle contained a 10% ethanol solution, whereas for the

final 13 days animals were provided with a bottle containing a 20% ethanol solution. Following the 24 hour two bottle choice paradigm, animals received 3-5 30 minute magazine training sessions before beginning the operant self-administration stage of the experiment. Within this stage animals were given 25 days of 1-hr access to nose-poke for delivery of 0.2 ml of 20% ethanol. Within this operant task two nose-poke ports are provided one active and one inactive. An active nose-poke by the animal elicits a 5-s presentation of an audiovisual stimulus (nose-poke light + tone) and then a 10-s presentation of the magazine light paired with activation of a solenoid (Lee Valves, WestBrook, CT, USA) for liquid ethanol delivery into the magazine bowl. A diagram of the cue-configuration can be found in Figure 1A. Inactive nose-pokes were recorded but never reinforced. Following the 25 days of access animals then received L-DOPA or saline I.P. injections. If animals failed to reach an average of 0.6 mg/kg of ethanol consumed (~10 nose-pokes) across 5 consecutive baseline operant sessions (20-25), they were removed from the study. This amount of intake was estimated to be about 0.02% blood alcohol content (Holgate et al., 2017).

Two-Bottle Choice:

Animals had access to liquid fentanyl (Fagron, Rotterdam, Netherlands) in addition to deionized (DI) water in separate bottles. Fentanyl and DI water were made available to orally self-administer for three hours a day (between 9 a.m. and 5 p.m.). All animals ran for a total of 18 sessions before starting treatment days. Body weight was recorded before each experimentation session. Fluid consumption of water (DI H2O) and fentanyl solution (50 µg/mL) dissolved in DI H2O was determined by weighing the drinking bottles. The positions of the fentanyl bottles were consistent through-out the entirety of the experiment and counterbalanced among animals in the left and right positions of the cage.

Oral Fentanyl Operant Self-Administration:

Animals learned to self-administer liquid delivery in a modular operant chamber (Med Associates, VT, USA) equipped with two nose-poke located on adjacent panels of the same

wall, a magazine light over a ~5 mL bowl liquid dispenser stationed between nose ports, a house light, and speakers to play a pure-tone. The operant chamber was placed within a sound-attenuated outer chamber. Rats were trained to obtain liquid fentanyl delivered into the dispenser following an operant response on FR1 reinforcement schedule. The dispenser is initialized to deliver 0.04 mL/kg of animal's weight. A nose poke in the active port (side counterbalanced between animals) immediately activates the solenoid (Lee Valves, West Brook, CT, USA) for liquid delivery into dispenser, and paired with a 10-second presentation of an audiovisual stimulus. The audiovisual stimulus consists of illumination of a red light inside the nose poke port for 1-second contingent with a 10-second tone and magazine light (conditioned stimulus, CS) overlapped on top of each other. During CS presentation, a 10-second time out period was imposed during which nose poking did not result in additional drug delivery or any other programmed consequences. Drug availability during the session was signaled by illumination of the house light (discriminative stimulus, DS). Refer to Figure 3 for cue-configuration diagram. To monitor response specificity, nose-poking of the second (inactive) port was recorded but was never reinforced. Rats were given daily access to either liquid fentanyl (50 µg/mL, 0.02 mg/kg/delivery) or DI H2O (0.04 ml/kg/delivery) for one hour per session for 24-31 days. Mg/kg or mL/kg was calculated by measuring the amount of liquid left at the end of every session and subtracting from the total amount of liquid delivered.

L-DOPA/Benserazide administration:

Administration of L-DOPA (L-3,4-dihydroxyphenylalanine) (Sigma Aldrich) was paired with the peripherally acting DOPA decarboxylase inhibitor Benserazide (Sigma Aldrich) to decrease peripheral breakdown of L-DOPA , and allowing it to reach the brain. Both L-DOPA (30 mg/kg) and Benserazide (15 mg/kg) were dissolved in saline and injected intraperitoneally (IP) at a volume of 1 mL/kg body weight 20 minutes prior to experimentation session. All animals in operant self-administration tasks received I.P. injections of both L-DOPA/Benserazide and saline. Each treatment was given in 3-5 consecutive days and the order in which saline or L-

DOPA was given was counterbalanced between animals. For two bottle choice, L-DOPA/Benserazide treatment was injected IP and given in sessions 19-22 of experimentation. Three of the females and three of the males received L-DOPA whereas the other three males and three females received saline control.

Results

L-DOPA decreases self-administration of ethanol

Animals learn to self-administer 20% ethanol in an operant self-administration task. Once animals interacted with the "active" nose-poke port an audiovisual stimulus would indicate a successful nose-poke lasting for 5 seconds followed by the onset of the magazine light and activation of the solenoid leading to drug delivery (Figure 1A). Prior to this operant task animals received 20 days of 24-hour access to an ethanol bottle in their home cage (Figure S1), and then up to 5 magazine training sessions. Animals had a significant increase in their active nose-pokes (NP) over sessions, whereas their inactive NPs did not change across the 25 pre-treatment operant sessions (Figure1B). After receiving 25 sessions with 20% ethanol access an additional 10 sessions were provided, 5 of which animals were pretreated with an intraperitoneal injection of L-DOPA and Benserazide (30mg/kg and 15mg/kg, respectively). The other 5 session's animals were treated with saline. Treatment order was counterbalanced across animals. Animals treated with L-DOPA decreased the amount of nose-pokes performed to get the delivery of ethanol (Figure 1D). The largest difference between vehicle and L-DOPA treatment days can be observed within the first 10 minutes of the session (Figure 1C). There was no statistical difference in the number of inactive NPs the animals made in treatment vs Vehicle days (Figure 1D). The observed effect persisted across all treatment days (Figure 1E). Interestingly, there was no correlation between how effective L-DOPA was in reduction of active NPs and the animals' ethanol intake in the 5 sessions just prior to the treatment sessions (i.e. sessions 21-25).

L-DOPA decreased fentanyl intake but not water intake in a free operant 2-bottle choice task

Rats underwent a two-bottle choice task in which they had access to fentanyl (50 µg/ml) and DI water for three hours every day. All animals had food and water *ad libitum* during this 3 hour period as well as the other 21 hours of the day. Animals were given 18 3-hr sessions before receiving any treatment. During this time the consumed approximately 0.6572 mg/kg of fentanyl (n=12 rats) (Figure 2A). Because there was no significant sex difference in fentanyl consumption (Figure S2), data from female and male rats were combined for all analyses. During the 18 sessions animals total liquid consumption within the 3 hour period was ~32% fentanyl (Figure 2B). Animals were split into two groups to test the hypothesis that L-DOPA will decrease volunteer consumption of fentanyl. One group received I.P. injections of vehicle (n=6, 3 males & 3 females) 20 minutes prior to the 3-hr behavioral session, the other group was administered with 30mg/kg L-DOPA and 15mg/kg Benserazide (n=5,). Each group received the injections for 4 consecutive sessions. The effect of the treatment was compared to the 4 sessions prior to treatment administration (baseline). The group that received L-DOPA treatment consumed significantly less fentanyl from baseline compared to the saline treated group (Figure 2C). Treatment of L-DOPA had no significant change on water consumption (Figure 2D), suggesting that the effect of L-DOPA is specific to fentanyl consumption.

L-DOPA decreases fentanyl consumption and operant responding in a fentanyl self-administration task

A total of 16 rats evenly balanced between sexes received 15 sessions of an oral fentanyl self-administration task before being administered L-DOPA/Benserazide to test its effects on self-administration behaviors. Once animals interacted with the active NP port the LED lights within the NP port would turn on for 1s to indicate a successful NP. Simultaneously, an audiovisual stimulus (Magazine light and a tone) would turn on for 10s, this was also paired with drug delivery through the opening of a solenoid valve (Figure 3A). Animals learned this task but had no statistical increase of nose-pokes over the course of 15 sessions. However, there

was a decrease in the number of inactive NPs being performed (Figure 3B). These animals received no magazine training or prior experience to a 2-bottle choice paradigm such as those in the Ethanol self-administration task (Figure 1). In sessions 16-25 animals were administered both L-DOPA/Benserazide (30mg/kg:15mg/kg) and saline IP injections for 3-5 sessions, with order being counterbalanced between animals. In sessions that L-DOPA was administered there is a visible change in the slope in the cumulative active NP plot (Figure 3C). There was an interaction between nose-poke type (i.e. active and inactive) and treatment. A Post-hoc analyses reveal there is a significant decrease in active nose-pokes whereas no significant change is detectable in the inactive NP port (Figure 3D). Looking at all animals 3 treatment days (half the animals received 5 treatment days but data is not shown) there is a main effect of treatment (L-DOPA vs saline) and post-hoc analyses reveal each day is significantly different from their matched vehicle treated session (Figure 3E). Previous work from the Phillips lab (Willuhn et al., 2014) demonstrated that only animals that were considered escalated cocaine consumers had a decrease of drug-intake from L-DOPA. Thus, I tested if animals' baseline intake, defined as sessions 10-15, had any correlation with how effective L-DOPA was at decreasing fentanyl consumption. There was a significant regression observed, in which animals with lower intake of fentanyl during sessions 10-15 had less reduction in intake during L-DOPA treated days (Figure 3F). Animals within this task did not drink all of the liquid in the bowl from every session, thus at the end of each hour session any remaining liquid was accounted for and subtracted from the amount delivered. Performing this allowed us to calculate the amount of fentanyl consumed. In Figure S3, this data is presented and shows that not only did animals treated with L-DOPA respond less for fentanyl delivery but they also drank less fentanyl as well.

Animals perform an operant task for water and L-DOPA decrease this operant responding

Seeing that systemic L-DOPA administration was therapeutically effective in decreasing fentanyl and alcohol consumption, we tested if this effect was specific to drugs of abuse by

having animals self-administer water. 4 male and 4 female rats underwent a similar paradigm as that of fentanyl operant self-administration where instead water was delivered at 0.4 mL/kg per active nose poke. Animals will readily nose-poke preferentially at the active port (Figure 4B) for delivery of water. Treating animals with L-DOPA had a similar effect on water self-administration as shown it did in fentanyl self-administration (Figures 4C, D, & E). Each animal received both vehicle and L-DOPA/Benserazide IP injections for 5 consecutive sessions. There is a clear separation of cumulative active nose pokes between saline and L-DOPA treatment as shown in Figure 7C. In other words, the rate of nose pokes within a session decreased after L-DOPA administration. L-DOPA also significantly decreased both average active nose pokes, but had no significant effect on inactive nose-pokes (Figure 4D). There was no correlation between animal's baseline operant self-administration of water and the effect L-DOPA had on water consumption. It is surprising at the amount of water these animals drink within a 1 hour operant task, especially given these animals had food and water *ad libitum.*

Discussion

Our results indicate that L-DOPA administration, given with a peripheral acting DOPA decarboxylase inhibitor, decreases drug consumption of both alcohol and fentanyl. The underlying model of these experiments hypothesize that diminished cue-evoked dopamine release within the NAc, promotes drug consumption, which has been previously shown during psychostimulant self-administration (Willuhn et al., 2014). Given that this treatment has reduced drug-consumption in all classes of drugs we tested, we reason that L-DOPA could be used as a therapy for substance use disorders in conjunction with behavioral and/or cognitive therapies.

We established a behavioral paradigm that achieves reliable self-administration of 20% ethanol in male Wistar Rats. Within this paradigm rodents intake is comparable to that of "drinking in the dark" paradigm (Holgate et al., 2017). Out of 28 rats only 5 animals did not reach a criteria of 0.6 g/kg of ethanol consumed during the 1-hour self-administration sessions.

Although our results indicate a similar neurochemical change of dopamine transmission's role in promoting ethanol consumption we are unaware if the NAc core is the site of action mediating the decrease of drug-consumption. Future studies will likely use a neurochemical detection technique such as fast-scan cyclic voltammetry or fiber photometry, to detect changes in cue evoked dopamine transmission during ethanol self-administration. Interestingly, in our ethanol self-administration paradigm we do observe a "load-up" phase (Figure 1C), similar to that observed in psychostimulant self-administration paradigms (Ahmed & Koob, 2005).

L-DOPA was effective at decreasing fentanyl consumption in two self-administration paradigms. We provided male and female Wistar Rats 3-hour fentanyl access in a two-bottle choice paradigm. We found that animals drank approximately 3 times more fentanyl in the two-bottle choice paradigm than the operant task, which suggests that animals are taking an equal amount of drug per hour, as the two-bottle choice task was also 3 times longer. During the fentanyl self-administration operant task animals showed a "load-up" phase of active nose-pokes. This behavior could be due to the animals reaching a hedonic set-point and the remainder of their drug-taking is to maintain a drug or dopamine concentration (Wise, Newton, et al., 1995; Wise, Leone, et al., 1995; Ahmed & Koob, 2005) . Within the two-bottle choice task animals did not show a preference for fentanyl, as only ~30% of their total liquid intake was from the fentanyl bottle. This outcome could potentially be interpreted that the animals find fentanyl as a punisher; however their intake after a few days of experience remained stable, and we did not alter which side of the cage the fentanyl bottle resided. Additionally, given the clear visual load-up during the operant fentanyl task, it is possible that animals in the two-bottle choice are modulating their high, and maintain a relatively stable hedonic set-point.

An inactive nose-poke port was utilized as a control operandum in the operant self-administration tasks, and a water bottle within the two-bottle choice task. In all operant tasks L-DOPA treatment did not statistically alter inactive nose-poke responding. Within the two-bottle choice task we show that animals treated with L-DOPA did not alter how much water they drank

during the 3-hour test period, indicating that L-DOPA reduction is drug specific. However, we ran a control operant experiment in which we replaced drug with water, allowing animals to nose-poke for the delivery of ~0.2 mLs of water. Interestingly, when provided L-DOPA animals decrease their responding and consumption for water, which contradicts the results we saw within the two-bottle choice experiment. Future experiments will need to be completed in order to better understand these contrary findings. It is likely that the cue paradigm being utilized in the operant task is contributing to our findings with a decrease in water consumption from L-DOPA. One follow-up experiment will be to include an operant task in which the animals can respond for the cues with no reinforcement. Another follow-up is to replace the inactive nose-poke port with an active water nose-poke, so activation of port 1 would elicit CS-1 and drug delivery, and responses on the 2nd port would elicit presentation of CS-2 and water delivery. Although this result is on the surface, worrisome because a treatment for substance use disorder should not decrease necessary water intake for survival, we only observe a decrease in water intake in an isolated and separate environment from where their normal water and food intake occur. Within their home-cage where the two-bottle choice experiments took place we did not see any effect of L-DOPA on water intake.

One interesting aspect observed by Willuhn and colleagues (Willuhn et al., 2014) is administration of L-DOPA to animals in cocaine self-administration only decreased drug consumption in animals who had escalated their intake over sessions, whereas in animals that did not escalate their intake L-DOPA had no effect. Thus, they saw a bimodal distribution of animals that were affected by treatment and those that were not. They classified these animals based on their drug-taking patterns and provided them with prolonged drug access and (Willuhn et al., 2014), which produces escalated intake over. Here to test if we see any effects on animal’s intake levels and how effective L-DOPA was at decreasing drug or water consumption we used the average intake of each individual animal the week before treatment occurred and compared it to the percent decrease in consumption from vehicle to L-DOPA treated sessions.

Surprisingly, only animals in the operant fentanyl task showed a significant relationship between baseline intake and effectiveness of L-DOPA. This could be explained by the amount of drug exposure animals had prior to L-DOPA testing. For instance, in fentanyl studies animals had only experienced fentanyl for 15 days prior to testing days, where as in alcohol studies they were introduced to ethanol for 20 days in a two-bottle choice paradigm and then another 25 operant sessions. Given the different histories of experience it is likely that the average intake of the 5 sessions before testing is not an accurate baseline. Further analyses on the drug-taking patterns of individual animals should be completed and may reveal parameters that predict which animal's drug-taking is effected by L-DOPA treatment.

In conclusion these experiments provide foundational preclinical evidence that L-DOPA treatment has potential to be used as a harm-reduction therapy for substance use disorders. Although not tested within the current experiments L-DOPA has already been through some clinical trials, where it was paired with contingency management therapy in cocaine abusers, and it was found to prolong abstinence beyond placebo (Mariani & Levin, 2012). Although here we show that L-DOPA decreases intake, rather than prevents intake, L-DOPA may have multiple uses in treating persons living with substance use disorders.

Figures

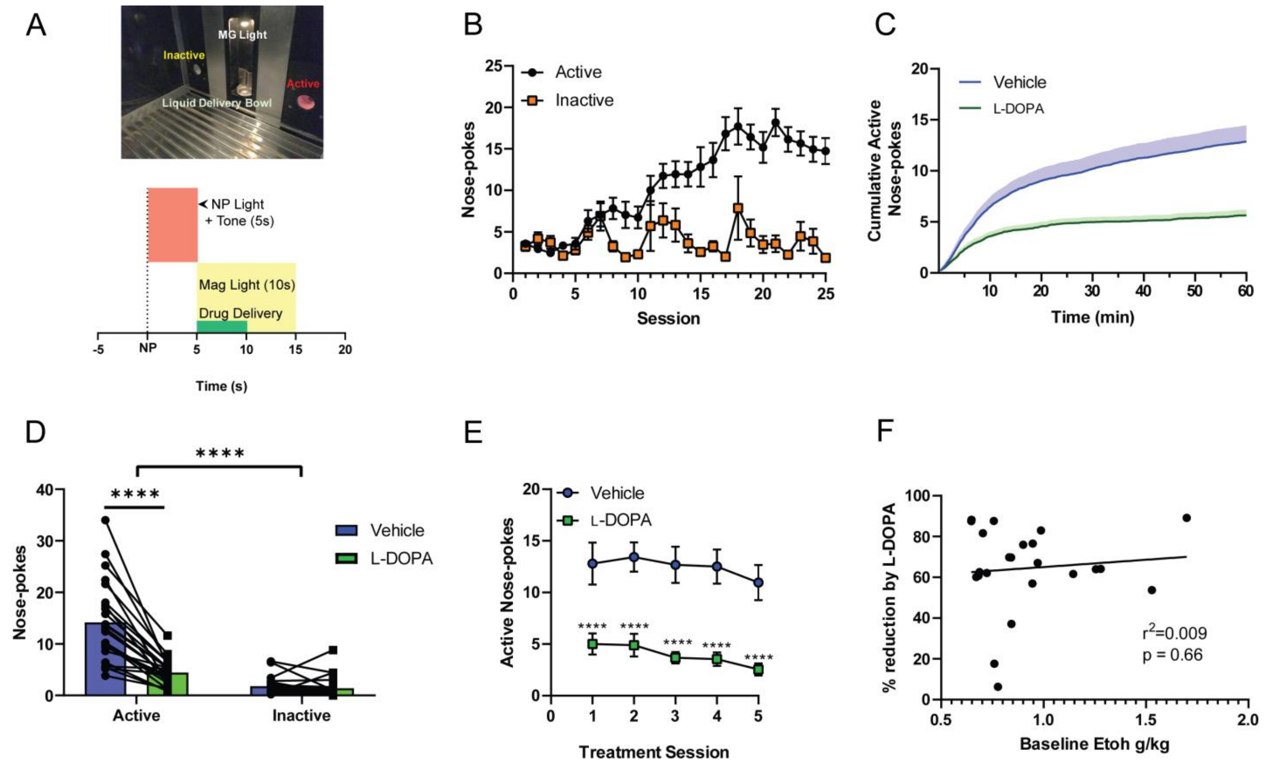

Figure 1. l-DOPA decreases ethanol consumption. A) Image of the operant chamber (top panel) and a schematic of the audiovisual stimulus paradigm when an animal performs an active nose-poke. **B)** Animals show a clear discrimination between active and inactive nose-pokes during the 25 sessions provided before any treatment tests. **C)** Animals were treated with L-DOPA or vehicle, cumulative nose-pokes were calculated and plotted. There is a clear visual difference in active nose-pokes across the session. **D)** The sum of animals active and inactive nose-pokes during treatment sessions were averaged. A two-way ANOVA revealed an interaction of nose-pokes between nose-poke type by treatment ($F(1,22)=44.07$, $p<0.0001$). A Sidak post-hoc test, revealed a significant difference in vehicle and L-DOPA active nose-pokes ($p<0.0001$). **E)** Animals were treated with L-DOPA and vehicle for 5 serial sessions, we analyzed how repeated treatment effects active nose-pokes. A two-way ANOVA found a main effect of treatment ($F(1,22)=48.51$, $p<0.0001$). A Sidak post-hoc analysis revealed a significant difference for each treatment day compared to the matched vehicle session ($p<0.0001$). **F)** A regression of baseline ethanol, calculated from sessions 20-25, and the % of reduction completed by L-DOPA was

completed. We found that baseline ethanol intake did not predict the amount of reduction in drug intake from L-DOPA.

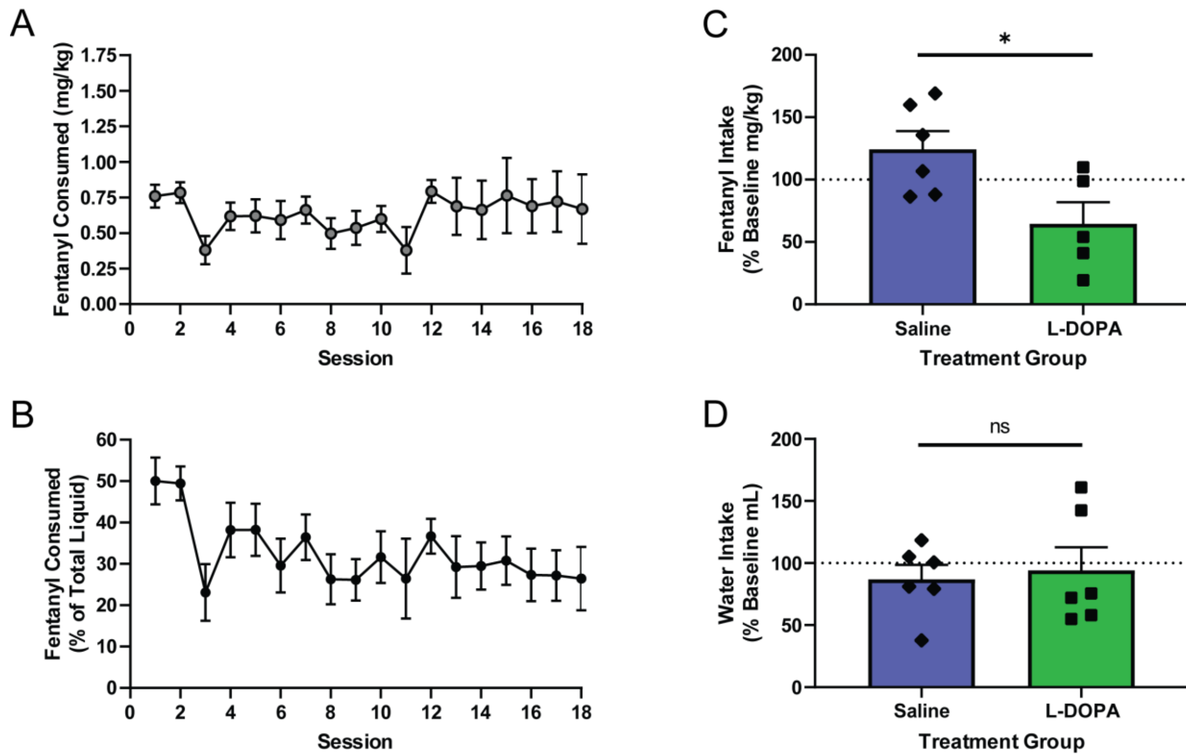

Figure 2. l-DOPA decreases fentanyl but not water consumption in two-bottle choice. A) Animals were provided with 18 3-hour sessions to access a fentanyl bottle within their home cage and drank about 0.63 mg/kg of fentanyl. **B)** Within these 3-hour sessions approximately 32% of their total volume intake was from the fentanyl bottle. **C)** Animals were treated with either L-DOPA or saline. Animals treated with L-DOPA had a significant difference in their change of intake from baseline (sessions 15-18) compared to saline treated animals ($t(9)=2.658$, $p<0.05$). **D)** The change in amount of water consumed from baseline sessions (15-18), was no different between saline and L-DOPA treated animals ($t(9)=0.6189$, $p>0.05$)

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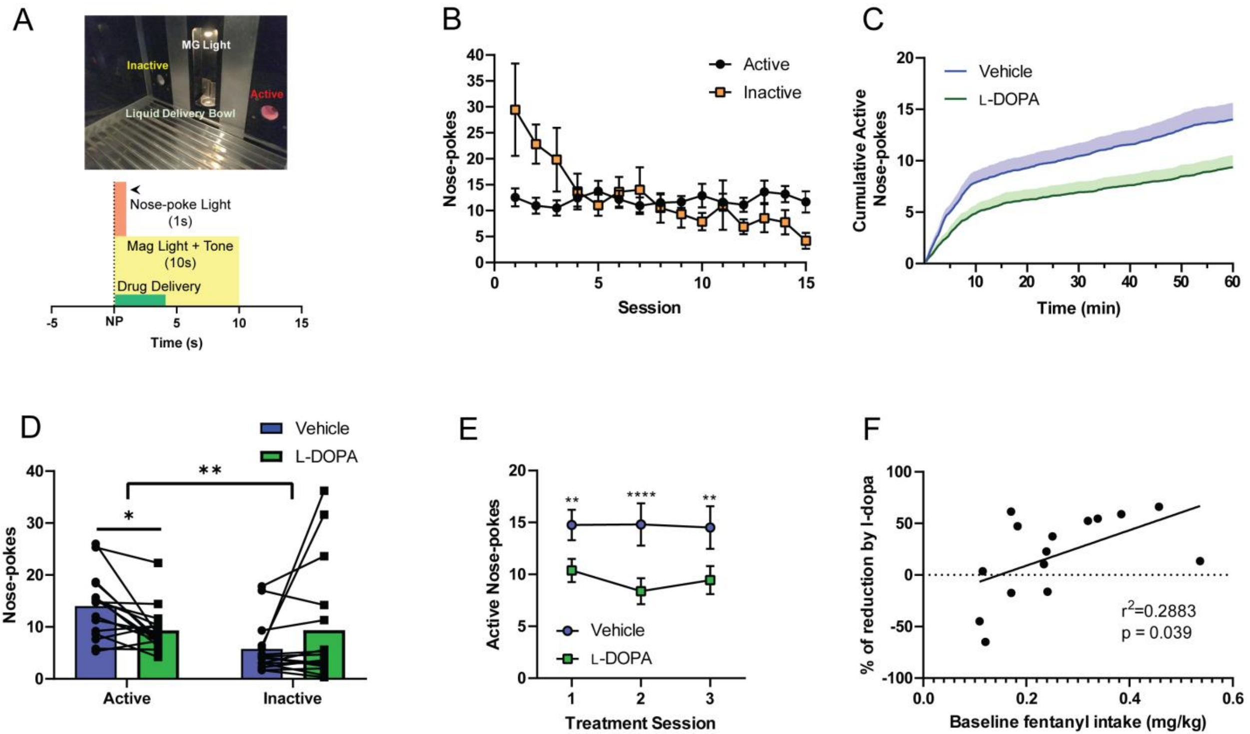

Figure 3. l-DOPA decreases fentanyl responding in an operant self-administration task. A) Schematic providing the cue configuration used for oral fentanyl self-administration. **B)** Animals were provided with 15 1-hr sessions with access to fentanyl. A significant interaction was observed between time and nose-poke port (F14,210)=4.341, $p<0.0001$). **C)** Animal's cumulative active nose-pokes were graphed and a clear separation of active nose-pokes between Vehicle treated and L-DOPA treated sessions can be observed. **D)** A comparison of the effect L-DOPA had on active and inactive nose-pokes was performed. A two-way ANOVA revealed a significant interaction between nose-poke type and treatment ($F(1,15)=13.32$, $p<0.01$). A Sidak post-hoc test revealed a significant decrease in active nose-pokes during L-DOPA treatment compared to vehicle treatment ($p<0.05$). **E)** Animal's received 3-5 days of each treatment (vehicle or L-DOPA). A comparison of individual treatment days was performed. A two-way ANOVA was calculated and found a main-effect of Treatment ($F(1,15)=8.877$, $p<0.01$). A Sidak post-hoc analysis was performed to see if L-DOPA treated days were significantly lower than the matched vehicle days. **F)** A linear regression was calculated on baseline fentanyl intake, which was defined as sessions 10-15, and the percent of reduction achieved by L-DOPA. A significant regression was found

and highlighted that high baseline fentanyl intake predicted a larger percent reduction in drug-consumption by L-DOPA (r^2- 0.2883, $p<0.05$). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

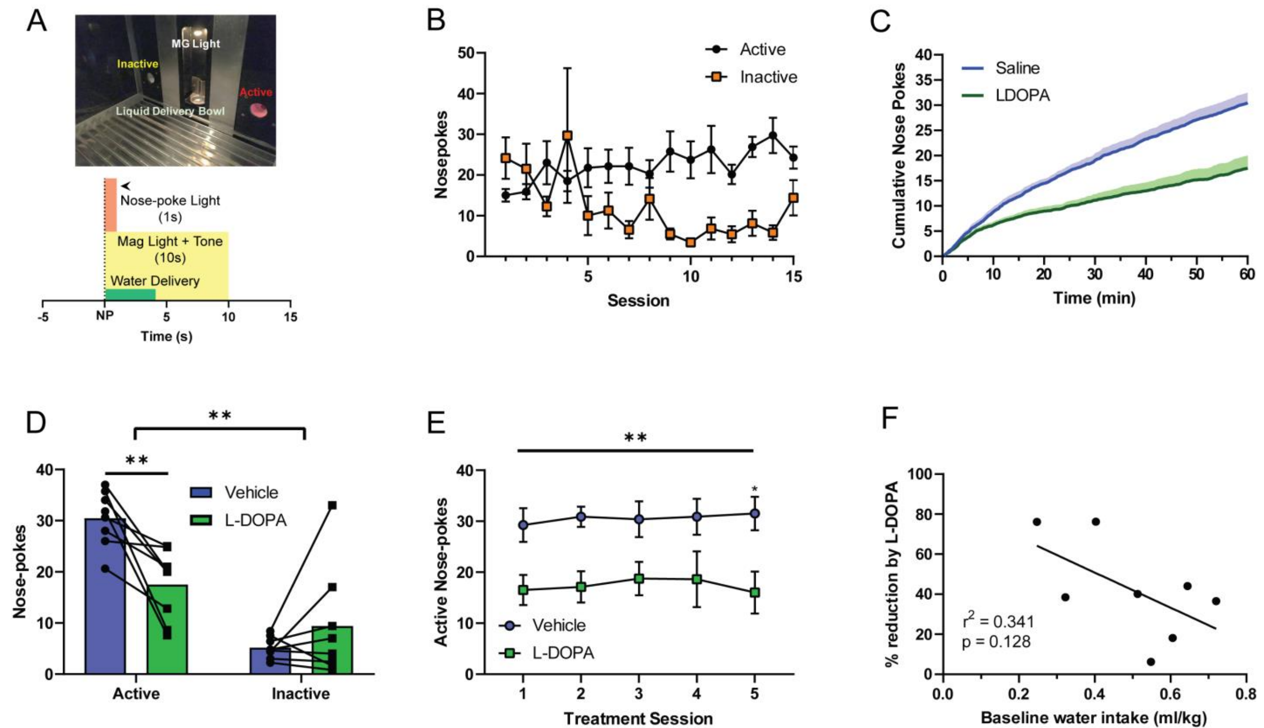

Figure 4. l-DOPA decrease water consumption in an operant task. A) Schematic with the CS configuration used during operant water self-administration task. **B)** Animals were given 15 1-hour sessions to self-administer water. An interaction of nose-pokes was observed between sessions and nose-poke type ($F(14,180)=4.891$, $p<0.0001$). **C)** Cumulative active nose-pokes were calculated for saline treated and L-DOPA treated sessions. **D)** Total nose-pokes within the 1 hour were used to analyze how L-DOPA affected nose-poking behavior. A two-way ANOVA was performed and a interaction of nose-poke type by treatment was observed ($F(1,7)=14.76$, $p<0.01$). A Sidak post-hoc test was completed and revealed a significant difference in active nose-pokes between the two treatment ($p<0.01$). **E)** The 10 sessions of treatment were separated into matching sessions. A two-way ANOVA revealed a main effect of drug treatment ($F(1,7)=16.05$), $p<0.01$). **F)** A linear regression was performed between baseline water intake (sessions 10-15), and % reduction in responding achieved from L-DOPA. Baseline water intake does not have a significant relationship with the reduction of responding by L-DOPA.

Supplemental Figures

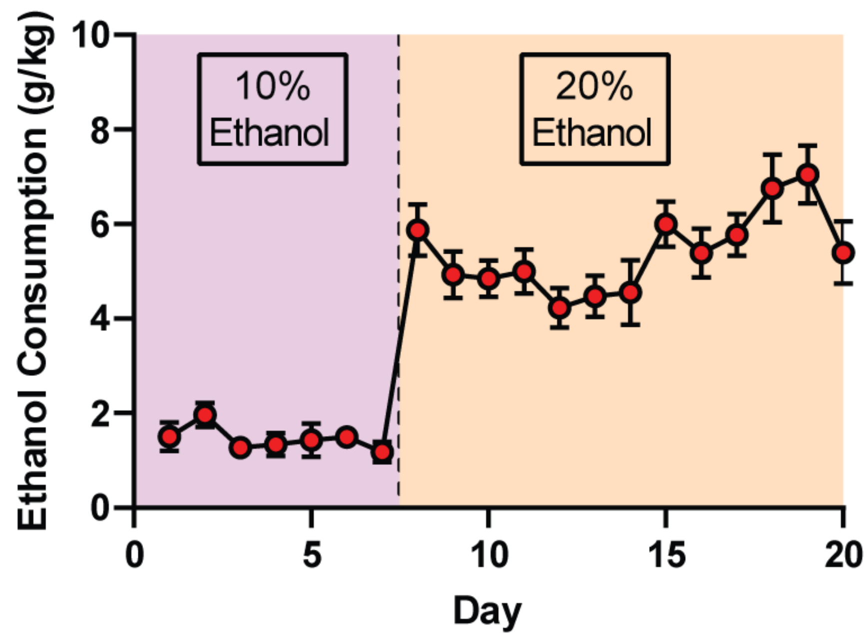

Figure S1. Ethanol consumption during 24-hour two-bottle choice prior to operant training. During the first 7 days animals were provided access to 10% ethanol (pink box). From day 8 to day 20 the concentration of ethanol was increased to 20%.

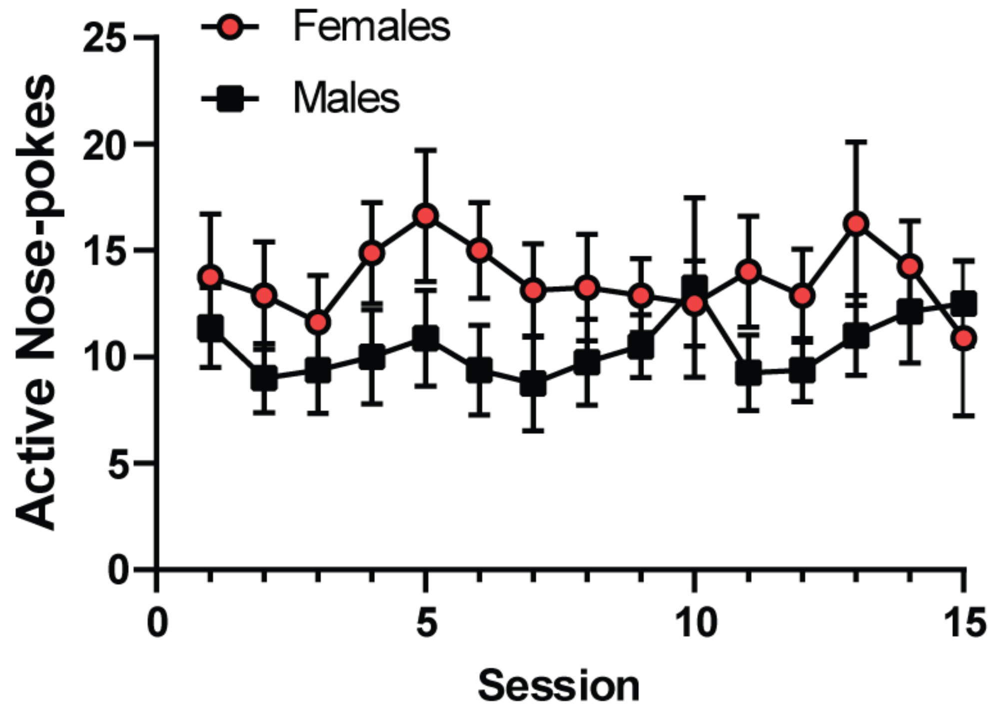

Figure S24. Males and females did not differ in their fentanyl intake during the first 15 sessions provided. A two-way ANOVA was completed and found no significant differences in active nose-pokes between sex ($F(1,14)=1.573$, $p>0.05$).

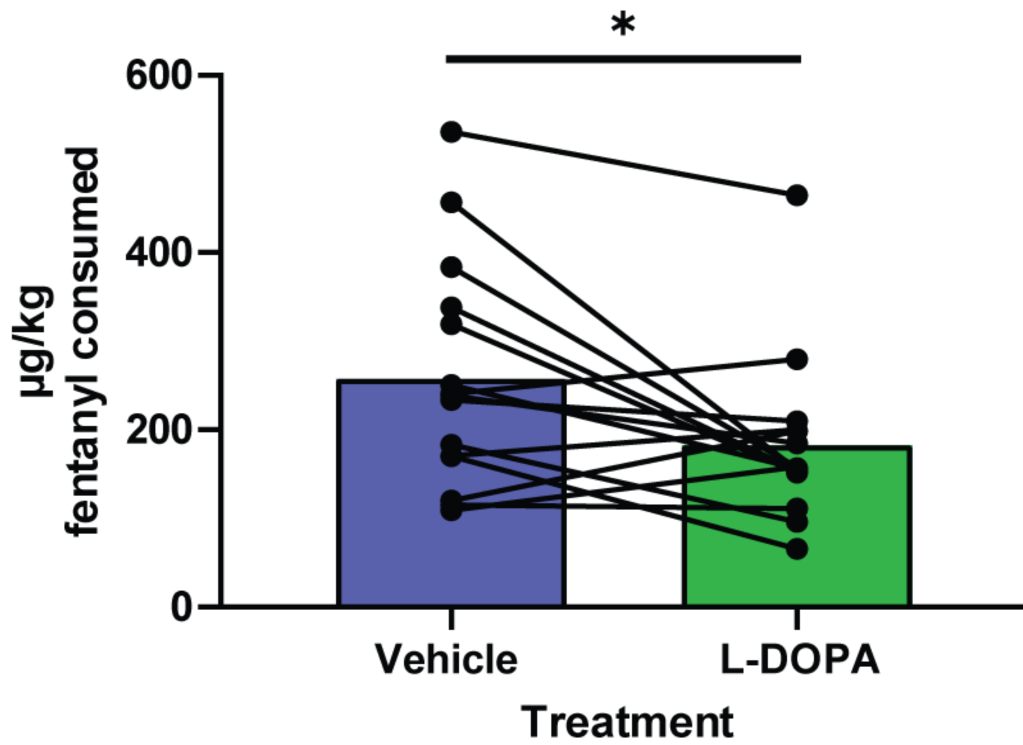

Figure S3. l-DOPA decreased total amount of fentanyl consumed. The amount of fentanyl consumed was calculated by subtracting the amount of liquid left over after the session. A significant decrease of fentanyl consumed was observed (paired t-test, $t(14)=2.655$, $p<0.05$).

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Chapter 5: The 5α-reductase inhibitor finasteride reduces opioid self-administration

Bosse, G. D., Cadeddu, R., Floris, G., **Farero, R. D.**, Vigato, E., Lee, S. J., Zhang, T., Gaikwad, N. W., Keefe, K. A., Phillips, P. E. M., Bortolato, M., & Peterson, R. T. (2021). The 5α-reductase inhibitor finasteride reduces opioid self-administration in animal models of opioid use disorder. *Journal of Clinical Investigation*, *131*(10). https://doi.org/10.1172/JCI143990

The entirety of this chapter has been published in the Journal of Clinical Investigation. I contributed to the overall conceptual framework, designed experiments, completed experiments, and analyzed data. I provided Figures 4,S8, S9, and S10, which were focused on testing the effect finasteride has on oral fentanyl self-administration in a rat model that included both males and females.

Abstract

Opioid use disorder (OUD) has become a leading cause of death in the US, yet current therapeutic strategies remain highly inadequate. To identify novel potential treatments for OUD, we screened a targeted selection of over 100 drugs using a recently developed opioid self-administration assay in zebrafish. This paradigm showed that finasteride, a steroidogenesis inhibitor approved for the treatment of benign prostatic hyperplasia and androgenetic alopecia, reduced self-administration of multiple opioids without affecting locomotion or feeding behavior. These findings were confirmed in rats; furthermore, finasteride reduced the physical signs associated with opioid withdrawal. In rat models of neuropathic pain, finasteride did not alter the anti-nociceptive effect of opioids and reduced withdrawal-induced hyperalgesia. Steroidomic analyses of the brains of fish treated with finasteride revealed a significant increase in dehydroepiandrosterone sulfate (DHEAS). Treatment with precursors of DHEAS reduced opioid self-administration in zebrafish in a fashion akin to the effects of finasteride. Our results highlight

the importance of steroidogenic pathways as a rich source of therapeutic targets for OUD and point to the potential of finasteride as a new option for this disorder.

Introduction

Over the last decade, the widespread abuse of prescription painkillers, such as oxycodone and hydrocodone, has led to a crisis of opioid use disorder (OUD) and a dramatic increase in opioid overdose in North America [1]. The extent of the crisis is such that opioids account for more than 60% of all drug overdoses in the United States, with an estimated 47 000 to 50 000 fatalities annually. Synthetic opioids, such as fentanyl, are the major drivers of opioid overdose[1,2]. Unfortunately, current therapeutic options for OUD are highly unsatisfactory. Existing treatments rely on replacement with long-acting opioids, such as methadone or buprenorphine[3]. While these options help patients cope with drug craving and manage withdrawal symptoms[3], they are not ideal due to their intrinsic liability for abuse and dependence[4]. This background highlights the urgent need for novel, therapeutic options to reduce the risk and severity of OUD, particularly in patients requiring opioid treatment for chronic and neuropathic pain syndromes (for which effective alternatives to these painkillers are not always available).

Rodent models have been successfully used to model substance use disorders and study the circuitry and neurobiological changes involved in drug abuse[5,6]. However, the viability of these models to screen for novel therapeutic candidates is limited by their low throughput and high costs. In recent years, the zebrafish (*Danio rerio*) has emerged as a new alternative to study a wide range of complex behavioral and neuropsychiatric disorders, such as schizophrenia and depression[7–9]. Importantly, zebrafish have been shown to develop conditioned place preference and withdrawal symptoms after exposure to opioids and other drugs of abuse, such as cocaine and alcohol[10–14]. Additionally, adult zebrafish possess a complex central nervous system, including a blood-brain barrier, and considerable similarities with the mammalian homolog. Furthermore, this model is well suited for the rapid testing of candidate drugs, as compounds of

interest can be dissolved directly into the water of the tank [15–17]. Therefore, the zebrafish affords a unique opportunity to combine drug-discovery screening and substance abuse research.

Using a recently described paradigm to condition adult fish to self-administer the opioid hydrocodone[18], we designed a behavior-based screen to identify novel compounds affecting opioid self-administration in zebrafish. We screened 110 unique molecules selected for their annotated activity against processes and pathways known to be involved in substance abuse disorders. From this screen, we identified the 5α-reductase (5αR) inhibitor finasteride[19,20] as one of the most effective compounds to reduce opioid self-administration. 5αR catalyzes the rate-limiting step of the conversion of several ketosteroids, including progesterone and testosterone, into their neuroactive metabolites dihydroprogesterone and dihydrotestosterone (DHT). In turn, these steroids are converted into the neurosteroids allopregnanolone and 3α-androstanediol (3α-diol). (Figure 5A)[21–24]. Finasteride has been approved for over 25 years as a clinically approved treatment for benign prostatic hyperplasia and male-pattern baldness[25]. These effects reflect the suppression of DHT synthesis.

Our results showed that the same finasteride doses that reduced opioid self-administration did not affect food-seeking behavior or overall locomotion. The effects on self-administration were confirmed in rats; furthermore, we found that finasteride opposes the physical effects and hyperalgesia associated with opioid withdrawal but did not reduce the pain-killing properties of opioids in rat models of neuropathic pain. Finally, we identified the neurosteroid dehydroepiandrosterone sulfate (DHEAS) as a likely mediator of finasteride's effects. Using zebrafish, we thus uncovered a role for neuroactive steroids in the control of opioid self-administration that is conserved in mammals.

Results

Validation of the screening method

To screen for novel modulators of opioid self-administration, we utilized our newly developed assay to condition fish with the opioid hydrocodone. As previously described, small groups of adult zebrafish are conditioned to swim across an active platform to receive a dose of the drug[18]. Each visit results in the delivery of hydrocodone directly at the platform. We used 15 animals per group of fish, so each n represents a unique group of 15 fish. Potential modulators of opioid self-administration are tested by training fish to self-administer hydrocodone for four days and treating with the compound of interest on the fifth day (Figure 1A). Before performing the small-molecule screen, we sought to validate the screening method by testing one of the only treatments used in the clinic, the slow-acting opioid methadone. Fish conditioned for four days to self-administer hydrocodone were treated with methadone (1 mg/L) for 60 min and then transferred to the self-administration arena. During each 30-min self-administration session, the number of triggering events for both the active and inactive platform was recorded and used as a readout of opioid intake. Methadone significantly reduced hydrocodone self-administration (Figure 1B), suggesting that the screening assay can identify molecules that reduce opioid self-administration.

Small molecule screening identifies modulators of opioid self-administration

We conducted a small-scale screen using a targeted collection of compounds selected based on hypotheses presented in the literature regarding neuronal pathways contributing to addiction, their ability to modulate pathways identified in GWAS studies on substance abuse patients, and molecular pathways affected during substance abuse. We hypothesized that focusing on this targeted collection would increase the probability of finding effective drugs within a smaller library of compounds. To test if the compounds reduce opioid self-administration, drug-conditioned animals were treated with 10 µM of each candidate compound 60 min before the 30-min self-administration session (Figure 1A). On average, control animals triggered the

release of drug more than 1800 times per session; to reduce the number of false-positive hits, we tested each compound in duplicate. Furthermore, only compounds with less than 1000 triggering events for both duplicates were considered hits.

Finasteride reduces opioid self-administration

After screening over 100 compounds, we identified the 5αR inhibitor finasteride as highly effective in reducing opioid self-administration. Incubation with a single dose of 10 μM of finasteride for 60 min was sufficient to reduce the number of triggering events at the active platform by 73% (Figure 1B). The complete list of tested compounds and their effects on opioid self-administration are presented in Figure S1 and Table S1).

To further validate that the inhibition of 5αR was responsible for the reduction in opioid self-administration, we tested a different 5αR inhibitor, dutasteride[26]. Similar to finasteride, dutasteride reduced the number of triggering events at the active platform (Figure S2). Although research on neuroactive steroids in zebrafish has been limited, the key steroidogenic enzymes are expressed in the adult brain, and the activity of 5αR has been detected in brain extracts[27–33]. Additionally, using a publicly available single-cell RNA seq library, we were able to detect 5αR transcripts in several different cell types in zebrafish brains[34] (Figure S3). Taken together, these results suggest that the inhibition of the enzyme 5αR reduces opioid self-administration in zebrafish.

To further characterize the efficacy of finasteride, we performed a dose-response experiment. We used 10-fold dilutions to test finasteride concentrations from 10 μM to 1 nM. A significant difference was detected with concentrations as low as 5 nM (Figure 1C). This dose-response experiment supports the idea that finasteride is highly potent and has a large therapeutic window in zebrafish.

Finasteride does not affect locomotion or food self-administration

To determine if the effect of finasteride was caused by sedation, we monitored the swimming speed of finasteride-treated animals. Finasteride did not reduce locomotion in comparison with DMSO (Figure S4). Importantly, we also did not observe any significant difference in the number of triggering events at the inactive platform between the finasteride-treated fish and control animals (Figure 1). These results suggest that finasteride specifically reduces the number of triggering events at the active platform without affecting overall locomotion.

Drugs of abuse are known to activate the reward pathway in the brain, which is a core contributor to motivated behaviors, such as feeding or reproduction[35]. We thus decided to test if finasteride was also affecting other motivated behaviors by measuring food self-administration in zebrafish. We first trained fish as previously reported for opioids but used food instead of opioid as the reward. Food-conditioned animals were then treated with DMSO or finasteride for 60 min, and the number of triggering events at the active platform was measured. As opposed to hydrocodone self-administration, finasteride-treated animals exhibited no decrease in food-seeking (Figure 2A). These data suggest that finasteride does not affect all motivated behaviors and further validate that this drug does not impair locomotion.

Finasteride reduces self-administration of different opioids

It has been described that each class of opioids has a different abuse potential[36,37]. Therefore, we decided to test the effect of finasteride on animals conditioned with the traditional opioid most commonly used in animal models, morphine, and one of the most potent and deadly opioids, the synthetic opioid fentanyl.

In order to test the effect of finasteride, we used the same conditioning protocol to train animals to self-administer morphine and fentanyl. As morphine and hydrocodone are closely related, we used the same dose of 6 mg/L. As for fentanyl, studies suggest that it is up to 50-100x more potent than morphine[38], so a dose 50X lower was used, 0.12 mg/L to take into account its greater potency. We first confirmed that animals conditioned with either opioid had similar self-

administration levels after 5 days of conditioning. There was no significant difference in the number of triggering events between fish trained with different opioids (Figure 2B). Therefore, this conditioning protocol is easily applicable to multiple classes of opioids. The conditioned animals were then treated with 10 μM finasteride for 60 min before performing a 30-min self-administration session. As observed with hydrocodone-trained animals, there was a reduction in the number of visits to the active platform without affecting the inactive platform (Figure 2B). This result suggests that finasteride reduces opioid self-administration behavior regardless of the opioid used during the conditioning phase.

Finasteride reduces opioid self-administration in rats.

The zebrafish model suggests that finasteride strongly reduces opioid consumption. To confirm this effect in mammals, we tested finasteride in a rat model of hydrocodone self-administration. Adult male Sprague-Dawley rats were first conditioned to press a lever to receive an intravenous infusion of hydrocodone (0.016-0.128 mg/kg/160μL). Operant conditioning consisted of three stages of fixed-ratio (FR) reinforcement schedules: FR1, FR2, and FR5 (i.e., FR1=each lever press resulted in a hydrocodone infusion). Animals progressed to the next stage of conditioning after reaching the criterion of >70% lever presses being on the lever on the previous schedule (Figure 3Aand S5). Importantly, to mimic the zebrafish conditions, rats had access to the drug for 60 min per day. To find the optimal concentration for hydrocodone conditioning, we tested a range of doses from 0.016 mg/kg to 0.128 mg/kg. We found that animals conditioned with 0.032 mg/kg and 0.064 mg/kg had the highest number of lever presses after 31 days of training (Figure 3B). We then treated animals conditioned with each concentration of hydrocodone by intraperitoneal injection of finasteride (50 mg/kg, IP) or vehicle before a self-administration session. That dose of finasteride was sufficient to significantly reduced the number of active lever presses at both 0.032 and 0.064 mg/kg/infusion of hydrocodone (Figure 3B).

To determine if finasteride was effective at different doses, we treated animals conditioned with 0.064 mg/kg of hydrocodone with 25 mg/kg or 50 mg/kg of finasteride. We confirmed that injection of 50 mg/kg significantly reduced the number of active lever presses, but there was no difference in animals treated with the lower dose (Figure 3C). Importantly, locomotion or inactive lever presses were not affected by the treatment with finasteride (Figure S6 and *S7).*

To further validate that finasteride can reduce opioid intake in mammals, we also tested the effect of finasteride on the potent synthetic opioid fentanyl. Adult male and adult female Wistar rats were used to perform the fentanyl self-administration assay*. Rats* were trained to perform a nose-poke to trigger the release of a drop of fentanyl drinking solution (0.02 mg/kg/delivery*) in a liquid magazine tray.* We trained both male and female animals over 15 sessions of 60 min. As the training progressed, we observed an increase in active nose-pokes per session, and animals rapidly learned to discriminate between the active and inactive nose-poke holes (Figure 4A). Following each self-administration session, the mg/kg of fentanyl consumed was calculated for each animal by subtracting the amount of fentanyl left in the magazine tray from the total amount of drug delivered. As observed for the number of active nose-pokes, we also measured an increase in ingested fentanyl over time (Figure S8A).

After the conditioning phase, animals were separated into two groups, and the effect of finasteride was tested over 10 sessions. Each group was treated with either *an* intraperitoneal injection of finasteride (50 mg/kg) or vehicle for five out of the ten sessions. Treatments were then inversed for the remaining five sessions so that both groups received five sessions of finasteride and five of vehicle. As observed for hydrocodone self-administration, acute treatment with finasteride significantly reduced the number of active nose-pokes (Figure 4B-C), as well as the total amount of fentanyl consumed per session (Figure S8B-E), without affecting the number of inactive nose pokes (Figure S9). This acute treatment with finasteride was effective during the five days of treatment, regardless of the order of treatment (Figure 4D-E).

Importantly, finasteride reduced opioid consumption in both male and female animals (Figure S10).

We also quantified the number of active nose-pokes over time during each session. In vehicle-treated animals, there was a rapid succession of active nose-pokes in the first 10 min before slowing down over the next 50 min. Finasteride treated animals followed a similar pattern, but they only acquired approximately half of the nose-pokes before slowing their rate of intake (Figure 4F).

These results collectively demonstrate that the activity of finasteride on opioid self-administration is conserved in mammals, even for animals conditioned with the synthetic opioid fentanyl.

Finasteride reduces the physical effects of opioid withdrawal.

Given that previous studies have shown that neurosteroids play a role in shaping opioid withdrawal[39], we tested whether finasteride may also modify the effects of opioid withdrawal, using the model of naloxone-precipitated withdrawal syndrome in adult Long Evans male rats. Finasteride significantly reduced the number of wet-dog shakes and the duration of grooming behavior induced by naloxone (Figure 5); however, it did not fully reverse the jumping and digging responses induced by the opioid antagonist (Figure S11). These results indicate that finasteride may reduce the severity of opioid withdrawal.

Finasteride does not affect the antinociceptive properties of opioids

Despite their high abuse potential, opioids are still invaluable as analgesics, and in the absence of an effective alternative, are still an essential treatment, especially for people suffering from chronic pain. Therefore, an ideal candidate for an opioid abuse treatment would be a drug that doesn't affect the pain-killing properties of opioids. A previous report demonstrated that finasteride did not reduce the efficiency of morphine; however, the authors used a lower dose of finasteride (25mg/kg) and tested a single nociceptive stimulus[40]. To further validate that

finasteride doesn't interfere with the antinociceptive effects of opioids in our conditions, we used the most rigorous test available in rats by testing the effect of opioids on a rat model of neuropathy.

We performed spinal nerve ligation (SNL) surgery on adult male Sprague-Dawley rats [41], and fourteen days after surgery, animals were separated into different groups. Mechanical allodynia and nociception were measured utilizing the von Frey Hair and Randall-Selitto tests, respectively[42,43]. We also measured thermal nociception (hot plate)[44]. We first validated the antinociceptive effects of opioids by testing different doses of morphine (1-3mg/kg, SC) or hydrocodone (1-10mg/kg, SC) every 30 min for 3 hours. For the Randall-Selitto test, an increase in mechanical force is applied to the paw until a withdrawal response is observed (Figure 6). Treatment with either morphine or hydrocodone significantly increased the force needed to trigger withdrawal compared with the same animals prior to treatment with the opioid (Figure 6A and Figure S12B). We then selected the most efficient dose for both opioids, morphine (3 mg/kg), and hydrocodone (10 mg/kg) and tested the effect of co-injection with finasteride (50 mg/kg, IP). The antinociceptive effect of neither opioid was reduced by the co-treatment with finasteride (Figure 6B and Figure S12C). We also performed the same set of experiments to test a different mechanical stimulus, i.e., the Von Frey test. As with the Randall-Selitto test, we did not detect any reduction in the antinociceptive effects of the opioids by treatment of the rats with finasteride (Figure S12).

In order to test a different type of nociception, we used the hot plate assay to test for a thermal stimulus on a different group of neuropathic rats. Animals were placed on a hot plate analgesia meter and the latency to lick their left hind paw was measured at different temperatures (48.5°C and 51.1°C). We selected the most effective dose of opioid, based on the Randall-Selitto test, and performed the assay 30 and 60 min after opioid injection. The latency for the first lick or paw retraction of animals treated with morphine (3 mg/kg) was significantly increased at both temperatures after 30 min (Figure 6C) and 60 min (Figure S14) when compared to untreated

animals. Interestingly, treatment with hydrocodone (10 mg/kg) did not reduce the latency at 51.5°C and was not very effective when given 60 min before the test. As with mechanical nociception, co-injection with finasteride (50 mg/kg, IP) did not reduce the antinociceptive efficiency of either opioid at any temperatures or pretreatment times (Figure 6C and Figure S14).

Finally, to test the effects of finasteride on the hyperalgesic effects of opioid withdrawal, we tested the effects of finasteride (50 mg/kg, IP) on the mechanical nociception induced by naloxone (1.5 mg/kg, IP) in rats subjected to SNL and treated with a 6-day escalating morphine treatment. Surprisingly, we found that finasteride significantly increased the force threshold for paw withdrawal only on the injured side (Figure 6D and Figure S15), indicating that finasteride reduced the hyperalgesia associated with opioid withdrawal.

The fact that finasteride does not inhibit the antinociceptive or antiallodynic effect of either morphine or hydrocodone in response to different painful stimuli in a rat model of neuropathic pain demonstrates that finasteride is unlikely to interfere with the principal beneficial activity of opioids. Furthermore, the finding that finasteride reduces the hyperalgesia associated with opioid withdrawal strongly suggests it may be used to reduce the liability for opioid abuse and the untoward consequences of opioid withdrawal while retaining the clinical utility of opioids.

To verify that finasteride would not interfere with the anti-nociceptive effects of opioids in healthy animals, we repeated both the mechanical and thermal nociception experiment in uninjured rats.

In healthy animals, injection of either morphine (3mg/kg, SC) or morphine (3mg/kg, SC)/finasteride (50mg/kg, IP) did not affect mechanical nociception (Figure S16A). However, in the hot plate assay, latency for the first lick or paw retraction of animals treated with morphine (3 mg/kg, SC) was significantly increased at 51.1°C (Figure S16B) which was not affected by co-treatment with finasteride (50mg/kg, IP). These data further suggest that finasteride does not diminish the anti-nociceptive properties of opioids.

Steroids regulate opioid self-administration

Because finasteride is a known 5αR inhibitor, we hypothesized that finasteride reduces opioid self-administration by altering the level of one or more neuroactive steroids in the brain. To investigate the landscape of changes induced by the treatment of opioid-conditioned animals with finasteride, and to identify candidate neuroactive steroids regulating opioid intake, we isolated whole brains from treatment-naive and opioid-conditioned zebrafish treated with either DMSO or finasteride (10 μM). We then performed steroid quantification using untargeted ultra-performance liquid chromatography-mass spectrometry (UPLC-MS).

In other models, finasteride induces changes in steroid levels in specific brain regions[45,46], but because of the small size of the zebrafish brain, we used whole brains and could not achieve the same level of regional specificity. Nonetheless, we identified interesting trends, including an accumulation of several 5αR precursors and a reduction in 5αR products after treatment with finasteride. We normalized the results (min-max) for each steroid and compared the levels between DMSO- and finasteride-treated conditioned animals. The only steroid that reached significance by itself was dehydroepiandrosterone sulfate (DHEAS), which was markedly increased in finasteride-treated animals (Figure 7A). We also observed that other precursor steroids, including testosterone and pregnenolone, also showed the same trend of accumulating in finasteride-treated animals. Surprisingly, we also detected a reduction in dehydroepiandrosterone (DHEA), which could suggest an increase in the conversion of DHEA to DHEAS in treated animals. Interestingly, we did not detect the same trend for other sulfated steroid species (Figure S17). The opposite trend of decreases in finasteride-treated animals was also observed for steroids downstream of 5αR, especially allopregnanolone (AP) and 3α-androstanediol, but these differences did not reach statistical significance (Figure 7A-B).

We tested how the accumulation of DHEAS affects opioid self-administration. Given that sulfated steroids are less effective at crossing the blood-brain barrier[47,48], we incubated conditioned zebrafish with 10 μM DHEA for 60 min before measuring opioid-self administration.

As observed with finasteride, incubation with DHEA drastically reduced the number of visits at the active platform (Figure 8A). We also tested the primary DHEA precursor, pregnenolone, and again observed a reduction in opioid self-administration (Figure 8A). Taken together, these results suggest that the accumulation of DHEAS and other precursors observed in finasteride-treated animals could play an important role in the reduction of opioid self-administration. The fact that DHEA-treated animals had a reduced number of visits at the active platform also suggests that the reduction in DHEA level observed in finasteride-treated animals is a result of an increased conversion to DHEAS.

Since we detected a reduction in some products of 5αR after treatment with finasteride, we decided to test whether their reintroduction could interfere with the activity of FIN. However, since we did not detect any significant changes for a single product, we decided to test if a combination of steroids from the same class could act together. We chose to co-treat conditioned animals with finasteride (10μM) and allopregnanolone (0.1μM), androsterone (1μM) and 3α-diol (1μM) for 60 min. Interestingly, the presence of these products was sufficient to partially block the effect of finasteride and significantly increase the number of visits at the active platform when compared to finasteride treatment alone (Figure 8B).

Taken together, these results suggest that the accumulation of DHEA or its sulfated form might play a critical role in mediating the effect of finasteride. However, other neurosteroids such as 5αR products may also be involved in the regulation of opioid self-administration.

Discussion

We have demonstrated that finasteride modulates opioid consumption, a critical aspect of OUD, in two different animal models, and for different classes of opioids. These results suggest that finasteride or finasteride-like molecules could be a viable therapeutic strategy to treat OUD. Although finasteride is typically used for treating non-neuronal indications, there is evidence in the literature to suggest it might also have beneficial effects in the nervous system. Rodents

treated with finasteride exhibit reduced reactivity towards both incentive stimuli and stress responses[49], both of which play important roles in substance abuse disorders[50,51]. Finasteride reduces risk-taking behavior, a behavioral feature typically associated with substance use disorders[49,52,53], and may help reduce pathological gambling[54]. Since finasteride is FDA-approved and its side effects and toxicology are well-studied, it may be possible to rapidly initiate clinical trials to test the therapeutic potential of this drug for OUD. Furthermore, since finasteride does not reduce the antinociceptive effects of opioids and even opposes the hyperalgesia and physical signs associated with naloxone-precipitated opioid withdrawal, it could potentially be used as a treatment for OUD or an adjunct therapy for patients using opioids for pain relief.

We currently do not know if finasteride influences opioid self-administration by reducing motivation for drug seeking or if treated animals are simply satisfied with a lower amount of drug.

Our results in the fentanyl self-administration assay reveal that finasteride treated rats initially perform nose-pokes to self-administer fentanyl at a rate similar to their untreated controls, but they slow down their intake sooner than controls, suggesting finasteride-treated animals may achieve satiation with a lower amount of fentanyl. While the precise mechanism of finasteride action in opioid self-administration remains unknown, it is likely to involve changes in steroid profile in the brain. Finasteride is an inhibitor of key enzymes in steroid production, the three 5α-reductase isoenzymes SRD5A1, SRD5A2, and SRD5A3, which are expressed in different tissues, including the nervous system[55–59]. Because the effects of finasteride on the steroid profile are pleiotropic[21,22,60], characterization of the specific steroid species involved in the regulation of opioid self-administration may eventually lead to the development of even more precise, targeted therapies for OUDs.

Our results suggest that accumulation of DHEAS plays an important role in opioid self-administration. Finasteride treatment causes a significant decrease in the non-sulfated DHEA and an accumulation of DHEAS in the brain. Importantly, treatment of opioid-conditioned zebrafish with DHEA alone reduces opioid intake, further supporting the hypothesis that DHEA/DHEAS plays an important role in opioid intake regulation. These neuroactive steroids have been shown to directly act on different neurotransmitters receptors; they can act as positive allosteric modulators of NMDA receptors, as negative modulators of the $GABA_A$ receptor, and as activators of other neuronal receptors such as σ1 and TrkA[61–63]. The modulation of these pathways has been shown to be important in the regulation of opioid abuse disorders[18,64–69] and could explain why modulation of DHEA reduces opioid intake. Furthermore, previous reports showed that repeated DHEAS treatment prevents the development of opioid tolerance without showing an effect on self-administration[70]. Additionally, chronic treatment with DHEA has also been shown to reduce cocaine self-administration and reinstatement in rats[71].

Although we believe that DHEA/DHEAS plays a key role in the effect of finasteride on opioid self-administration, we also have evidence that other steroid species could be involved. Similarly, to DHEA, these other steroids have been shown to affect key neuronal pathways relevant to substance abuse. For instance, the products of 5αR, 3α-diol and allopregnanolone can act as positive allosteric modulators of $GABA_A$[72–76] and have thus been linked to neuronal stress response[77,78]. Therefore, by reducing allopregnanolone production, finasteride may reduce the negative affective state that contributes to opioid self-administration.

In this study, we performed most of the rat experiments with males since finasteride is mainly prescribed in men (although there is some history of use to treat hirsutism in women)[79] but our data suggest that both male and female rats showed reduced fentanyl self-administration upon finasteride treatment, raising the possibility that finasteride could be used as a treatment for OUD in both males and females, despite the differences in steroids levels between males and females[80].

Finasteride has been clinically used since the 1990s for the treatment of androgenic alopecia and benign prostate hyperplasia. Although it is considered a well-tolerated and relatively safe drug, there is evidence of sexual disfunction in 3.4 to 15.8 percent of men. A rare but serious side effect known as post-finasteride syndrome (PFS) has also been reported[81–83]. PFS prevalence is unclear but it manifests as a range of persistent physical and neuropsychiatric disorders such as depression and anxiety that develop during or after discontinuation of finasteride use. Clinical studies will be needed to fully elucidate the treatment regime and understand the side-effects associated with the use of finasteride for the treatment of OUDs, and careful clinical consideration must be given in weighing potential risks and benefits of finasteride use. The optimal human dose for OUD would also have to be determined. The finasteride doses we used in rats (25-50 mg/kg, IP) are considerably higher than the dose regimens of 1-5 mg/day used in humans for alopecia and benign prostatic hyperplasia, respectively. The rat doses were selected empirically based on previous studies conducted in the Bortolato lab, showing that these doses are necessary to produce a significant reduction in 5α-reduced metabolites in rodents after acute treatment. It has also been shown empirically that the doses used in this study produce ameliorative effects in rodent models of Tourette syndrome (TS) akin to those observed within a 3-6 week period of 5 mg/day dosage in TS patients[84–86]. It is important to note that the rat doses are single acute doses, whereas human dosing of finasteride is typically chronic. Finasteride is known to accumulate slowly with repeat dosing in humans, and the effects of finasteride persist for several days beyond what would be predicted based on compound half-life alone, which is attributed to persistent tight binding of finasteride to 5aR[87]. Taken together, these factors suggest that the human dose necessary to treat OUD would be much lower than the acute dose used in our rat studies, but the optimal dose would need to be established empirically.

In conclusion, the present study identifies the widely-used drug finasteride as an effective agent for reducing opioid intake in both zebrafish and rats and self-administration

paradigms. The data further indicate that the DHEA/DHEAS pathway is a major mediator of finasteride's effect. These findings point to a promising potential therapeutic strategy in the fight against OUD and open new avenues for investigating the role of specific steroids in regulating opioid use behaviors.

Materials and Methods

Zebrafish

Fish handling

Adults zebrafish (*Danio rerio*) of the wild type Ekkwill strain were maintained in the fish facility at 28–29°C with a 14/10 hours light/dark cycle. All zebrafish experiments were approved by the University of Utah Institutional Animal Care and Use Committee. All fish experiments were performed on mixed population (50% male/female).

Adult fish treatment

Adult fish were transferred to a small treatment chamber (USplastic, USA) with 100 mL of fish water, and the compound of interest was injected directly into the water. Fish were allowed to swim in the treatment solution for one hour prior to the self-administration assay.

Zebrafish self-administration

The same protocol as detailed in Bosse et Peterson, 2017*(89)* was used to condition fish in small groups of 15 animals. For the screen, fish were conditioned for 4 days and treated with the different compounds on day 5, before being tested in the arena for 30 min. For opioid conditioning, we used the following doses: hydrocodone and morphine 6 mg/L and fentanyl 0.12 mg/L. Opioids were diluted in fish water. Between 60 to 100 animals were conditioned simultaneously and randomly assigned to different treatment conditions.

Chemicals used

Finasteride (Sigma-Aldrich, USA), Hydrocodone bitartrate (Spectrum Chemicals, USA and NIH), Morphine (Spectrum chemicals), Fentanyl (Spectrum Chemicals, USA), Dutasteride (Cayman chemical company, USA), Pregnenolone (Sigma-Aldrich, USA), DHEA(Sigma-Aldrich, USA), Methadone (Sigma-Aldrich, USA), Allopregnanolone (Tocris, USA), 3α-diol (Steraloids, USA), Androsterone (Steraloids, USA), Fentanyl (Sigma-Aldrich, USA). Compounds were either re-suspended in DMSO or water according to manufacturer recommendation.

Food conditioning

The same apparatus was used as for hydrocodone conditioning. For food conditioning, fish were trained directly in the arena without performing the pre-conditioning protocol. Fish were trained for 50 min daily in a small group. Larvae food Ziegler #4 (VWR, USA) was suspended in fish water.

Fish locomotion

A high-speed infrared camera (Point Grey, Canada) was used to record 1-min videos of animals in the self-administration arena. Zebrafish tracking and movement quantification were performed with the software Actualtrack (United Kingdom).

Statistical analyses

For zebrafish self-administration data, R graphic programming was used to generate the plots. ANOVA tests were run on plot data to test significance. ANOVA tests were performed first on inactive platforms for each dataset to validate that there was no difference between the different conditions, and then active platform values were used to test for significance. All boxplots were generated using R graphic programming and the *ggplot* module. The lower and upper hinges correspond to the first and third quartiles. The line is the median. The whiskers extend from the hinged to the maximum or minimum value at most 1.5x the inter-quartile range (IQR) from the hinge. Data points beyond that are considered outliers. No data points were excluded from the statistical analysis.

Single-cell data analysis

Processed single cell data from *Raj et al. Nat Biotechnol(90)* from 6 individual whole zebrafish brains (f1 n=6,759, f2 n=7,112, f3 n=15,156, f4 n=12,121, f5 n=9,919, f6 n=6,009) as well as manually dissected fore- (n=3,615), mid- (n=1,504) and hindbrains (n=3,894) were downloaded as an R data object from Gene Expression Omnibus (GSE105010). The R package Seurat (v3.1.1) was used to create a new Seurat object from the raw counts data found within the downloaded InDrops object. Samples with less than 200 genes detected were already removed from these data, but we additionally removed outliers with more than 6000 genes detected and those higher than 25% mitochondrial genes. This further processing removed an additional 371 samples. We followed the SCTransform workflow as recommended in the Seurat vignette (https://satijalab.org/seurat/v3.1/sctransform_vignette.html) for scaling, normalization, and finding variable genes. Mitochondrial mapping percentage was regressed during normalization. A principal component analysis was first performed, then clustering using the Seurat FindNeighbors (using the first 30 dimensions) and FindClusters function (resolution of 2.5). The data were visualized using t-SNE dimensionality reduction using the first 30 dimensions. Similar to the published analysis in *Raj et al. Nat. Biotechnol.*, we observed that the 65,718 samples produced 61 clusters. t-SNE coordinates, expression values from the SCT “data” slot, and metadata were exported from the Seurat data object to create t-SNE figures using data.table and ggplot2 demonstrating the cluster identities, broad brain regions (fore-, mid- and hindbrain) and srd5a family expression. We plotted all family members highlighting only those cells in the foreground with relatively medium to high expression values where expression cut-offs were determined for each marker individually based on expression distribution (srd5a1 0.8-3.0; srd5a2a 1.0-3.0; srd5a2b 1.0-3.0; srd5a3 0.8-3.0)[34,88–93].

Steroid quantification

Brain extraction

Conditioned fish were transferred to a small treatment chamber (USplastic, USA) with 100 mL of fish water and treated with either DMSO (0.02%) or finasteride (10μM). Fish were allowed to swim in the treatment solution for one hour. Treated animals were then transferred to a water bath with ice-water for euthanasia. The brain of each animal was then extracted in PBS 1X. The head was cut using a razor blade behind the gill, the skull was then carefully peeled to expose the brain using surgical forceps. The brain was then extracted by performing a cut at the base of the cerebellum. The extracted tissue was then placed in 1.5 mL self-standing microcentrifuge tube, (USAscientific, USA) on ice and the brains of 10 animals were pooled together in the same tube. Any liquid was then removed from each tube before weighing the tissues. Samples were then flash frozen in liquid nitrogen and placed at -80°C until extraction.

Chemical

Reference standards were purchased from Steraloids (Newport, USA). All solvents were HPLC grade, and all other chemicals used were of the highest grade available. Stock neurosteroid standard mixture was prepared by mixing 5 μL of 1mg/mL solution of each steroid and adjusting the final volume to 1mL by using methanol*(97-99)*. All the stock solutions were stored at -80°C.

Sample preparation

Tissue samples were extracted as described previously*(97-99)*. Briefly, tissue samples were extracted with 1 mL chloroform. The mixture was vortexed for 30 sec and centrifuged for 5 min; the chloroform layer was transferred to 2 mL tube and dried. The resulting residue was extracted with 1 mL MeOH. The MeOH layer mixture after 5 min centrifugation was added to the above chloroform extract. This mixture was dried and re-suspended in 125 μL MeOH and filtered using 5kD membrane filters. Filtrates were transferred to vials for UPLC-MS analysis.

UPLC-MS Analysis

Tissue sample extracts were subjected to UPLC-MS analysis for the measurement of neurosteroids, as described previously(97-99). UPLC analyses were carried out using a Waters Acquity UPLC system connected with the high-performance triple quadrupole mass spectrometer. Analytical separations on the UPLC system were conducted using an Acquity UPLC C18 1.6 μ column (2.1 x 150 mm) at a flow rate of 0.15 mL/min and C18 1.7 μ column (2.1 x 50 mm) at flow rate 0.2mL/min. For the first column, the gradient was started with 100% A (0.1% formic acid in H_2O) and 0% B (0.1% formic acid in CH_3CN), after 0.1min changed to 80% A over 1 min, and then 45% A over 5min, followed by 20% A in 2min. Finally, over 0.5 min, it was changed to 0% A, then after 13 min, it was changed to the original 100% A over 1 min, resulting in a total separation time of 13 min. For the second column, the gradient was started with 100% A (0.1% formic acid in H_2O) and 0% B (0.1% formic acid in CH_3OH), after 0.1min changed to 80% A over 2 min, and then 45% A over 2 min, followed by 20% A in 2min. Finally, over 1 min, it was changed to 0% A, then after 8 min, it was changed to the original 100% A over 2 min, resulting in a total separation time of 10 min. The elution from the UPLC column was introduced to the mass spectrometer. All MS experiments were performed by using electrospray ionization (ESI) in both positive ion (PI) and negative ion (NI) mode, with an ESI-MS capillary voltage of 3.5 kV, an extractor cone voltage of 3 V, and a detector voltage of 650 V. The following MS conditions were used: desolvation gas at 400 l/h, desolvation temperature at 350ºC and source temperature 150ºC. Pure standards of all targeted neurosteroids were used to optimize the UPLC-MS/MS conditions prior to analysis and performing calibration curves [94–96]. Reference standards were run before the first sample, in the middle of the runs and after the last sample to prevent errors due to matrix effect and day-to-day instrument variations. In addition, spiked samples were also run before the first sample and after the last sample to calibrate for the drift in the retention time of all neurosteroids due to the matrix effect. After standard and spiked sample runs several blanks were injected to wash the injector and avoid carry-over effects. Resulting data were processed by using Target Lynx 4.1 software (Waters) [94–96].

Data normalization

Steroids counts were first normalized using the initial weight of the tissue before extraction. To compare the levels of the steroids, we then used min-max normalization for steroid count in each sample.

Rats

Hydrocodone self-administration and nociception

Animals

Adult male Sprague-Dawley and Long-Evans rats (Charles River, Roanoke, USA) were group-housed (3-4/cage) within rooms maintained at 22 ± 2 °C and 55% humidity, on a 12/12 h light/dark cycle (lights on at 7:00 AM). Food and water were available *ad libitum.* Following a 7-day acclimation to the housing facilities, animals were handled daily for 5 min. Behavioral measurements were carried out and analyzed by trained experimenters in a blinded fashion.

Chemicals

Hydrocodone (Spectrum Chemical, USA), morphine (Spectrum Chemical, USA), and naloxone (Tocris, Bio-Techne) were dissolved in a solution of 2% DMSO and 98% saline. Finasteride (Astatech, Bristol, USA) was suspended in a solution of 5% DMSO, 5 % Tween 80 and 90% saline (5:5:90).

Hydrocodone self-administration.

Apparatus: The apparatus consisted of 8 operant conditioning chambers (Habitest, Coulbourn, USA), measuring 30.48 cm (W) x 25.4 cm (D) x 30.48 cm (H), and enclosed in sound-attenuating cubicles with ventilation fans. Each chamber was equipped with two retractable levers: an active lever coupled to the intravenous delivery of hydrocodone, and a control (inactive) lever. Active lever placement on the left or right side followed a counterbalanced order. Three cue lights were placed over the active lever. The apparatus was controlled by Graphic State 4 software (Coulbourn, USA).

Experimental procedure: Opioid self-administration was performed using a modified version of the protocol described by Mavrikaki et al[97]. Rats weighing 225-250 g were used. Rats were anesthetized with ketamine and xylazine and underwent catheterization surgery. Briefly, a polyurethane catheter was inserted through the external jugular vein, passed under the skin, and fixed in the mid scapular region. Post-operative care included buprenorphine and enrofloxacin for analgesic and antibiotic management, respectively. Catheter patency was maintained through daily flushing with a heparin (500 IU/mL) / 50% dextrose solution.

Ten days after surgery, all rats were gently handled and kept under a food restriction regimen that maintained them at 90% of their initial body weight and was continued throughout the whole behavioral procedure. A syringe containing a hydrocodone solution was placed in an infusion pump located outside the chamber and connected to the rat's catheter via a fluid swivel and spring-covered Tygon tube suspended through a counterbalanced swivel. The solution was administered at a dose of 0.016-0.128 mg/kg/infusion, in a volume of 160 µL/kg/infusion. Operant training began three days later and consisted of three stages of fixed-ratio reinforcement schedule: FR1, FR2, and FR5. Rats underwent daily, 1 h-long experimental sessions, between 9:00 AM and 3:00 PM and for 7 days/week, consisting of a sequence of trials (Figure 3). Each trial began with a 5-s period, during which the house light was turned off and the cue light blinked three consecutive times. Subsequently, the house light was turned on and both levers were extended. Once the rat completed the fixed ratio on either lever , both levers retracted, and a new trial began after a 15-s time-out period. Each rat progressed from FR1 to FR2 and from FR2 to FR5 after reaching stability, defines as >70% of total lever presses on the active lever for three consecutive days. FR1 and FR2 stability criteria were reached from day 12 through 19 and form day 15 through day 23 of training, respectively. All animal reached FR5 stability by day 31 of training and were then treated with either finasteride or its vehicle.

Locomotor activity

Rats were tested for locomotor activity in an open field surrounded by black Plexiglas walls (47 cm x 47 cm x 47 cm). Comparisons were drawn between self-administering animals (immediately after their performance in the operant chamber) and control animals not receiving any opioids. The locomotor analysis was performed using EthoVision XT 14 pathway tracking software (Noldus Instruments, Wageningen, The Netherlands).

Naloxone-precipitated opioid withdrawal.

Long Evans male rats (180-225 g) received subcutaneous injections of morphine with the regimen previously described (cumulative doses of 5, 10, 20, 30, and 40 mg/kg per day within five days). On day 6, rats received an acute dose of morphine (40 mg/kg, SC), followed by either finasteride (50 mg/kg, IP, 100 min later) or its vehicle, and naloxone (1.5 mg/kg, IP, 120 min later), and were immediately placed inside a Plexiglass chamber with bedding. Animals were video-recorded for 30 min, and blinded observers monitored their opioid-withdrawal signs, including wet dog shakes, jumps, grooming, and digging.

Neuropathic pain assessment

Sprague-Dawley rats weighing 150-180 g were used. Following 2-3 days of handling, rats underwent mechanical nociception testing via von Frey Hair and Randall-Selitto tests. Neuropathy was then induced by SNL surgery, as previously described[41]. Rats were anesthetized using xylazine and ketamine (10/75 mg/kg, IP), and their left L5 spinal nerve was exposed and tightly ligated with 4.0 silk suture (Mersilk®, Ethicon thread, Johnson). Muscle, fascia, and skin were then sutured, and the rats were treated with enrofloxacin (10 mg/kg, SC) and carprofen (5 mg/kg, SC) for post-operative care. Fourteen days after surgery, nociception was re-tested, and allodynia was confirmed in rats exhibiting a >30% reduction of their pain threshold. Rats were then assigned to different treatment groups to receive either morphine (1-3 mg/kg, SC), hydrocodone (1-10 mg/kg, SC) or saline. The antinociceptive effects of opioids were tested every 30 min for 6 consecutive observations. The analgesic effects of morphine and

hydrocodone (at their most effective doses) were also tested in combination with finasteride (50 mg/kg, IP) or its vehicle, to ascertain whether finasteride altered the antiallodynic properties of opioids. The effects of opioids and finasteride were also tested for thermal nociception in a separate group of rats with SNL using the hot plate procedure.

von Frey Hair Test: Tactile allodynia was assessed using a set of 8 von Frey monofilaments (Bioseb, Vitrolles, France) with logarithmic incremental stiffness (of 1.4, 2, 4, 6, 8, 10, 15 and 26 g). Paw-withdrawal threshold was measured, and 50% response threshold was calculated using the Up-Down method and Dixon´s formulae, as previously described[42]. Behavioral assessments were run prior to and 14 days after SNL surgery. Rats were individually placed in plexiglass compartments (17 x 11 x 13 cm) with a wire mesh bottom that allowed full access to paws. After 20-30 min of acclimation, a first 6-g hair was perpendicularly applied against the plantar surface of the left hind paw for 6 s. Paw withdrawal and/or licking reflex was considered as a positive response. Depending on the positive or negative response, the next filament with either lower or higher force was tested, respectively. Testing continued until either four consecutive negative or five consecutive positive responses were recorded after the first change of direction.

Randall-Selitto Test: Nociceptive withdrawal threshold was assessed using the Randall-Selitto algesimeter (Ugo Basile, Varese, Italy), as previously described[43]. Following daily handling and acclimation to the apparatus, rats were wrapped into a cotton cloth and immobilized. The medial portion of the plantar surface of the left hind paw was carefully placed on the device's tip. An increasing mechanical force was applied until a withdrawal response was observed. Paw withdrawal threshold for Randall-Selitto experiment is set at 25g of force applied. Rats were tested every 30 min for three consecutive hours following treatment (6 applications in total). For the assessment of morphine withdrawal-induced hyperalgesia, rats with SNL were subjected to a cumulative morphine treatment (as described above) for five days. On day 6, the effects of finasteride on naloxone-precipitated opioid withdrawal were tested using the Randall-Selitto algesimeter immediately before naloxone treatment, as well as 30 and 60 min later.

Hot Plate Test. Thermal nociception was assessed using the hot plate analgesia meter (IITC Life Science, Woodland Hills, USA). The rat was placed on a plate maintained at different temperatures (48.5 and 51.5 °C), and their progressive latencies to lick the left hind paw were measured.

Fentanyl self-administration

Subjects

A total of 20 adult male and adult female Wistar rats (Charles River, USA) weighing 200 – 475g at the start of the experiment were individually housed and kept on a 12-h light/ 12-h dark cycle in a temperature and humidity-controlled room. Animals were provided food and water *ad libitum*.

Drugs

Fentanyl Citrate (Medisca, USA) was dissolved in deionized water at a concentration of 50μg/mL. Finasteride (Astatech, USA) was suspended (50mg/mL) in a solution of 2.5% ethanol, 5% Tween80, and 92.5% saline.

Fentanyl self-administration

Apparatus: Oral fentanyl self-administration tasks were completed in eight modular operant chambers (Med Associates, USA), equipped with a liquid magazine tray stationed between two nose-poke devices. Additionally, the operant chamber was outfitted with a solenoid controlled liquid valve (Lee Valves, USA) and a set of audiovisual cue equipment [house light, magazine light, and a tone generator].

Experimental procedure: A operant oral self-administration behavioral assay described by Shaham and colleagues*(104)* was utilized. Rats were trained to obtain liquid fentanyl delivered into a liquid magazine tray following an operant response on an FR1 reinforcement schedule. During the self-administration sessions, a nose-poke in the active port (counterbalanced between animals) resulted in fentanyl delivery (0.02mg/kg/delivery). Concurrent with drug

delivery was a 10s audiovisual conditioned stimulus (CS) comprised of a 1s illumination of a light inside the nose-poke port, a 10s tone, and a 10s illumination of a light stationed above the liquid magazine tray. Any additional nose-pokes during the 10s CS were without consequence. Drug availability at the start of each session and following CS presentations was signaled by illumination of the house-light placed on the wall opposite of the nose-poke ports. All nose-pokes in the inactive port were without consequence. Animals were given two 30-min magazine training sessions, during which any active responses resulted in fentanyl and CS presentation; however, if the animal made no responses within 2-3 min of the last drug delivery (or start of the session) a non-contingent fentanyl and CS delivery occurred. Following these two training sessions, animals had 15, 1-hour sessions to self-administer fentanyl for 5 days/week. During these 15 sessions, all animals reached a response criterion of >70% of nose-pokes occurring at the active nose-poke port. To test the effect of finasteride on fentanyl consumption animals received 10 additional self-administration sessions across 12 days, in which finasteride (50mg/kg, 1mL/kg) or vehicle (1mL/kg) was administered intraperitoneally 45 min prior to the session. Each treatment (vehicle or finasteride) was given for 5 consecutive days, with a 48-hour period before they received the opposite treatment. The order of treatment administration was counterbalanced across animals. Following self-administration sessions, mg/kg of fentanyl consumed was calculated for each animal by subtracting the amount of fentanyl left in the magazine tray from the total amount of drug delivered.

Study approval

All animal studies were approved by local Institutional Animal Care and Use Committees (IACUC). All zebrafish experiments were approved by the University of Utah Institutional Animal Care and Use Committee. Hydrocodone self-administration studies and nociception studies in rats were compliant with the National Institute of Health guidelines and approved by the IACUC of the University of Utah. The fentanyl self-administration studies in rats were conducted under

the guidance and permission of the Institutional Animal Care and Use Committee at the University of Washington and pursuant to federal regulations regarding work with animals.

Author contributions

G.D.B designed the experiments and performed the zebrafish assays, analyzed the data and wrote the manuscript with R.T.P. and M.B. R.C. designed the nociceptive experiments in rats and performed the experiments. G.F. designed and performed the hydrocodone self-administration assay in rats assisted by E.V. T.Z. contributed to experiment design. R.T.P. designed and supervised zebrafish experiments. M.B. designed, analyzed and supervised the experiments on rat hydrocodone self-administration experiments and nociception. N.W.G. performed steroid extraction and quantification R.D.F. J.S.L designed and performed the Fentanyl self-administration experiment in rats. R.D.F and P.E.M.P. designed and analyzed the Fentanyl self-administration in rats. All authors contributed to data interpretation and commented on the manuscript.

Acknowledgments

We thank the NIDA Drug Supply Program for providing hydrocodone bitartrate powder. We thank C. Herdman for helpful comments and bioinformatic support. We thank Karen Odeh and Stefanos Loizou for their technical support. We would like to acknowledge the Centralized Zebrafish Animal Resource (CZAR) at the University of Utah for providing Zebrafish husbandry, laboratory space, and equipment to carry out portions of this research. Expansion of the CZAR is supported in part by NIH grant # 1G20OD018369-01. This work was supported by the Charles and Ann Sanders Research Scholar Award, the L.S. Skaggs Presidential Endowed Chair, and NIH grant R21DA049530 (to M.B. and R.T.P). Oral rodent fentanyl supported by National Institute of Drug Abuse (R01-DA039687. G.D.B. was supported by the Canadian Institutes of Health Research (CIHR) fellowship program. R.D.F. was supported by NIDA (T32-DA007278). T.Z. was supported by NHGRI (T32 HG008962).

Figures

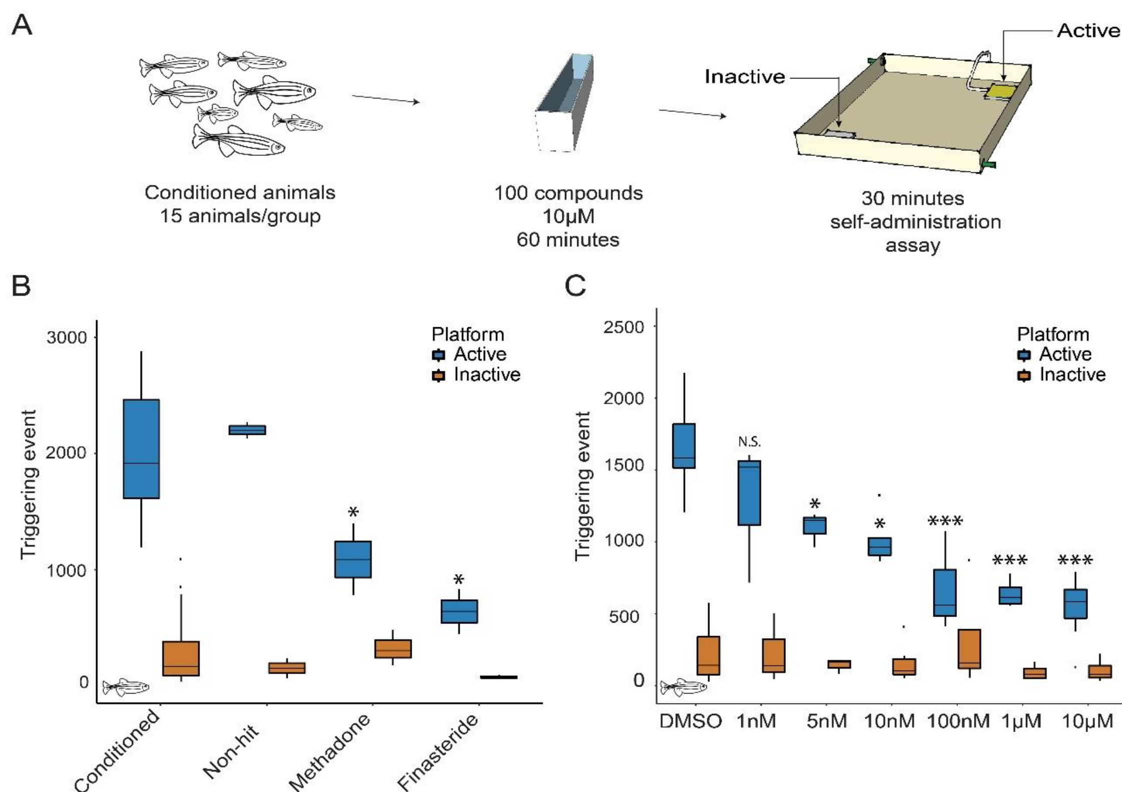


Figure 1. Small molecule screen for modulators of opioid self-administration.

A. Conditioned animals were treated with the different molecules at 10μM for 60 min before assessing their opioid self-administration for 30 min. **B**. Known small molecules affect opioid self-administration. Treatment with methadone (n=3), and Finasteride (n=2) significantly reduces the number of triggering events at the active platform. Conditioned fish n=56. No difference was observed at the inactive platform. *p*-value calculated with Student's T-test compared to conditioned animals. **C**. Dose response experiment for Finasteride. Three doses, 100nM, 1μM, 10μM, reduce the number of triggering events below our threshold of 1000 activations. *p*-value computed by Tukey HSD on one-way ANOVA, Inactive platform (F(6,36)=1.06) p=0.40 and Active platform (F(6,36),24.60) p=2.31E-11, compared to the DMSO control. No significant difference detected for the inactive platform. DMSO n=16, 1nM n=3, 5nM n=5, 10nM n=6, 100nM n=5, 1μM n=4, 10μM n=7. * *p*-value <0.05, ** *p*-value <0.01, *** *p*-value <1E-5. Each n represents a group of 15 animals. These experiments were performed using a between subject design.

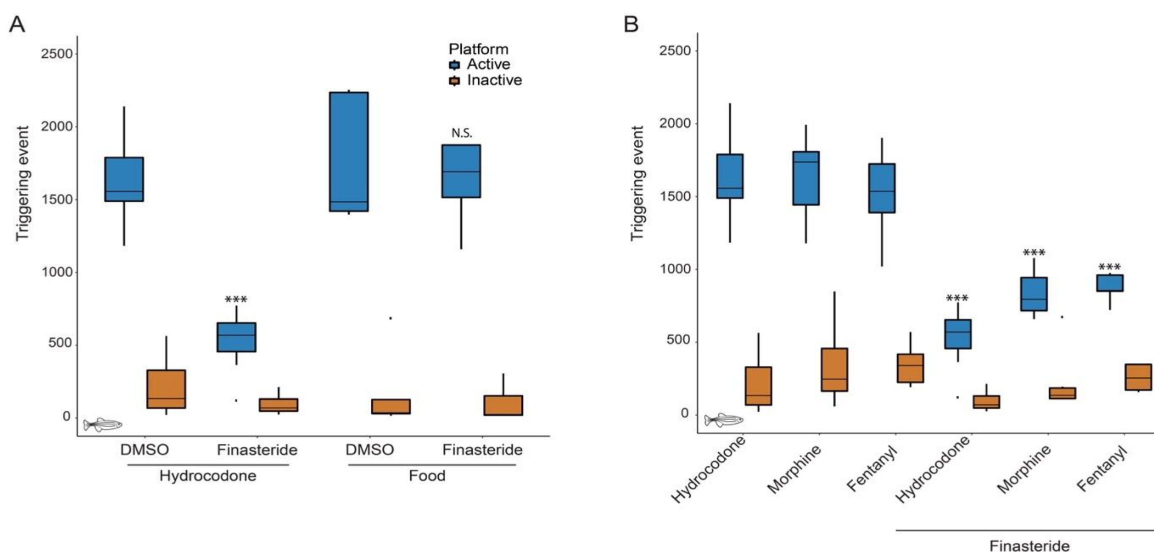


Figure 2. Finasteride effectively reduces the self-administration of different opioids, but does not affect all motivated behaviors.

A. Finasteride reduces opioid self-administration without affecting food-seeking behavior. Finasteride significantly reduces the number of triggering events at the active platform compared to DMSO control for hydrocodone. No difference was detected for fish conditioned to self-administer food. *p*-value computed by Tukey HSD on one-way ANOVA, Inactive platform (F(3,29)=1.05)p=0.38 and Active platform (F(3,29)=19.37)p=4.32E-07, compared to respective DMSO control. Opioid trained animals: DMSO n=16, Finasteride n=7. Food seeking: DMSO n=5, Finasteride n=6. These experiments were performed using a between subject design. **B**. Finasteride affects opioid self-administration for animals conditioned with 3 different opioids. *p*-value computed by Tukey HSD on one-way ANOVA, Inactive platform (F(5,81)=1.57)p=0.18 and Active platform (F(5,81)=31.68)p=4.52E-13, compared to respective control. Hydrocodone control n=16, Hydrocodone+Finasteride n=7, Morphine control n=7, Morphine+Finasteride n=6, Fentanyl control n=7, Fentanyl+Finasteride n=5. No significant difference was detected for the inactive platform in any condition. * *p*-value <0.05, ** *p*-value<0.01, *** *p*-value <1E-5. Each n represents a group of 15 animals. Data for hydrocodone treatment alone reproduced from Figure 1C for comparison. These experiments were performed using a within subject design.

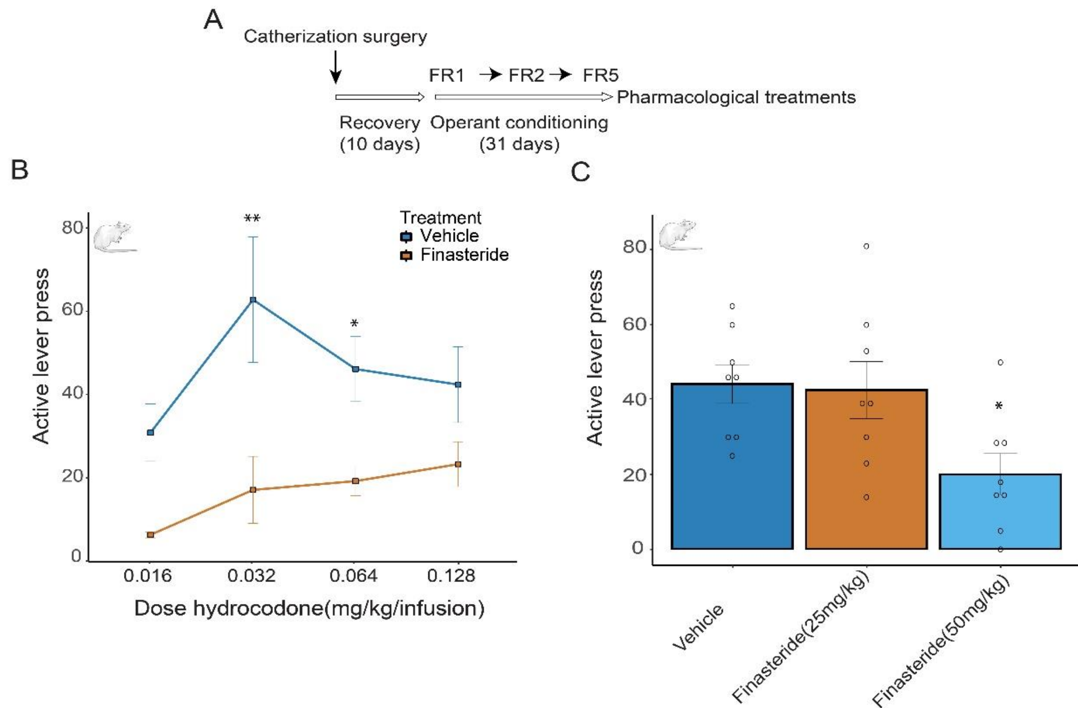


Figure 3. Opioid self-administration is reduced by finasteride treatment in rats.

A. Rats were conditioned to self-administered hydrocodone and after establishing robust FR5, they were treated with either vehicle or finasteride. **B**. Active lever presses for animals trained with different concentrations of hydrocodone, a total of 6 animals per conditions were tested. Treatment with finasteride (50mg/kg) reduces opioid self-administration of hydrocodone for animals conditioned with 0.032 and 0.064 mg/kg. *p*-value corrected for multiple comparison two-way Anova ($F(1,38)=30.5$)$p=0.0001$). *p* value compared vs vehicle-treated animals **p*-value < 0.05, ** *p*-value<0.01. **C**. IP injection with 50 mg/kg, but not 25 mg/kg finasteride reduces the number of active lever presses for *0.064 mg/kg hydrocodone*, 8 animals were treated for each dose of finasteride. *p*-value corrected for multiple comparisons on Anova ($F(2,21)=4.69$)$p=0.02$. Error bars represent the mean +/- s.e.m. Adult male Sprague-Dawley rats were used to perform the hydrocodone self-administration assay. These experiments were performed using a within subject design.

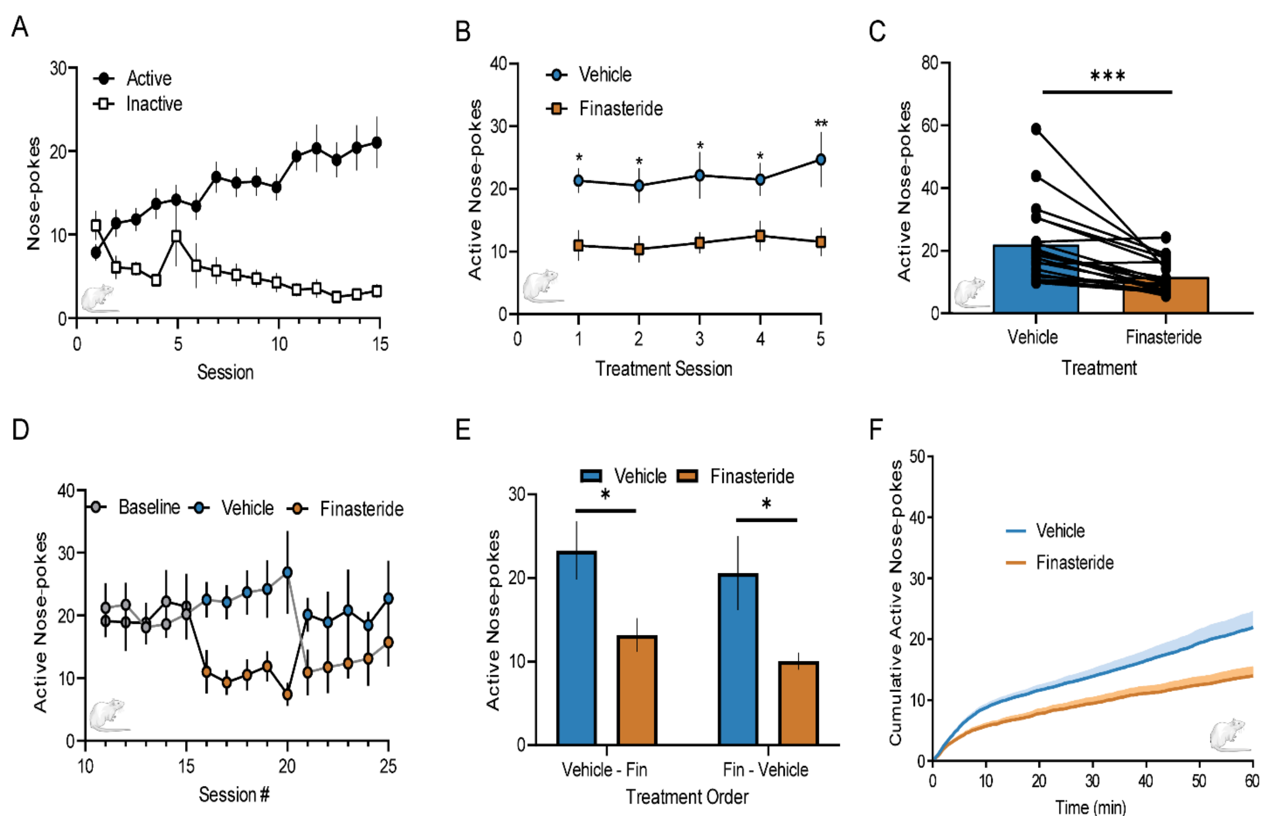


Figure 4. Finasteride decreases operant responding for oral fentanyl self-administration.

A. Operant nose-poke responses at the active (blue circles) and inactive (orange squares) nose-poke ports during baseline fentanyl self-administration sessions (n=20 rats). **B.** Daily finasteride injections (IP, 50mg/kg) (orange squares) decreased active nose-pokes compared to vehicle injections (blue circles). A main effect of drug treatment was observed, Sidak post-hoc analysis was performed for multiple comparisons on mixed-effect model $(F(1,19)=20.77) p=0.0002$. **C.** Average operant responses during vehicle (blue) and finasteride (orange) treatment for each individual animal. A paired t-test revealed finasteride significantly decreased animals' active responding for fentanyl delivery $(t(19)=4.481) p=0.0003$. **D & E.** Animals received daily injections of finasteride during sessions 16-20 (n=10 rats, D: black line, E: Finasteride-Vehicle) or during sessions 21-25 (n=10 rats, D: gray line, E: Vehicle - Fin). **D.** Baseline refers to animals responding during sessions 10-15. **E.** Order of treatment had no interaction with the effect of the treatment $(F(1,18)=0.007) p=0.9321$. A main-effect of treatment was observed $(F(1,18)=19.03) p=0.0004$. Sidak analysis was performed for multiple comparisons on two-way ANOVA.

F. An average cumulative plot of active nose-poke responses during the vehicle- (blue) or finasteride- (orange) treated sessions (n=20). *p-value < 0.05, ** p-value<0.01, ***p-value<0.001. Error bars represent the mean +/- s.e.m. Adult male and adult female Wistar rats were used to perform the fentanyl self-administration assay. These experiments were performed using a within subject design.

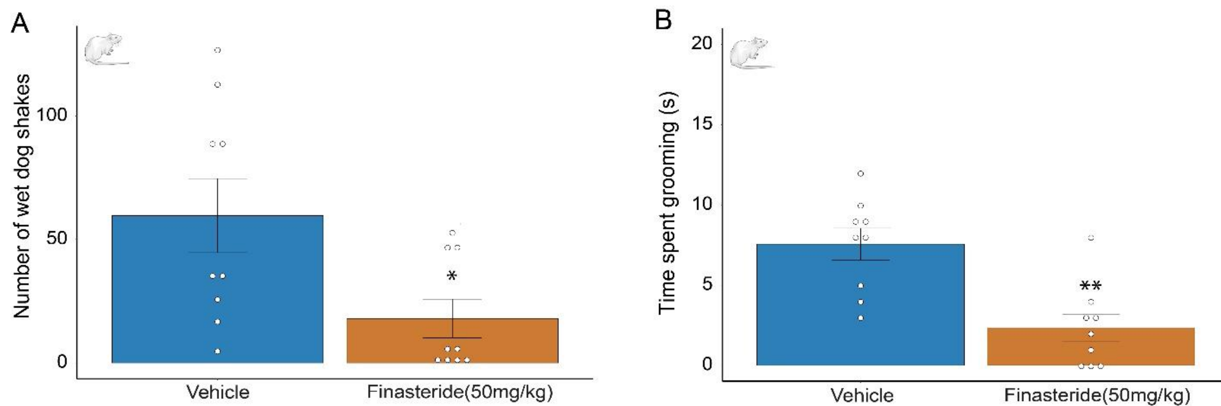


Figure 5. Finasteride reduces the physical effects of opioid withdrawal.

Animals were treated with escalating doses of morphine for five days. On day 6, rats received an acute dose of morphine (40 mg/kg, SC) followed by either finasteride (50mg/kg, IP) or vehicle. The opioid receptor antagonist naloxone (1.5mg/kg, IP) was administered 20 minutes later and animals were placed in a plexiglass chamber and their behavior was then recorded for 30 minutes. **A**: Acute injection of finasteride reduces the number of wet-dog shakes in animals in morphine withdrawal. **B**: Animals in withdrawal spent less time grooming after finasteride treatment. *p-value < 0.05, ** p-value<0.01. Error bars represent the mean +/- s.e.m. n=9 for both conditions. p-value calculated using unpaired t test with Welch's correction between finasteride- and vehicle-treated animals. Adult male Long Evans male rats were used to test naloxone-induced withdrawal. This experiment was performed with a between-subject design and blind analysis.

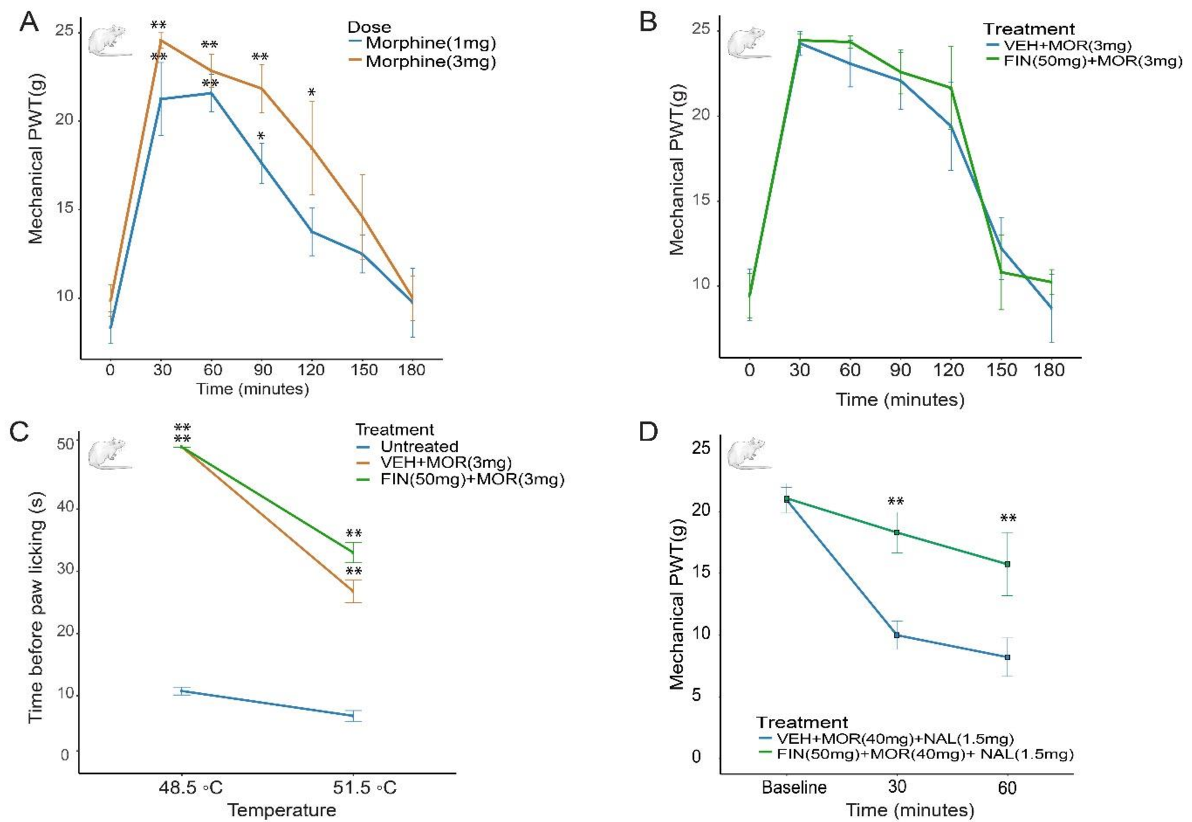
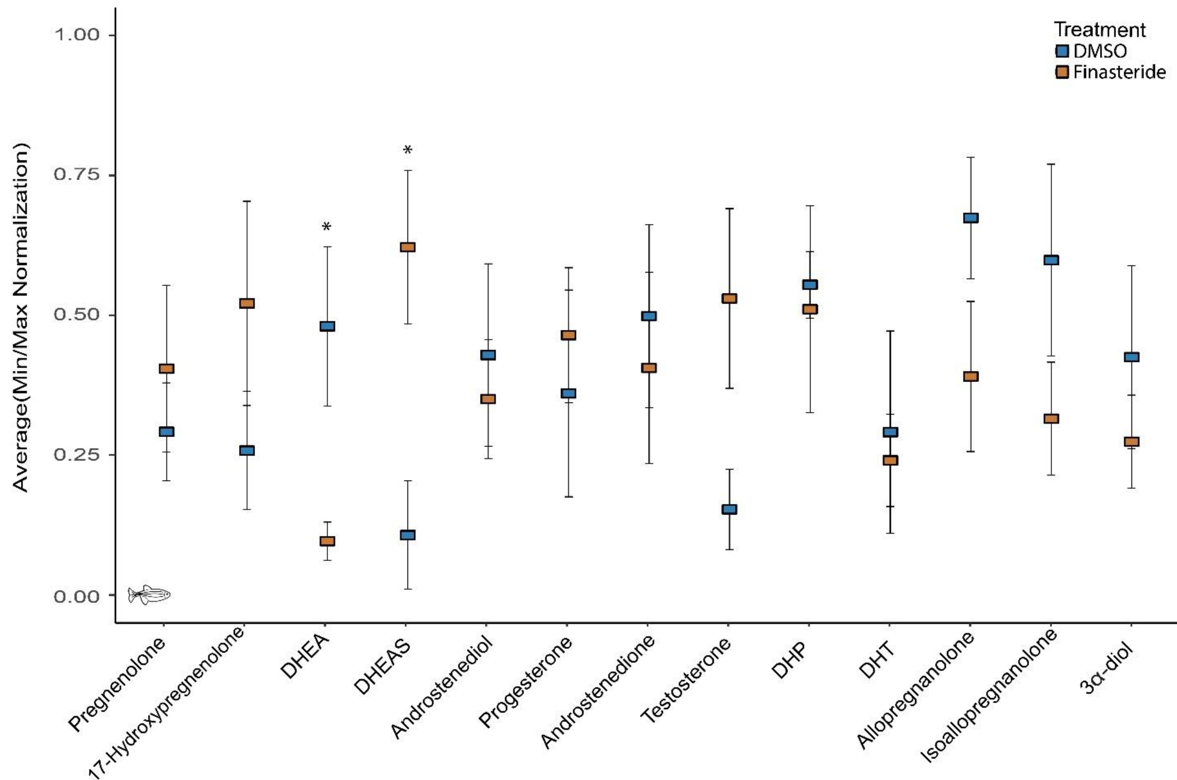


Figure 6. Finasteride does not affect the antinociceptive effect of opioids in a neuropathic pain model.

A. Paw withdrawal thresholds (PWT) to the Randall-Selitto test. Pain tolerance was measured over 180 min after treatment with different doses of morphine. Both doses of morphine significantly increased PWT compared to animals tested immediately after injection. Two-way ANOVA significant effect of Time ($F(6,72)=31.09$) $p<0.0001$. Morphine 1mg/kg, n=6, Morphine 3mg/kg, n=8 per condition. **B**. Co-treatment with finasteride (50mg/kg) did not block the antinociceptive effect of morphine (3mg/kg). Two-way ANOVA significant effect of Time ($F(6,48)=45.31$)$p<0.0001$, but no significant effect of treatment ($F(1,8)=0.1875$)$p=0.6764$ and no effect of interaction ($F(6,48)=0.3727$, $p=0.8927$. N=5 per condition. **C**. Finasteride did not affect the thermal antinociceptive effect of morphine as measured by the time before paw lick in response to different temperatures 30 min after treatment with morphine. Two-way ANOVA 48.5°C significant effect of Treatment $F(2,9)=770$)$p<0.0001$, 51.5°C significant effect of Treatment $F(2,9)=33.12$)$p<0.0001$. Both vehicle+morphine and finasteride+morphine are significantly different from untreated animals. No difference observed between vehicle+morphine and finasteride+morphine. N=4 per condition. **D:** Finasteride reduces hyperalgesia associated with opioid withdrawal. Rat subjected to SNL

and treated with a 6-day escalating morphine treatment were injected with finasteride (50mg/kg, IP) or vehicle. Mechanical nociception was measured immediately, on the injured paw, after naloxone treatment and repeated 30 and 60 min later. Two-way ANOVA significant effect of Interaction $F(2,20)=4.53)p=0.0238$, Time $F(2,20)=19.4)p<0.0001$, Treatment $F(1,10)=13.1)p=0.0046$.* p-value < 0.05, ** p-value<0.01. error bars +/- s.e.m. Adult male Sprague-Dawley rats were used to test the effect of finasteride treatment on morphine antinociceptive effect and adult male Long Evans rats were used for the naloxone-precipitated withdrawal experiment . These experiments were performed using a between subject design.



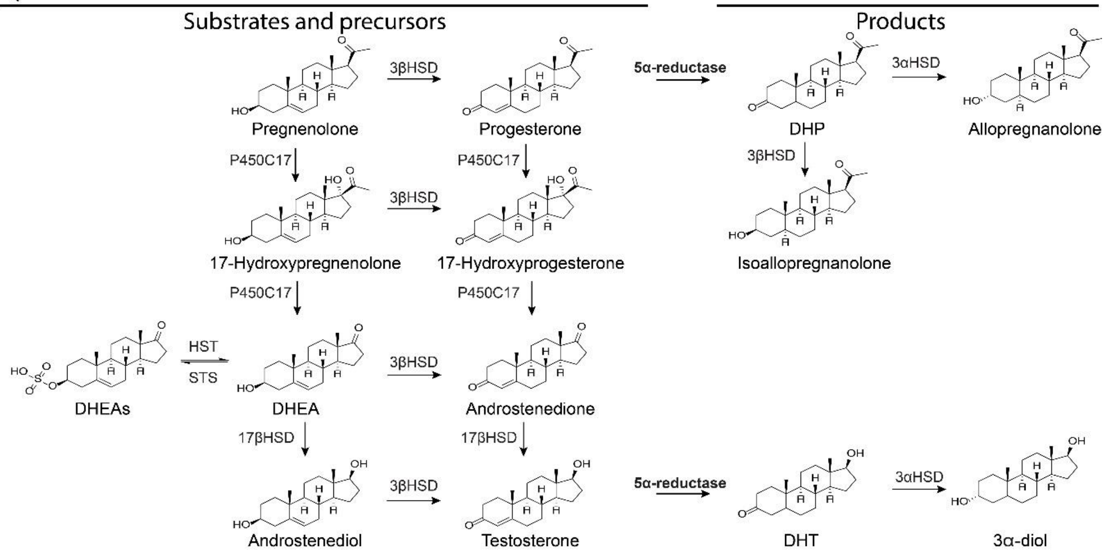


Figure 7. Finasteride treatment changes neurosteroid levels in the conditioned animal brain. A. Normalization score for the quantification of steroids in conditioned brains treated with DMSO or finasteride (10 µM). p-value calculated with Student's T-test. n=5 set of 10 brains per condition. *p-value<0.05 B. Partial neurosteroidogenesis pathways. Finasteride blocks the rate limiting enzyme, 5α-reductase, causing accumulation of upstream neurosteroid species.

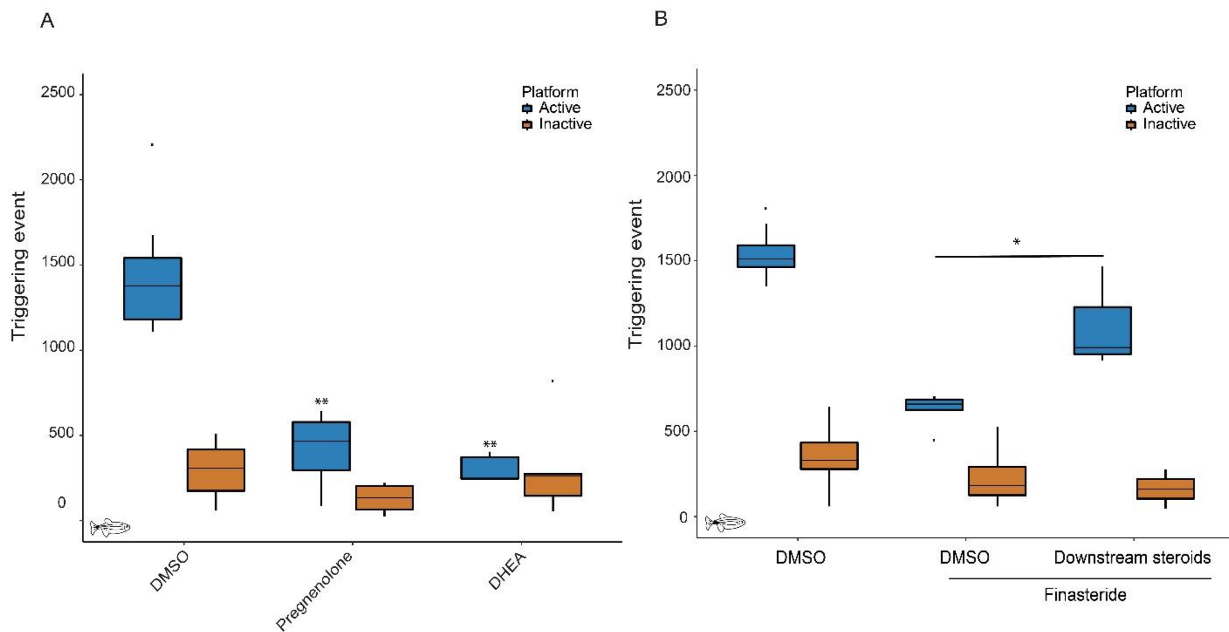


Figure 8. Specific neurosteroids also affect opioid self-administration.

A. Incubation with steroids upstream of 5αR, DHEA (10μM) and pregnenolone (10μM) reduces the number of triggering events at the active platform. *p*-value computed by Tukey HSD on one-way ANOVA, Inactive platform (F(2,21)=2.175)p=0.139 and Active platform (F(2,21)=51.09)p=8.57E-09. No significant difference was detected for the inactive platform compared to the DMSO control. DMSO n=12, Pregnenolone n=8, and DHEA n=5. **B**. Co-treatment with finasteride (10 μM) and a selection of steroids downstream of 5αR partially blocks the reduction in opioid self-administration induced by finasteride. *p*-value computed by Tukey HSD on one-way ANOVA, Inactive platform (F(2,16)=1.575)p=0.239 and Active platform (F(2,16)=68.93)p=1.37E-08. No significant difference was detected for the inactive platform. DMSO n=8, finasteride n=8, finasteride+downstream steroids n=3. **p*-value < 0.05, ** *p*-value<0.01. Each n represents a group of 15 animals.

Supplemental Materials

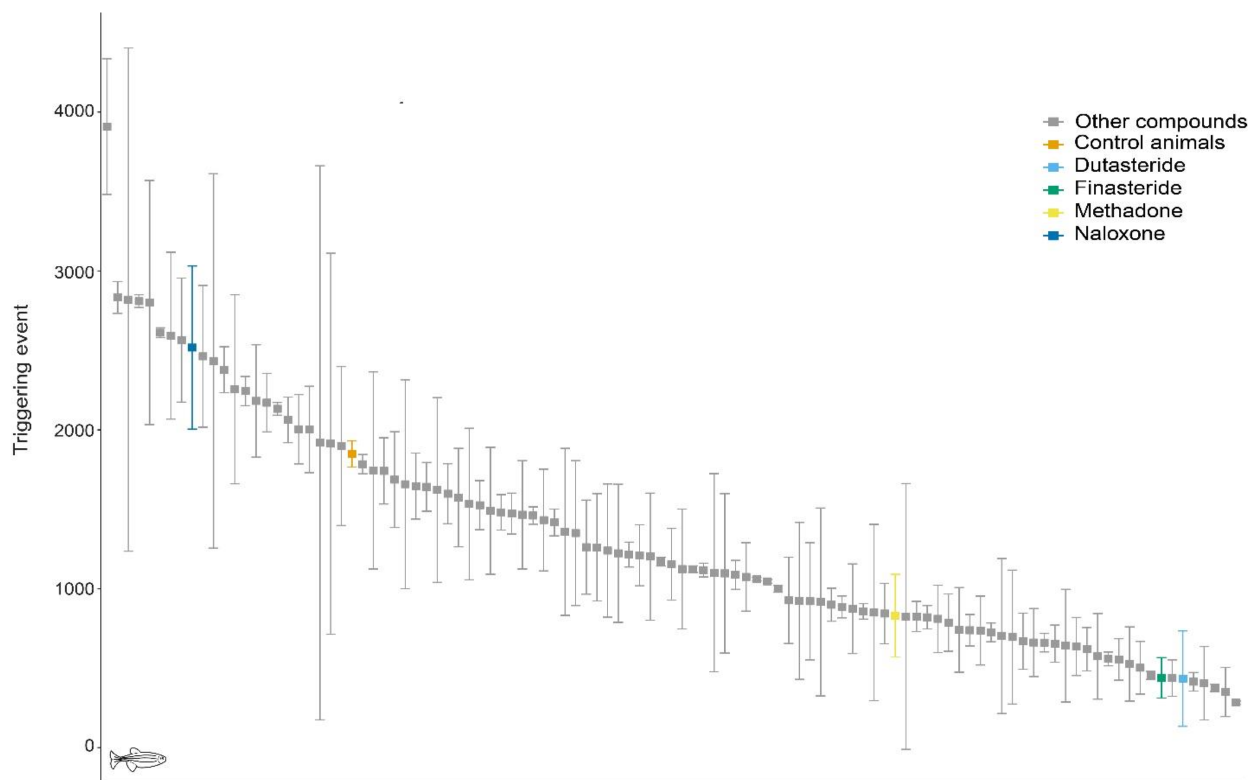


Figure S1: Results from the small molecule screen

Overview of the effect on the number of triggering events at the active platform for each of the 110 molecules tested during the screen. The average of triggering events for untreated conditioned groups of fish (15 fish per group, 46 groups tested) compared to the average for conditioned fish treated with each candidate molecule (15 fish per group, 2 groups per compound). Each compound was tested at 10 µM. Error bars represent mean +/- s.e.m.. These experiments were performed using a between-subject design. Two molecules were omitted from this figure due to a drastic effect on normal fish behavior (hypolocomotion, lethality). The name of each molecule is presented in Table S1.

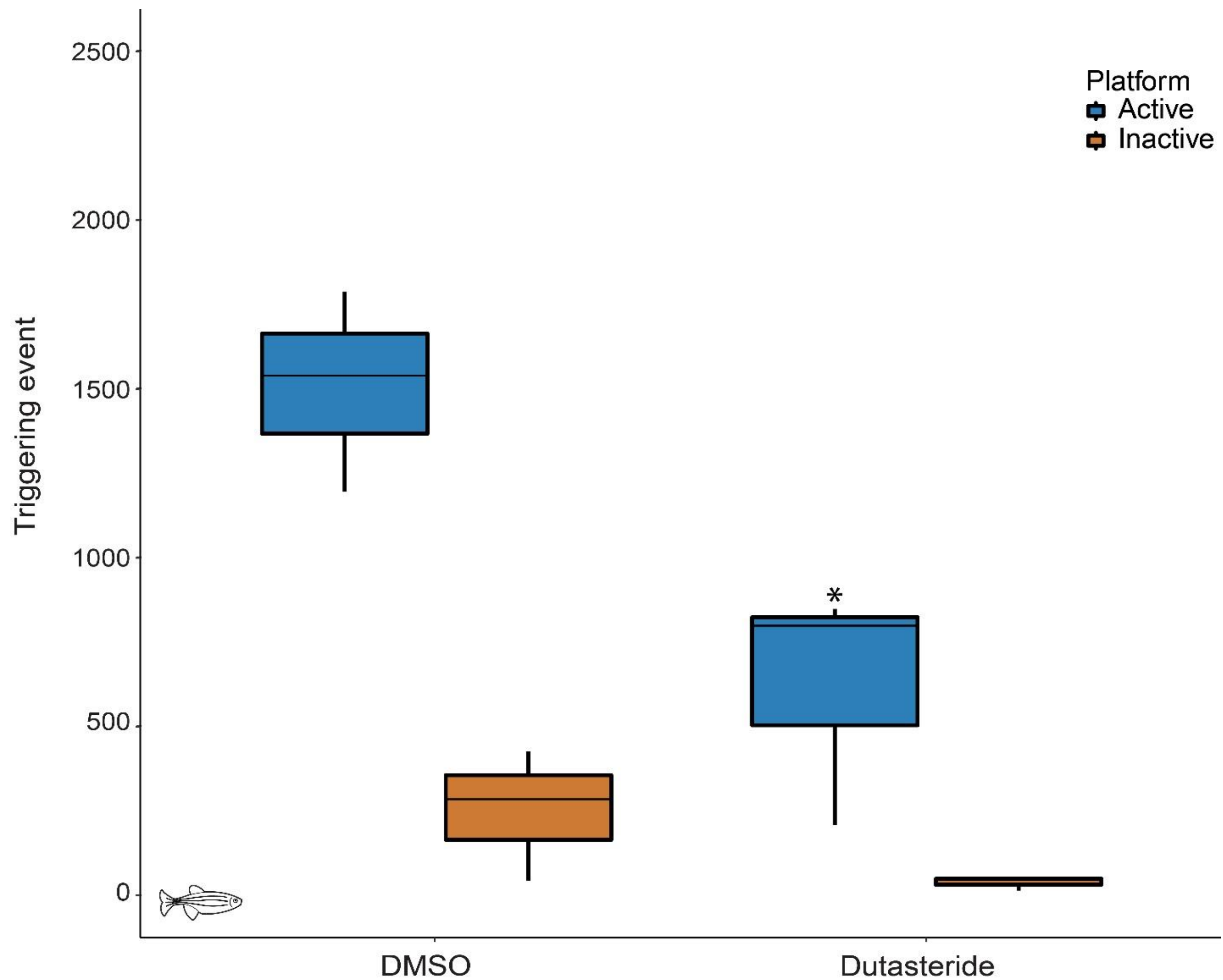


Figure S2: Dutasteride reduces opioid-self-administration

An additional 5αreductase inhibitor, dutasteride (10µM) also reduces the number of triggering events at the active platform. *p*-value computed by TukeyHSD on one-way ANOVA, no significant difference was observed at the inactive platform $(F(1,4)=3.65)p=0.13$ and reach significance for the active platform $(F(1,4),11.01)p=0.03$. DMSO n=3, dutasteride n=3. *p-value < 0.05. Each n represents a group of 15 animals. These experiments were performed using a within-subject design.

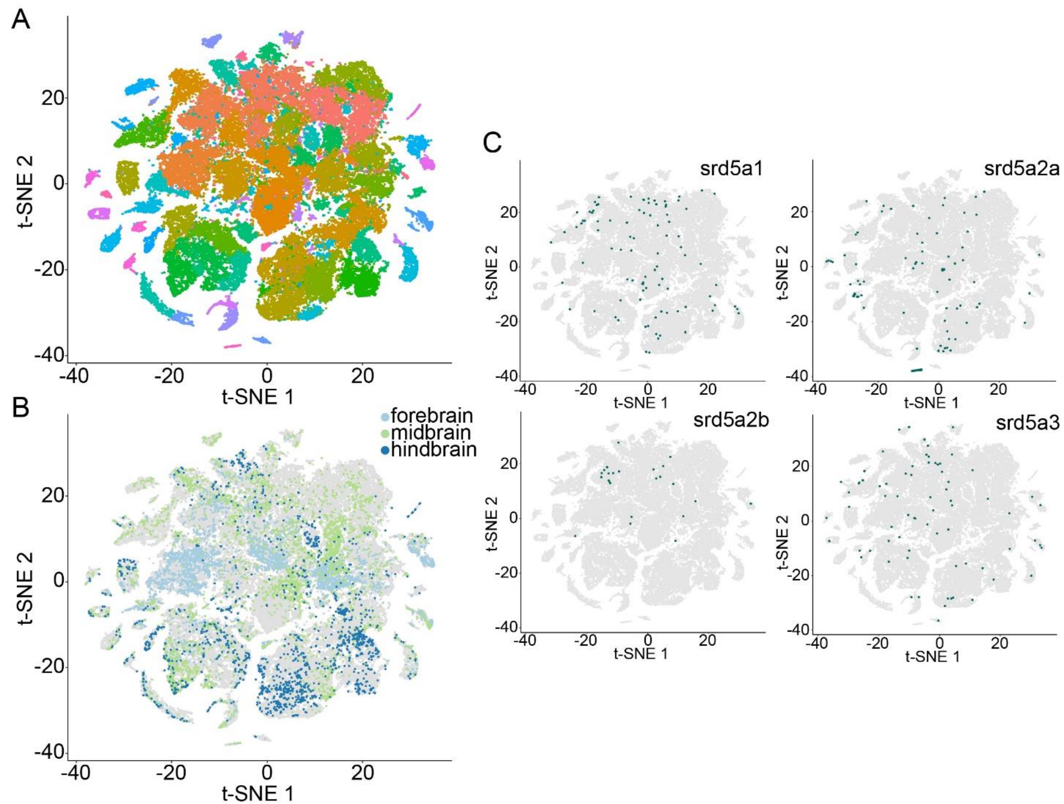


Figure S3: Srd5a family members display scattered expression in multiple brain regions.

Single Cell RNA-seq data from *Raj et al. Nature Biotechnology* was reanalyzed to investigate Srd5a family member expression in specific regions of the zebrafish brain. Individual cells are visualized as unique dots on the above plots using t-SNE dimensionality reduction. **A.** t-SNE plot representing the 61 clusters produced by the 65,718 cells from the zebrafish brain. Cells cluster together based on gene expression and the various clusters are shown by color. **B**. To demonstrate the broad brain regions across the single-cell data, samples originating from manually dissected fore-, mid- and hindbrains are highlighted in light blue, green, and dark blue respectively in this t-SNE plot. The cells colored grey originate from whole-brain samples (n=6) **C**. t-SNE plots, as in A and B, are shown here with cells expressing relatively medium to high levels of srd5a family members highlighted in green showing broad expression of srd5a across brain regions and cell types. Other cells displaying little to no expression detected for srd5a are colored grey. Expression cut-offs were determined for each marker individually based on expression distribution of scaled normalized counts (srd5a1 0.8-3.0; srd5a2a 1.0-3.0; srd5a2b 1.0-3.0; srd5a3 0.8-3.0).

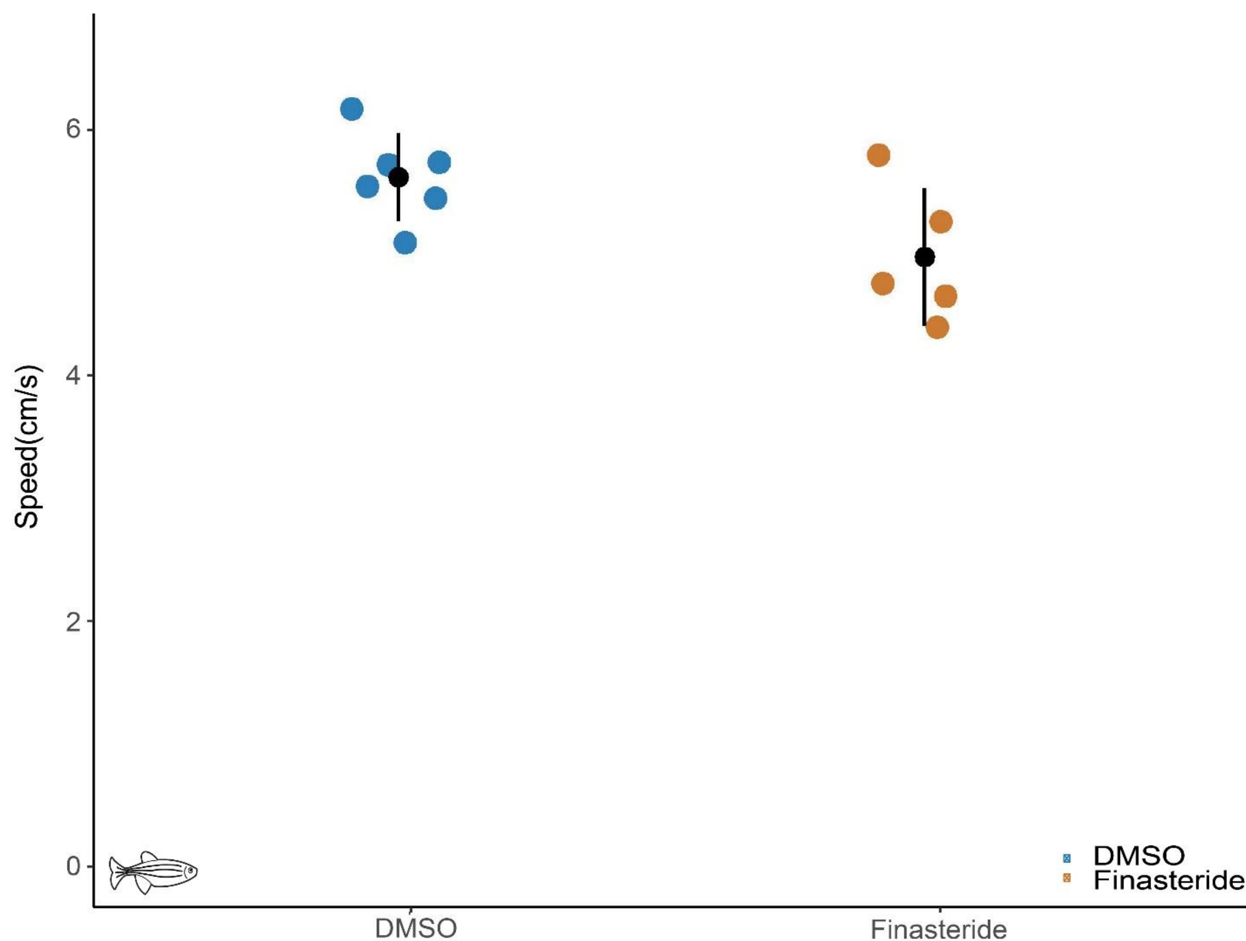


Figure S4: Locomotion is unaffected in finasteride-treated fish.

Data are the average speed of the animals in the arena (cm/s) from the different treatment conditions. DMSO n=6, Finasteride n=5. No significant difference. *p*-value computed by TukeyHSD on ANOVA ($F(2,14)=2.77$)$p=0.1$. Each n represents a group of 15 animals. These experiments were performed using a between-subject design.

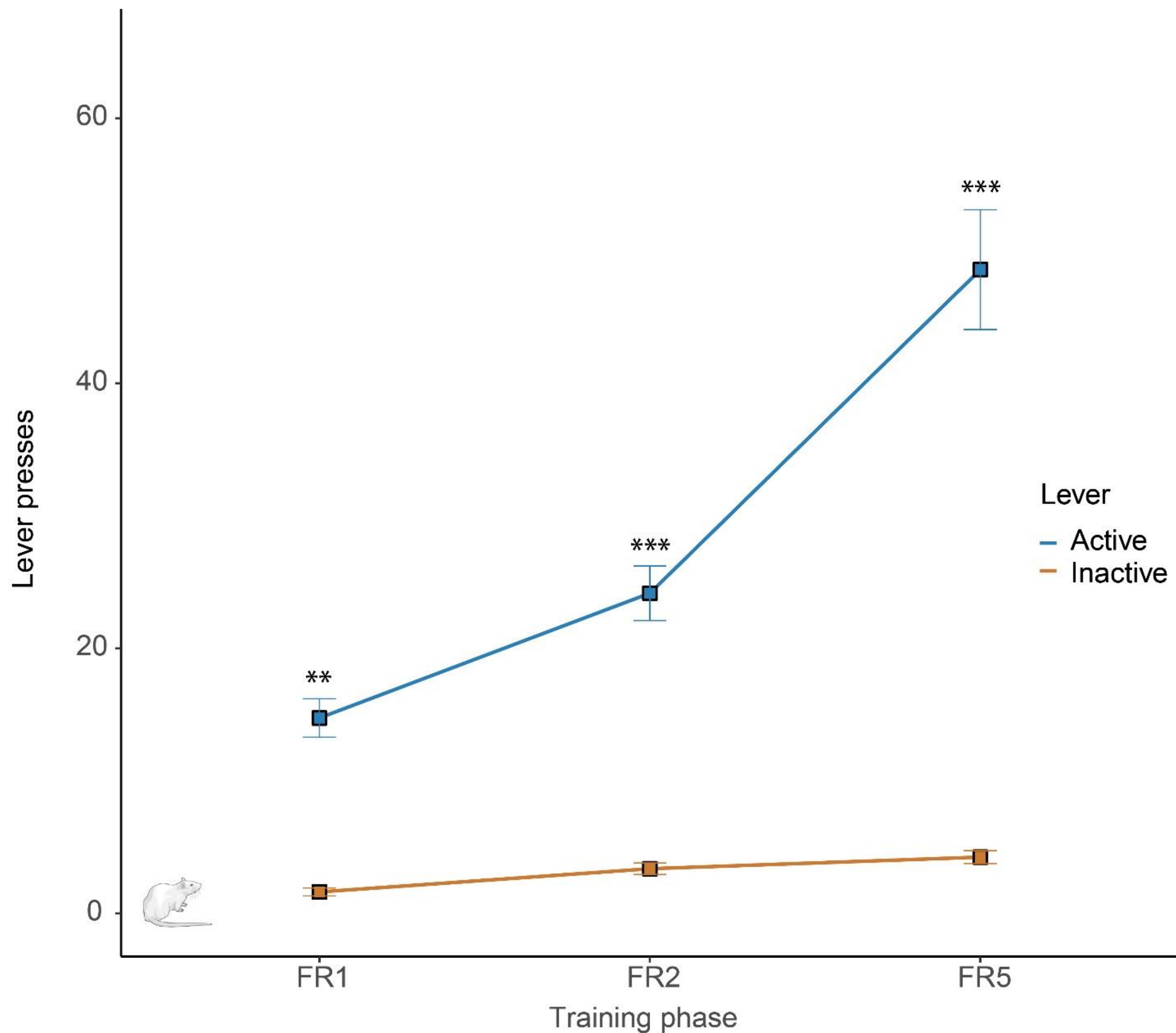


Figure S5: Number of lever press during the hydrocodone self-administration training phase.

Rats were conditioned to self-administer hydrocodone by pressing a lever. Each rat progress from FR1 to FR2 and from FR2 to FR5 after reaching stability, defined as >70% of total level presses on the active lever for three consecutive days. FR1 and FR2 stability criteria were reache from day 12 through 19 and from day 15 through d3 of training, respectively. All animal reached FR5 stability by day 31 of training and were then treated with either finasteride or its vehicle. Two-way ANOVA significant effect of interaction (F(2,138)=29.02, p<0.0001) lever (F(2,138)=38.29, p<0.0001), and phase (F(1,138)=224<p0.0001). *p*-value < 0.05, ****p*-value<0.001, *****p*-value<0.0001. Error bars represent the mean +/- s.e.m. N=24 animals. Adult male Sprague-Dawley rats were used to perform the hydrocodone self-administration assay.

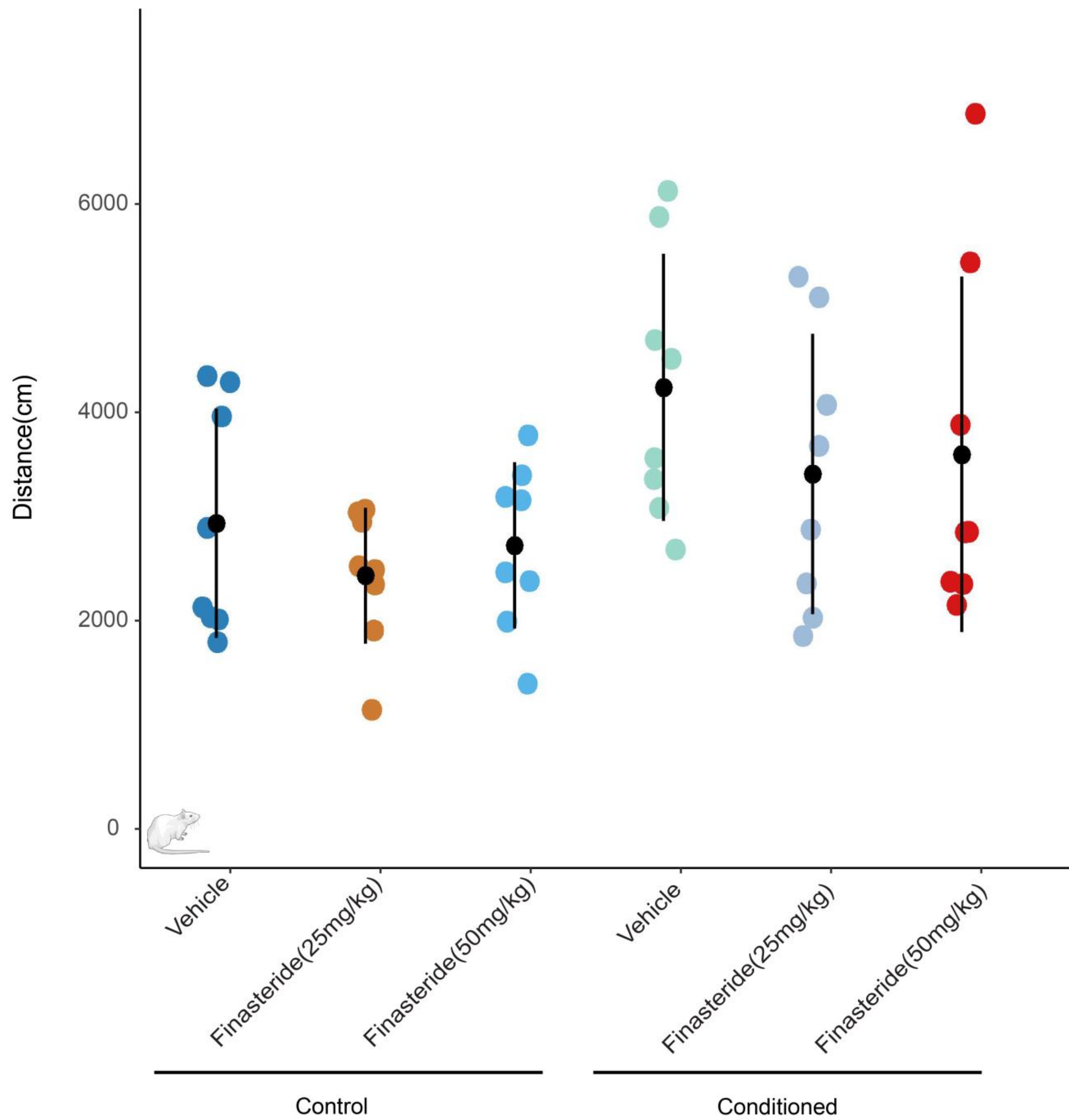


Figure S6: Locomotion is unaffected in finasteride-treated rats

Locomotion in the open-field test of rats treated with finasteride at 25 or 50 mg/kg, i.p.is not changed compared to corresponding vehicle-treated animals. N=8 per condition. Errors bars represent mean +/- s.e.m. Adult male Sprague-Dawley rats were used to measure locomotion. These experiments were performed using a within-subject design.

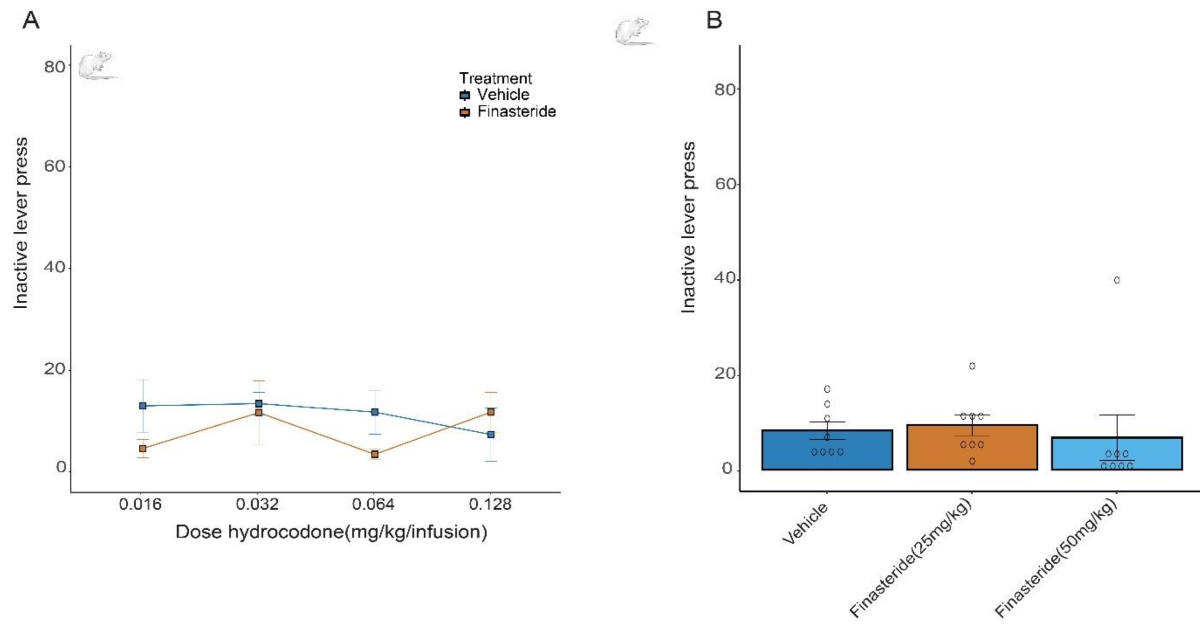


Figure S7. No difference in inactive lever press during hydrocodone self-administration in rats

A. Pretreatment with 50mg/kg finasteride did not alter the number of inactive lever presses, regardless of the dose of hydrocodone. n=6 per condition Two-way ANOVA($F(3,38)=0.07, p=0.97$) **B.** Different doses of finasteride did not affect inactive lever presses for animals conditioned with 0.064mg/kg of hydrocodone. ANOVA($F(2,21)=0.19$)$p=0.82$. error bars represent mean +/- s.e.m. Adult male Sprague-Dawley rats were used to measure hydrocodone self-administration. These experiments were performed using a within-subject design.

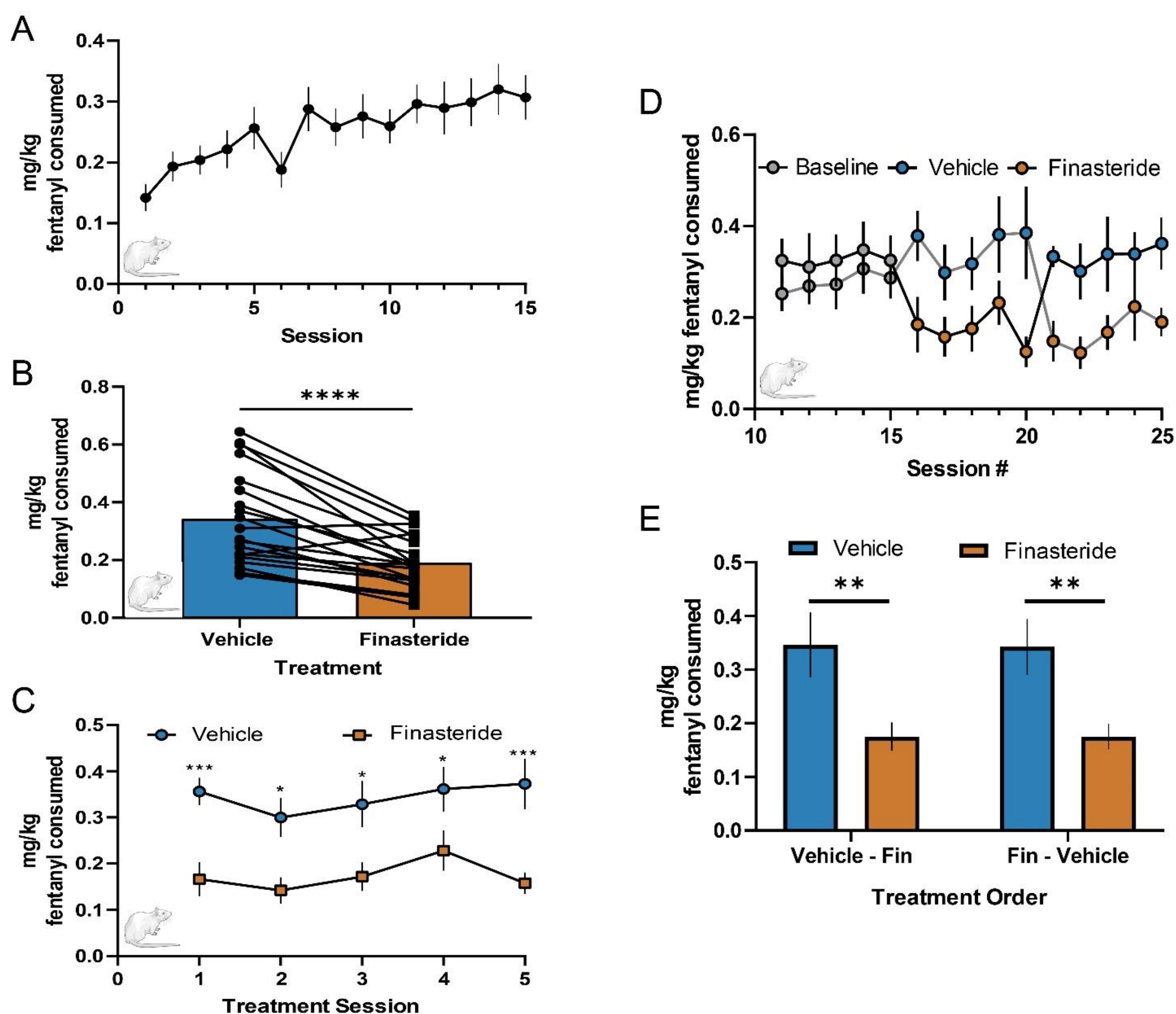


Figure S8. Finasteride decreases fentanyl consumption in rats.

A. Average fentanyl consumed in mg/kg for each baseline fentanyl self-administration session (n=20 rats). **B.** Average mg/kg of fentanyl consumed during vehicle (blue) and finasteride (orange) pretreated sessions. A paired t-test revealed finasteride treatment decreased the amount of fentanyl consumed compared to vehicle ($t(19)=5.720$)$p<0.0001$. **C.** Daily injections of finasteride (50mg/kg, i.p.; orange squares) significantly decreased fentanyl consumption compared to daily injections of vehicle (blue circles). A main effect of drug treatment was observed, Sidak post-hoc analysis performed for multiple comparisons on mixed-effect model ($F(1,19)=26.94$)$p<0.0001$. **D & E.** Animals either received injections of finasteride during self-administration sessions 16-20 (n=10 rats, D: black line, E: Fin–Vehicle) or during

sessions 21-25 (n=10 rats, D: gray line, E: Vehicle–Fin). **D.** Baseline refers to animals responding during sessions 10-15. **E.** The order of treatment had no effect on fentanyl consumed ($F(1,18) = 0.0017$, $p=0.9670$). No statistical interaction was observed between treatment and treatment order ($F(1,18)=0.0025$)$p=0.9604$. A main-effect of finasteride treatment was present ($F(1,18)=22.85$)$p = 0.0001$. *p*-values corrected for multiple comparisons on two-way ANOVA. *p*-value < 0.05, ****p*-value<0.001, *****p*-value<0.0001. Error bars represent the mean +/- s.e.m. Adult male and adult female Wistar rats were used to perform the fentanyl self-administration assay. These experiments were performed using a within-subject design.

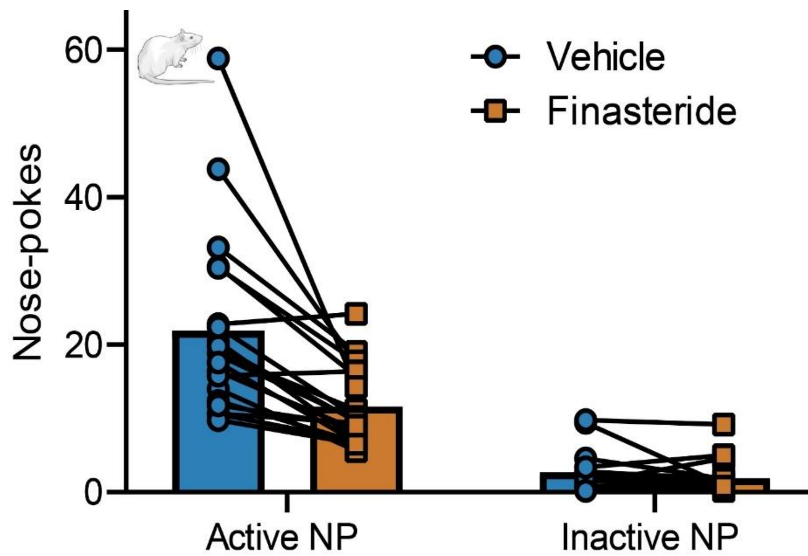


Figure S9: No difference in inactive nose pokes in fentanyl-conditioned rats

Finasteride didn't affect the number of nose pokes (NP) at the inactive port. Average operant nose-poke responses at both the active and inactive ports during finasteride (orange) and vehicle (blue) treatment sessions. N=20. Adult male and adult female Wistar rats were used to perform the fentanyl self-administration assay. These experiments were performed using a within-subject design.

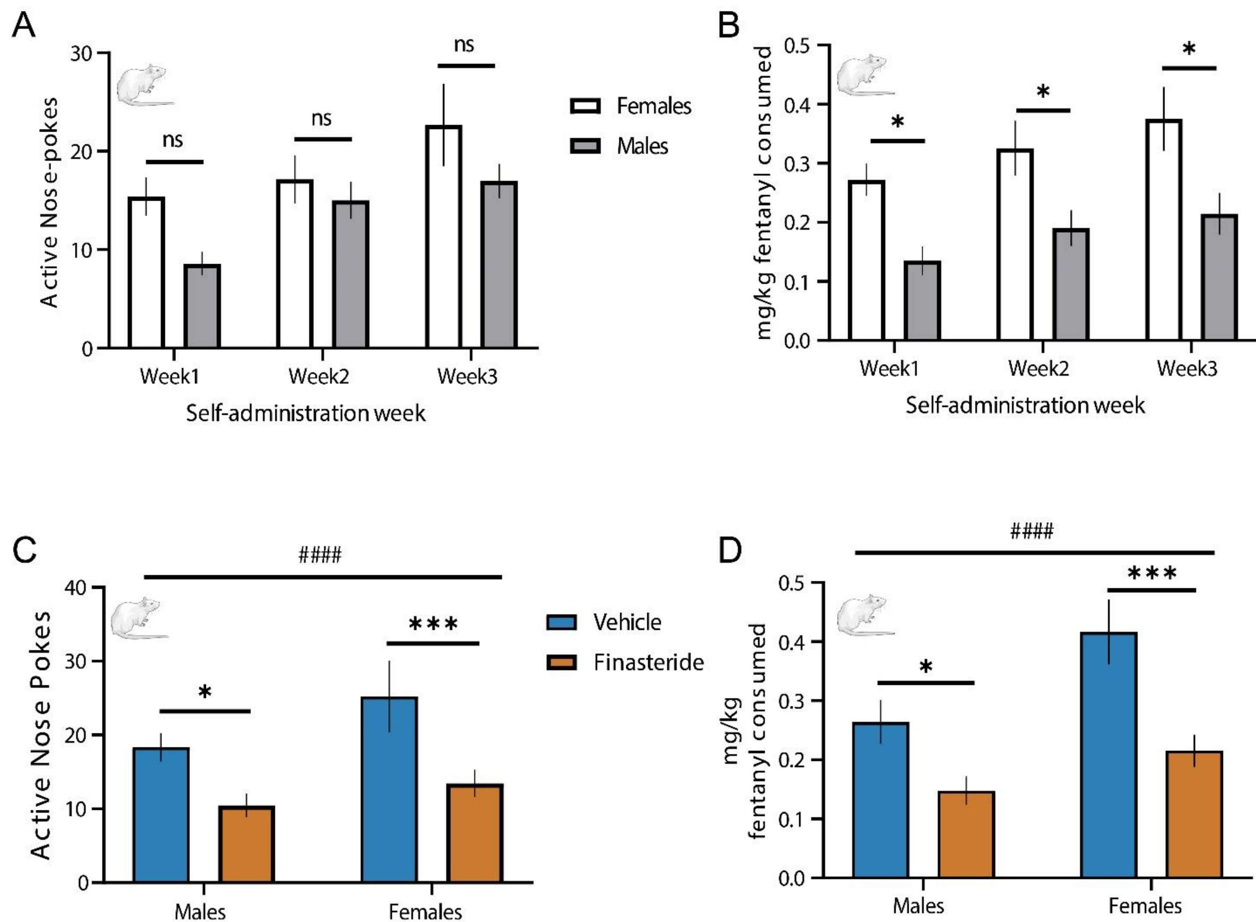


Figure S10. Finasteride decreases oral fentanyl self-administration behaviors in male and female rats.

A. Average operant nose-poke responses per session at the active port within each week of baseline fentanyl self-administration in male (filled bars) and female (open bars) Wistar rats. There was no observed statistical effect of sex on active nose-pokes $(F(1,17)=2.821)p=0.1113$ nor was there a significant self-admin week by sex interaction $(F(2,34) = 0.9610)p=0.3927$. A significant main effect of self-admin week was observed $(F(2,34) = 9.848)p = 0.0004$. **B.** Average mg/kg fentanyl consumption per session during each baseline week of oral fentanyl self-administration grouped by sex. There was no significant interaction between self-admin week and sex $(F(2,34) = 0.1499)$. Statistical significance was observed in both main effects of sex $(F(1,17) = 10.00)p = 0.0057$ and self-admin week $(F(2,34) = 6.299)p = 0.0047$. A Sidak post-hoc analysis revealed female rats on average consumed more mg/kg of fentanyl in each session (?) during each week of baseline fentanyl self-administration. **C.** Average operant nose-poke responses during finasteride (orange) and vehicle (blue) treatment sessions. There was no

difference in the effect of finasteride on active nose-pokes between the sexes, as evidenced by the lack of a significant sex x treatment interaction in the two-way ANOVA ($F(1,18)= 1.148$)$p= 0.2981$. An overall main effect of drug treatment was observed ($F(1,18)= 29.22$)$p < 0.0001$. **D.** Average mg/kg fentanyl consumption during finasteride (orange) and vehicle (blue) treatment sessions. There was no observed sex difference in the effect of finasteride on mg/kg of fentanyl consumed as no significant interaction was present between sex and treatment in a two-way ANOVA ($F(1,18)=2.069$, $p=0.1674$). There were main effects of both sex ($F(1,18)=6.110$, $p=0.0237$) and treatment ($F(1,18)=28.82$, $p<0.0001$). **p*-value < 0.05, ****p*-value<0.001, ##*p*-value<0.01 and indicates a main effect of treatment. Error bars represent the mean +/- s.e.m. Adult male and adult female Wistar rats were used to perform the fentanyl self-administration assay. These experiments were performed using a within-subject design.

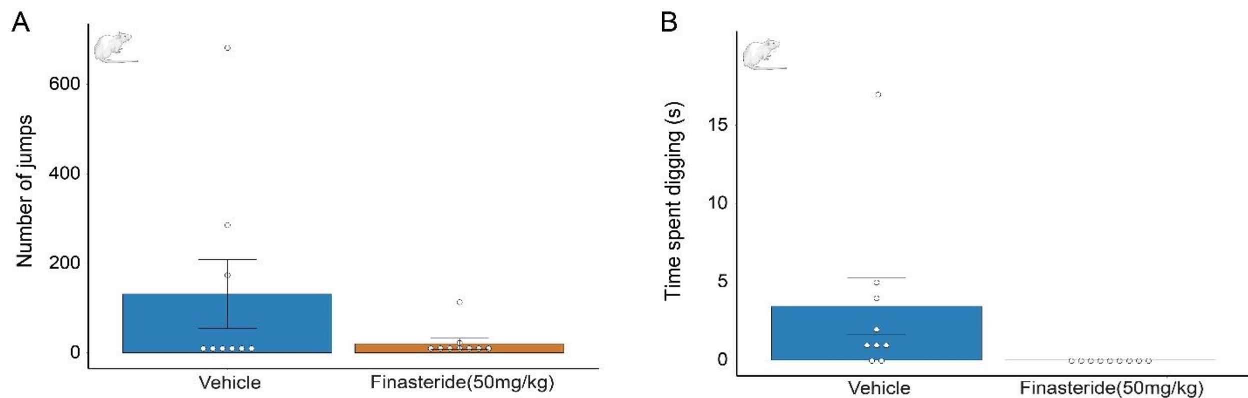


Figure S11. Finasteride did not fully reverse the jumping and digging responses induced by the opioid antagonist.

Animals were treated with an escalating dose of morphine for five days. On day 6, rats received an acute dose of morphine (40 mg/kg, s.c) followed by either finasteride (50mg/kg, i.p) or vehicle. The opioid receptor antagonist naloxone (1.5 mg/kg, i.p.) was administered 20 minutes later and animals were placed in a plexiglass chamber. Behavior was then recorded for 30 minutes. **A**: Acute injection of finasteride did not fully reverse the number of jumps in animals experiencing morphine withdrawals. **B**: Although finasteride reduces the time spent digging, it did not reach significance. Error bars represent the mean +/- s.e.m. n=9 for both conditions. *p*-value calculated using Unpaired t-test with Welch's correction between finasteride and vehicle-treated animals. Adult male Long Evans male rats were used to test naloxone-precipitated withdrawal. This experiment was performed with a between-subject design and blind analysis.

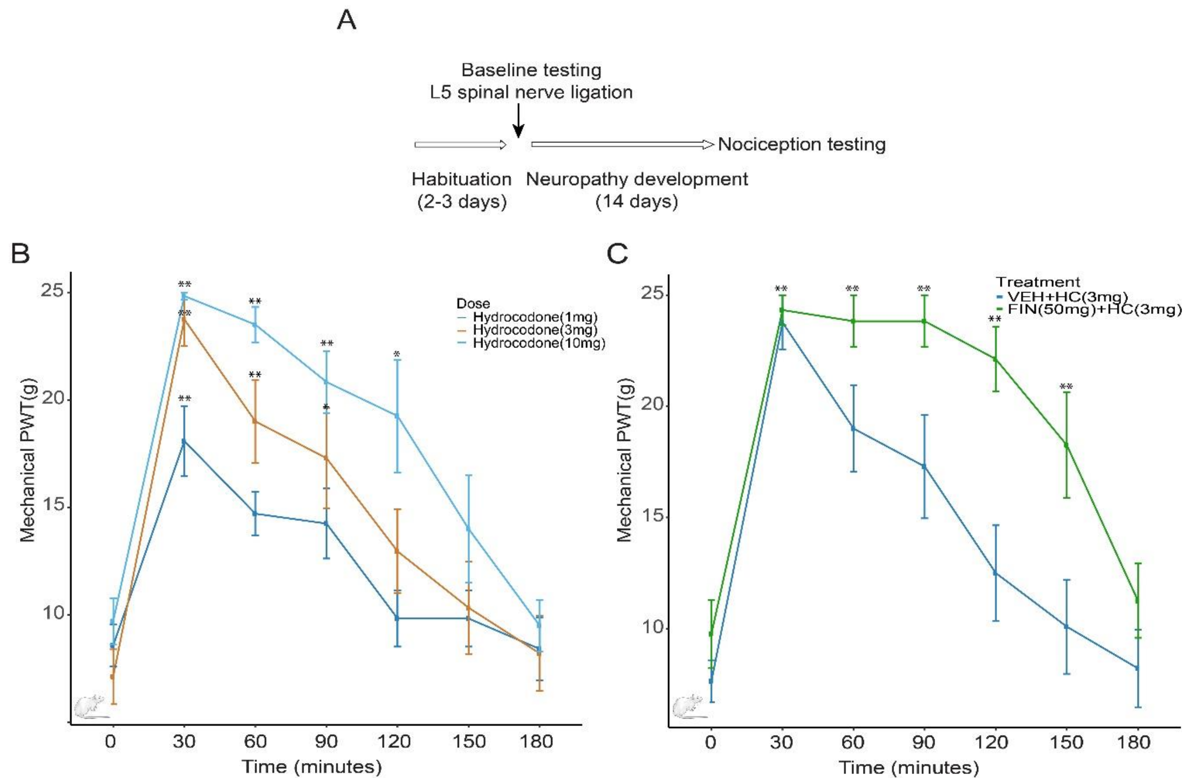


Figure S12. Randall Sellito hydrocodone and finasteride.

A. After a 2-3 day habituation period, animals were assessed for baseline nociception tolerance followed by surgical L5 spinal nerve ligation. Neuropathy was established for 14 days before animals were tested for nociception with the different treatments. **B**. Paw withdrawal thresholds (PWT) Randall-Selitto assay. The withdrawal thresholds to paw pressure were measured up to 180 minutes after treatment with different doses of hydrocodone. Different doses of hydrocodone significantly increase latency to withdrawn the paw when a pressure in g is applied, compared to animals tested immediately after injection. Two-way ANOVA significant for TimeF(6,90)=53,90)p<0.0001 and Treatment F(2,15)=4.499)p=0,0295. N=6 per conditions **C**. Co-treatment with Finasteride (50mg/kg) did not block the antinociceptive effect of hydrocodone(10mg/kg) N=6 per condition. P compared with values measured before injection(Time 0)*p-value < 0.05, ** p-value<0.01. Adult male Sprague-Dawley rats were used to test the effect of finasteride treatment on morphine antinociceptive effect. These experiments were performed using a between-subject design.

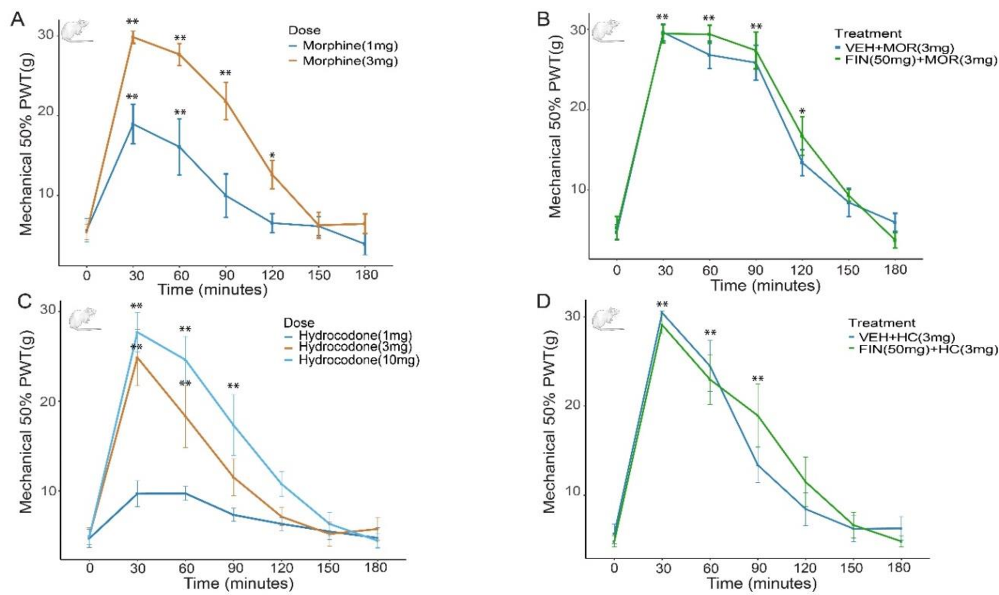


Figure S13. Von-Frey morphine and hydrocodone and finasteride

Finasteride does not affect the antinociceptive effect of opioids in a neuropathic pain model; paw withdrawal thresholds (PWT) to Von Frey filament. **A**. Different doses of morphine increase the PWT threshold. Two-way ANOVA revelaed a significant effect of time (F(6.72)=56.41)p<0.0001 and treatement (F(1.12)=14.40)p=0.0026. 1mg/kg n=6, 3mg/kg n=8 **B**. Co-treatment with finasteride(50mg/kg, IP) did not block the antinociceptive effect of morphine(3mg/kg). Two-way ANOVA significant effect of time (F(6.72)=125.4)p<0.0001, but no effect of treatment (F(1.12)=0.3659)p=0.5565 and no significant interaction (F(6,72)=0.4152)p=0.8666. Vehicle n=6, Finasteride n=8 **C**. Hydrocodone at doses of 3 and 10 mg/kg increased the PWT to Von Frey filament. N=6 per condition Two-way ANOVA identified significant main effects of time (F(6.126)=56.67)p<0.0001 and treatment (F(2,21)=9.488)p=0.0012. **D**. Co-injection with finasteride(50mg/kg) did not affect the antinociceptive activity of hydrocodone (10mg/kg), as the two-way ANOVA revealed a significant effect of time (F(6.72)=60.06)p<0.0001, but no main effect of treatment (F(1,12)=0.04560)p=0.8345 and no significant treatment x time interaction (F(6,72)=1.374)p=0.2368. Vehicle n=6, Finasteride n=8. P compared with values measured before injection(Time 0), **p*-value < 0.05, ** *p*-value<0.01. Adult male Sprague-Dawley rats were used to test the effect of finasteride treatment on morphine antinociceptive effect. These experiments were performed using a between-subject design.

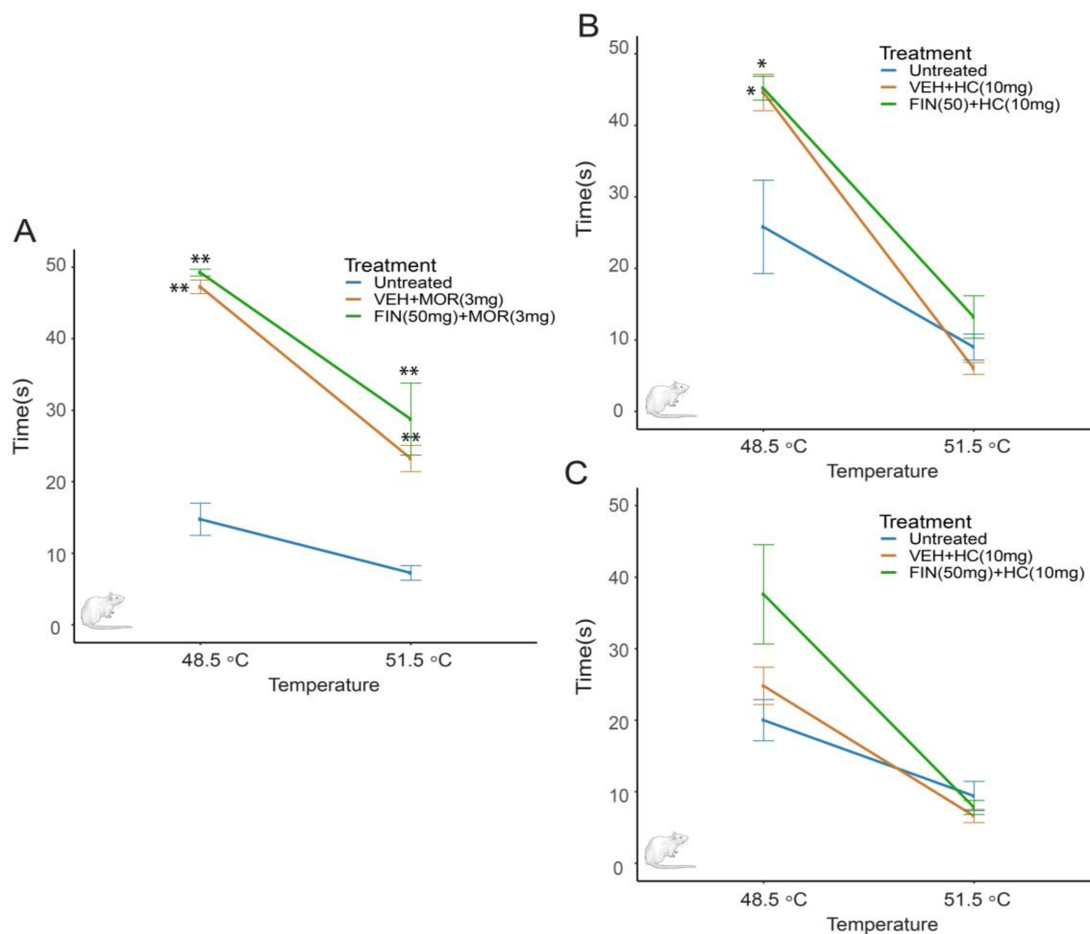


Figure S14. Hot-plate morphine and hydrocodone and finasteride.

Finasteride didn't affect the thermal antinociceptive effect of hydrocodone, as measured by the time before paw lick/retraction at different temperatures. *p* value compared with untreated animals. **A**. Finasteride didn't affect the thermal antinociceptive effect of morphine administered 60 min prior to the test, which measured the time before paw lick at different temperatures. 48.5°C :Two-Way ANOVA significant effect of treatment (F(2.9)=770.3)p<0,0001. 51.5°C two way-ANOVA significant effect of treatment (F(2.12)=7.66)p=0.0072. Both vehicle+morphine and finasteride+morphine are significantly different from untreated animals. No difference observed between vehicle+morphine and Finasteride-Morphine. **B**. Finasteride did not affect the thermal antinociceptive effect of hydrocodone administered 30 min prior to the test, which measured the time before paw lick at different temperatures. Two-Way ANOVA significance effect of treatment (F(2.12)=6.1119)p=0.0147 N=5 per condition. Both vehicle+hydrocodone and finasteride+hydrocodone are significantly different from untreated animals at 48.5°C. No difference observed between vehicle+hydrocodonde and finasteride+hydrocodone. **C**.

Hydrocodone given 60 min prior to the test did not have a thermal antinociceptive. N=5 $*p$-value < 0.05, $**p$-value <0.01. Adult male Sprague-Dawley rats were used to test the effect of finasteride treatment on morphine antinociceptive effect. These experiments were performed using a between-subject design.

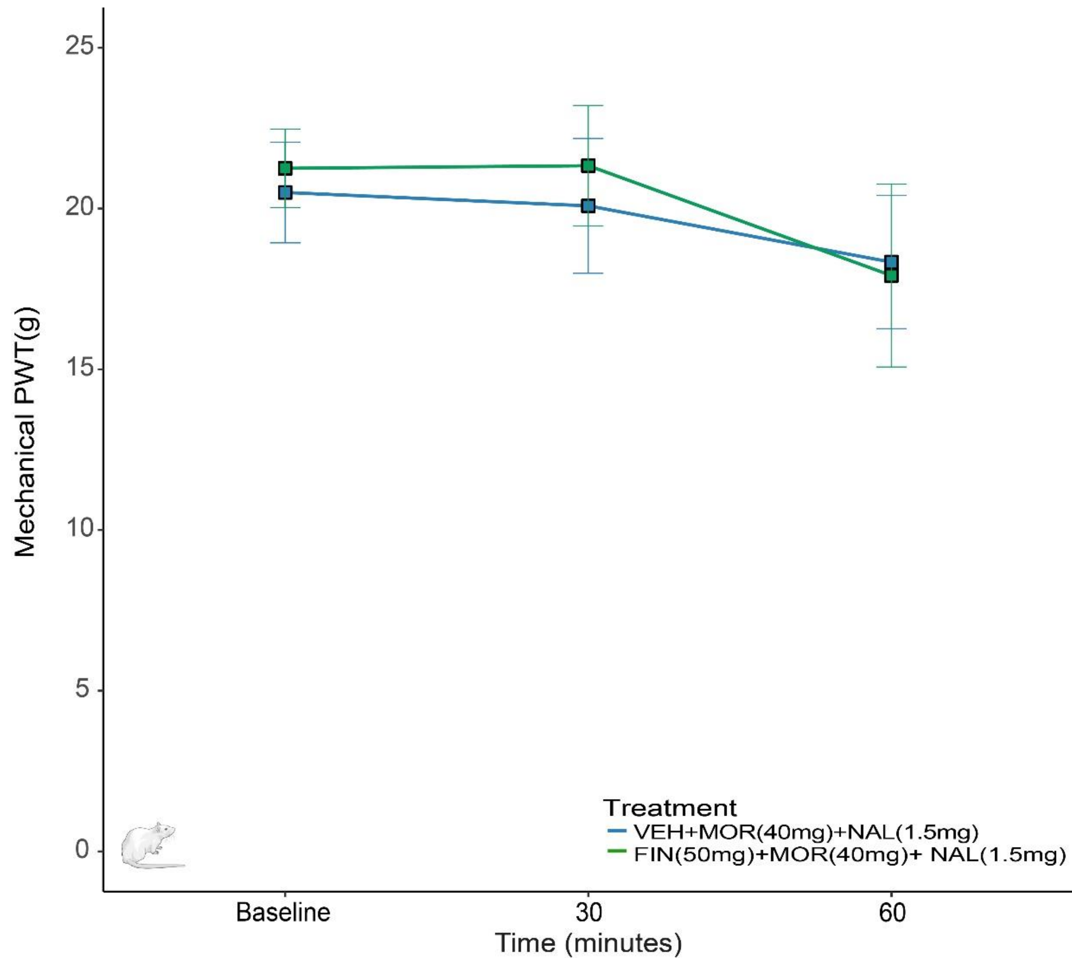


Figure S15: Finasteride does not affect pain tolerance on the un-injured paw

Rats subjected to SNL and treated with a 6-day escalating morphine treatment were injected with finasteride (50mg/kg, i.p.) or vehicle. Mechanical nociception was measured immediately after naloxone treatment and repeated 30 and 60 min later. Two-way ANOVA no significant effect of Interaction $F(2,20)=0.09334$)$p=0.9113$, Time $F(2,20)=1.213$)$p=0.3182$, Treatment $F(1,10)=0.09746$)$p=0.09746$ error bars +/- s.e.m. n= 6 per condition. Adult male Sprague-Dawley rats were used to test the effect of finasteride treatment on morphine antinociceptive effect. These experiments were performed using a between-subject design.

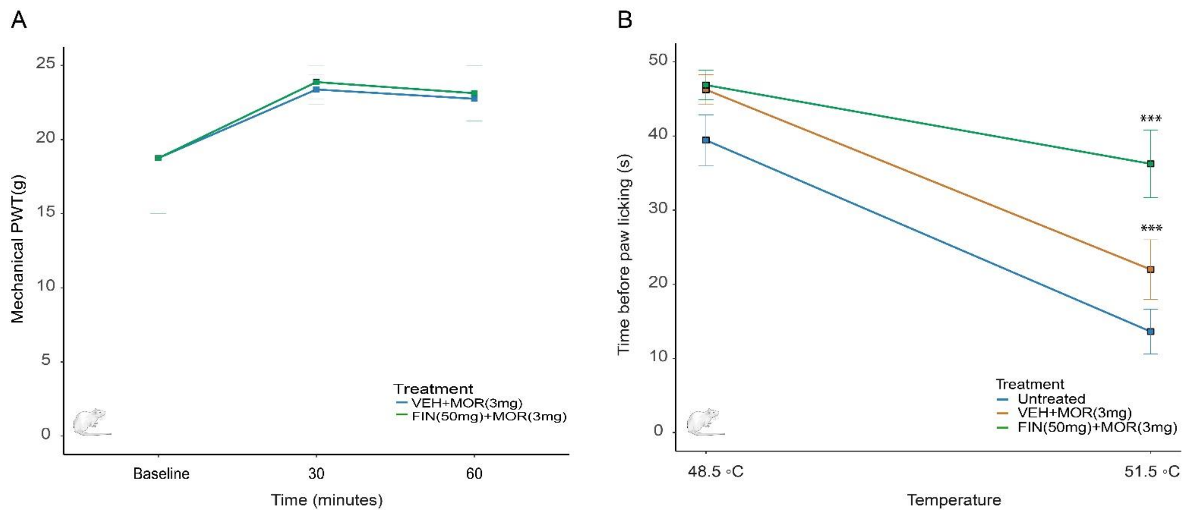


Figure S16 Finasteride affects heat sensation in healthy rat

A. Paw withdrawal thresholds (PWT) to the Randall-Selitto test. Pain tolerance was measured over 60 min after treatment with morphine and either finasteride (50mg/kg) or vehicle. Treatment with either morphine+vehicle or morphine+finasteride does not affect mechanical pain tolerance in healthy rats. Two-way ANOVA no significant effect of Time (F(2,12)=2.587)p=0.1164, treatment (F(1,6)=0.01465)p=0.9076 and no effect of interaction (F(2,12)=0.006290, p=0.9937. N=4 per condition. **B**. Finasteride did not affect the thermal antinociceptive effect of morphine as measured by the time before paw lick in response to different temperatures 30 min after treatment with morphine. Two-way ANOVA significant effect of Treatment F(2,21)=8.814)p=0.0117. Both vehicle+morphine and finasteride+morphine are significantly different from untreated animals. No difference was observed between vehicle+morphine and finasteride+morphine. N= 8 per condition. **p*-value < 0.05, ** *p*-value<0.01, *** *p*-value<0.001. error bars +/- s.e.m. Adult male Sprague-Dawley rats were used to test the effect of finasteride treatment on morphine antinociceptive effect. These experiments were performed using a between-subject design.

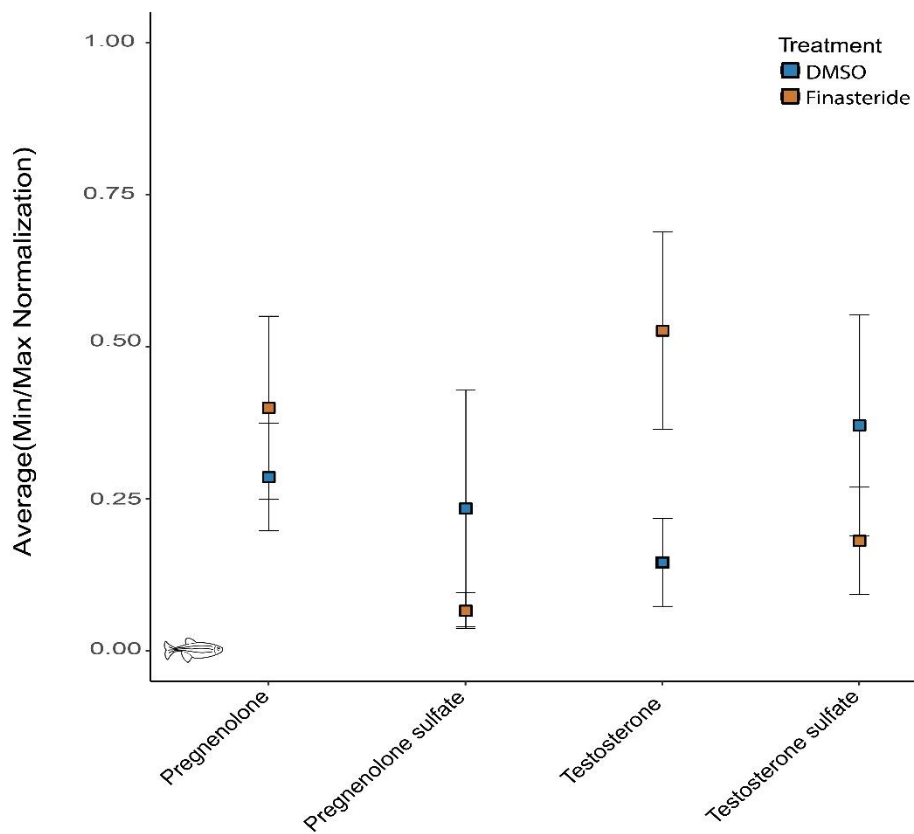


Figure S17. Other sulfated steroids are not accumulating in finasteride-treated fish.

Other sulfated steroids were not affected by the treatment with finasteride. Data are the normalization scores for the quantification of steroids in conditioned brains treated with DMSO or finasteride (10 μM). N=5 per condition. Each n represents a set of 10 brains.

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Chapter 6: Finasteride reduces cocaine self-administration and decreases incubation of cue craving

This chapter consists of preliminary data that was collected in collaboration with Lauren Burgeno and Nicole Murray. This data was one of the underlying motivations of the work shown in Chapter 5.

Introduction

The diametrically opposed changes that occur throughout the pathology of addiction as indicated by Chapter 2; highlight the difficulties in establishing a treatment that would be beneficial regardless of the stage of addiction. For instance, we observed a decrease in Nucleus Accumbens (NAc) cue evoked dopamine transmission, which has an inverse relationship with drug-consumption. A large portion of the work presented within this text has been dedicated to elucidate and treat the mechanisms responsible for this attenuation. However, we also demonstrate a causal relationship between increases in cue evoked dopamine transmission and drug-seeking behaviors. Thus, an ideal pharmacological treatment would stabilize dopamine transmission, preventing attenuated dopamine signaling to keep drug consumption moderate, and inhibit increases in dopamine transmission to prevent drug-seeking.

Through collaboration with Dr. Marco Bortolato we became aware and interested in the 5α-reductase inhibitor, finasteride, as a potential therapeutic for substance use disorders. This medicinal drug is currently FDA approved for male patterned hair loss, and benign prostatic hyperplasia. Interestingly, finasteride decreases CRF expression within the paraventricular nucleus, (Godar et al., 2019), and antagonists of CRF has been shown to decrease drug consumption [Chapter 2, Figure 4B] (Specio et al., 2008; Greenwell et al., 2009; Roberto et al.,

2010). As such we tested if finasteride had an effect on drug consumption within a rodent model of cocaine self-administration.

Another interesting outcome of finasteride is that during hypodopamienrgic states, which lead to upregulated D3 expression (Fanni et al., 2019; Lanza & Bishop, 2021), it decreases striatal D3 and D1 co-expression (Fanni et al., 2019). Moreover, it has been demonstrated that there are low dopamine levels during abstinence (PARSONS ET AL., 1991; CHEFER & SHIPPENBERG, 2002; PICKENS ET AL., 2011), methamphetamine users have increased D3 rECEPTOR ACTIVATION (BOILEAU ET AL., 2016), and D3 receptor antagonists block the incubation OF CRAVING (XI ET AL., 2013). Given all of these lines of evidence we tested if finasteride would act similarly to D3 receptor antagonists and block the incubation of cue craving.

Methods

Experiment 1

Male Wistar rats were equipped with indwelling intravenous jugular catheters. Animals were trained to self-administer cocaine (0.5mg/kg/infusion) during 1-hour short-access (ShA) sessions. Following acquisition animals were provided an additional 5 days of ShA to establish baseline drug-consumption, and then transitioned into 15 six-hour long-access (LgA) sessions. In sessions 11-15 animals were either treated with daily finasteride (25 mg/kg), or its vehicle (95% saline, 5% Tween80) 30 minutes prior to cocaine self-administration (Figure 1).

Experiment 2

All animals within this experiment had the same experience of cocaine self-administration as animals in Experiment 1. The day after the last LgA cocaine self-administration session, animals were provided with a 30-minute drug-seeking probe session to test the incubation of craving. These sessions were identical to self-administration sessions, except that responses on the active nose-pokes elicited the CS without the reinforcement of

drug infusion. Animals had 5 daily injections of finasteride (25mg/kg) or its vehicle in incubation days 26-30. On incubation day 30 animals underwent a second 30-minute extinction test (Figure 1; gray box).

Results & Discussion

Finasteride decreases cocaine consumption

At the time of running these experiments we were unaware if repeated finasteride administration would have lasting effects, as such we designed the experiment for between animal comparisons. We found that administration of Finasteride prevented further escalation of drug consumption, whereas vehicle treated animals continued to escalate their intake (Figure 2). Increasing the sample size of these experiments would allow us to test if finasteride differently effects escalators and non-escalators, an outcome we previously observed with the Crhr1-receptor antagonist, CP154,526 (Chapter 3, Figure 4), L-DOPA (WILLUHN ET AL., 2014) and optogenetic stimulation of dopamine transmission (Chapter 2, Figure 4). Overall, these data highlight that finasteride has effects on cocaine consumption and further investigation is needed to elucidate a mechanism. Our current hypothesis is that finasteride's inhibition of CRF expression decreases activation of the Crhr1-receptor which is responsible for the decrease in drug consumption.

Finasteride blocks incubation of craving following prolonged abstinence

We tested if finasteride had any effect on the incubation of craving, a phenomenon in which animals responding for non-reinforced cues increases the longer the animals is in abstinence (Grimm et al., 2001). We found that animals treated with vehicle significantly increased their extinction responding after 1 month of abstinence. This increase in responding typically observed was blocked by finasteride administration (Figure 3).

Conclusion

These data show promising results and indicate finasteride has potential to be utilized as a pharmacotherapy with multiple classes of substance use disorders (see Chapter 5). Finasteride treatment could possibly help stabilize someone's' consumption of drugs and potentially help diminish their drug-seeking behaviors during abstinence. Further investigations should focus on the mechanisms mediating the effects observed, as well as replicating the results demonstrated here.

Figures

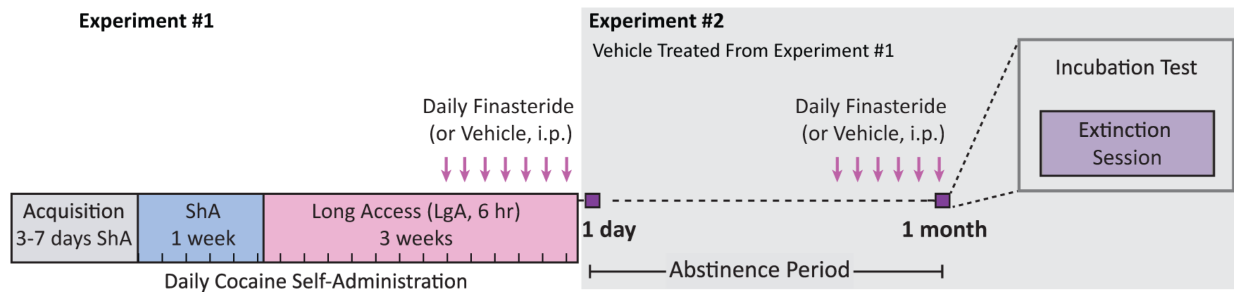

Figure 1. Schematic showing experimental timelines.

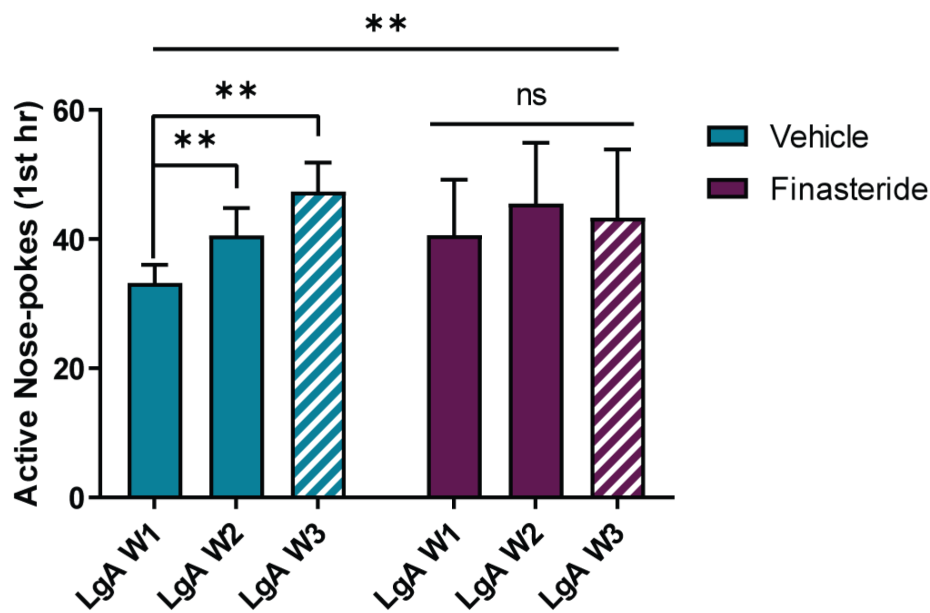

Figure 2. Finasteride blocks further escalation of cocaine intake.

A two-way ANOVA revealed a significant interaction between Week and Treatment Group ($F(2,18)=6.074$, $p<0.01$). A Sidak post-hoc analysis further revealed that animals treated with vehicle had a significant increase in drug-consumption in LgA week 2 compared to LgA week 1 ($p<0.01$), a similar result was observed in LgA week 3 ($p<0.01$).

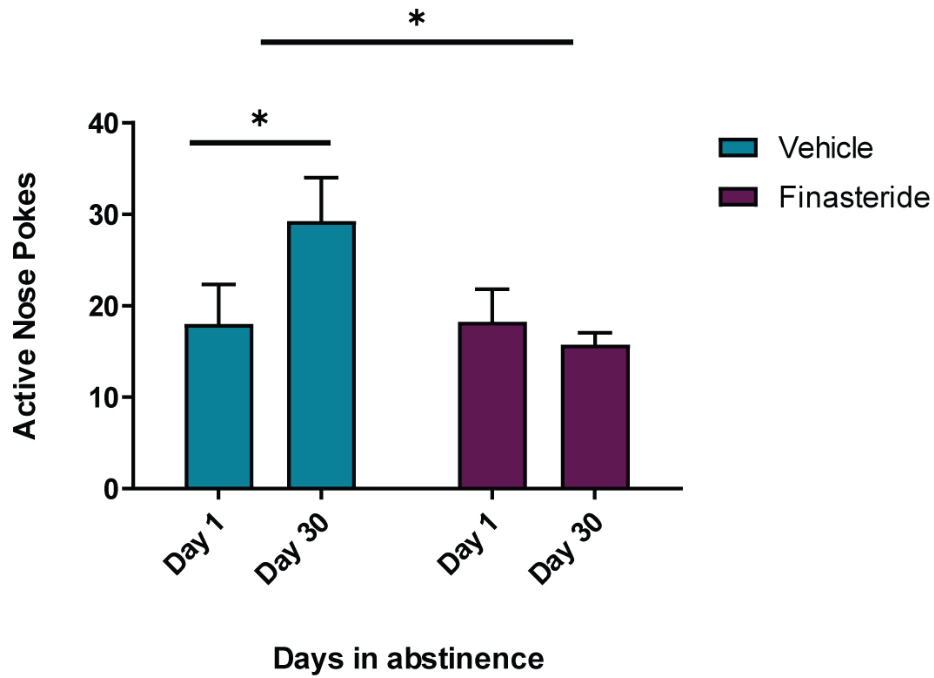

Figure 3. Finasteride blocks incubation of craving.

A two-way ANOVA revealed an interaction between Day of Abstinence and Treatment Group ($F(1,6)=6.880$), $p<0.05$). A Sidak's post-hoc test revealed a significant difference between Day 1 and Day 30 in the vehicle Treatment Group ($p<0.05$). There was no significant change of nose-pokes across abstinence days in the Finasteride treated animals ($p>0.05$).

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Chapter 7: Discussion

Summary of Findings

The data presented within this dissertation expand our knowledge of neurobiological mechanisms promoting and maintaining consumption of drugs of abuse. Substance use disorders can lead to tragic outcomes on an individual's well-being, including death. One approach in addiction treatment is to help reduce harm and alleviate negative consequences of drug addiction. The primary focus of this dissertation was to elucidate the neural mechanisms associated with excessive drug intake and explore treatments that decrease consumption to moderate levels.

In Chapter 2, I investigated the role cue evoked phasic dopamine transmission has in drug taking and drug seeking behaviors. I demonstrate that the timing and context of nucleus accumbens (NAc) dopamine release is important in its behavioral output. For instance, when a drug paired CS is presented following a successful action to obtain drug, dopamine is released and acts as a feedback to suppress further actions. As such a decrease in dopamine release during this response-contingent CS fails to suppress further actions and an increase in responses and drug consumption is observed. Conversely, if the same drug paired CS is presented non-contingently, meaning unexpected and not dependent on the agents behavior, dopamine release promotes an increase in drug seeking behavior and the motivation to obtain drug. As the addiction cycle progresses we observed a decrease in dopamine transmission to response-contingent CS and an increase in drug consumption. Simultaneously, an increase in dopamine release to a non-contingent CS was observed, which coincided with an increase in drug-seeking behaviors. We additionally used optogenetics to artificially stimulate dopamine and found causal relationships, in which increasing dopamine during response-contingent CS decreased overall response to obtain drug, whereas increasing dopamine to non-contingent cues enhanced drug seeking behavior. These data demonstrate diametrically opposed changes

in dopamine which differentially drive drug taking and drug seeking behaviors through-out the progression of addiction.

The work in Chapter 3 tests the hypothesis that activation of Kappa opioid receptors (KOR) on dopamine terminals in the NAc disrupts dopamine release during drug-taking and promoting escalation of cocaine consumption. Inactivation of KOR in the NAc, achieved by intracranial injection of a long-lasing KOR antagonist, disrupted the typical observed escalation of cocaine intake overtime. I then utilized a viral vector delivered CRISPR/SaCas9 targeting the Oprk1 gene to genetically knock-out KOR from separate populations of KOR that reside in the NAc. Removing expression of KOR from ventral tegmental area (VTA) dopamine neurons and their terminals, including those in the NAc, blocked the development of escalated drug consumption. Removing KOR from the cell bodies in the NAc (Medium Spiny Neurons and interneurons) did not prevent escalation of consumption. These findings reveal that KOR on dopamine terminals serve a critical role in the development of escalated cocaine consumption. I additionally found that disruption of the CRF receptor Crhr1 through intracranial injection of a Crhr1 antagonist decreased previously escalated drug consumption but had no effect on animals with stable drug-intake. The work in this chapter indicates that the neuromodulators dynorphin and CRF act within the NAc to establish and maintain escalated drug consumption. Future studies will examine the interactions these neuromodulators have with one another and their direct impact on dopamine signaling during drug taking sessions.

Because of the causal relationship found between dopamine release and its control of drug consumption was based on CS evoked dopamine, we hypothesized that this was a common mechanism across multiple classes of substances of abuse. As such in Chapter 4 I tested if L-DOPA, a precursor of dopamine, would decrease oral consumption of ethanol and fentanyl. As predicted, L-DOPA decreased consumption of ethanol, and fentanyl. Interestingly, we found that if animals were provided water and fentanyl in a free operant two-bottle choice task that L-DOPA has no effect on water consumption, and selectively decreased fentanyl

consumption. However, in animals naïve to fentanyl but were provided self-administration operant sessions in which responses led to water delivery, L-DOPA significantly decreased their responses and water consumption during the 1-hour task. Animals during this time were also provided *ad* libitum water and no negative consequences typically associated with dehydration were observed. Because this effect was specific to operant tasks involving conditioned stimuli, a future experiment should assess if animals will respond for cues alone with no liquid reinforcement and test the effect of L-DOPA on those responses. These data overall suggest that response-contingent cue evoked dopamine regulate consumption behavior across multiple classes of drugs of abuse. These results show promising harm-reduction properties from a pharmacological agent that is already FDA approved for human use.

In Chapters 5 and 6 we explored the outcomes from inhibition of 5α-reductase on addiction behaviors. This collaborative project utilized multiple animal models of addiction. We show that finasteride, a 5α-reductase inhibitor, decreases opioid consumption in zebrafish, and rats. Finasteride treatment did not reduce food-seeking behavior or have motoric effects. Males and female rats both reduced their opioid consumption following treatment. The neurosteriod dehydroepiandrosterone sulfate (DHEAS), was identified as a mediator of finasteride's effects on drug taking behavior. Finasteride also prevented further cocaine escalation when given to rats experienced in cocaine self-administration. Moreover, finasteride blocked the incubation of cue craving in cocaine addicted rats. These data provide promising results that finasteride may be able to reduce harm by decreasing the amount of drug consumed, as well as prolong abstinence. Future studies should investigate if there are interactions between DHEAS and CRF synthesis or signaling, as it has been shown that finasteride decreases CRF signaling within the paraventricular nucleus. Further study into the mechanisms responsible for finasteride's effect on incubation of craving will be pertinent into understanding the role 5α-reductase plays in relapse behavior. Overall these two chapters highlight a medicinal drug, finasteride, that is

currently FDA approved for human use, could be used as a pharmacological therapeutic through-out the addiction cycle.

Collectively, this dissertation presents novel findings that contribute to our knowledge in the mechanisms promoting to drug consumption behaviors. The results presented within each chapter suggest a local neuromodulator circuit within the NAc that mediates drug taking behavior. Furthermore, I present data that reveals two effective pharmacological treatments, in reducing the consumption of multiple classes of substances of abuse. Importantly, both of these treatments are already FDA approved and used by humans.