# Investigating the Impact of Drug History and Cortical Circuitry on Substance Use and Decision-Making

Elizabeth A. Crummy

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Reading Committee:

Susan Ferguson, Chair

Sheri Mizumori

Paul Phillips

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**Abstract**

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Elizabeth A. Crummy

Chair of the Supervisory Committee:

Associate Professor Susan M. Ferguson

Psychiatry & Behavioral Sciences

While substance use is commonplace within the United States, a subset of users will develop a substance use disorder (SUD). SUDs are characterized by binge-like intake with compulsive seeking and consumption, even in the face of adverse consequences. Extensive research has characterized phenotypes underlying various drugs on a behavioral, pharmacological, and circuit level. However, these models do not necessarily capture the patterns and combinations typical to clinical populations, leaving a significant gap in our understanding of the risks and unique alterations of substance use pathology that translates to SUDs. The public health significance of developing translatable models is highlighted and discussed in Chapter 1. Furthermore, an overview of relevant circuitry in regulating motivated behaviors impacted by substance use are described. In Chapter 2, one proposed behavioral model for investigating behavioral differences with different patterns of cocaine and heroin consumption, including polysubstance use, is explored. Here, we find that, largely, drug class produces the starkest differences in motivated consumption and cue sensitivity, but that polysubstance use produces subtle phenotypical differences in these measures.

A significant area of study in substance use models examines the delineation between nucleus accumbens and dorsal striatum projection subtypes. The striatum is known to be involved in the processing of motivated behaviors and reward and is altered in substance users, as well as in preclinical models. Two types of medium spiny neurons (MSNs) – D1 and D2 MSNs – form direct and indirect pathways, respectively. Their opponent regulation of behavioral

outputs and changes to activity and behavior impacted by manipulation of these subtypes in SUDs models has illuminated the role of circuit specificity in the SUDs pathology. The striatum integrates many inputs, including innervation from glutamatergic afferents from the prefrontal cortex (PFC). It is also known that cortical activity is altered in substance users, making regional and circuit characterization of cortex critical for fully understanding the development and progression of SUDs. However, while parsing the role of MSN subtypes is extensively investigated, further delineation of cell types in other parts of the cortico-basal-ganglia (CBG) pathway have largely been uncharacterized. Of note are the glutamatergic projection subtypes within cortex, intratelencephlaic (IT) and pyramidal tract (PT) neurons, which are known to have distinct morphology, firing patterns, and projections, but how these correspond to their function roles in behavioral outputs is not well understood. Previous work demonstrates that these cortical subtypes indeed play unique roles in the processing of rewarding and aversive aspects of drug use. Chapters 3 and 4 extend this role to our understanding of subtype-specific modulation in the anterior cingulate cortex (ACC) from psychomotor sensitization to cocaine, as well as the impact of cortical neurons on volitional cocaine use. Our results demonstrate that dampening activity of IT neurons in the ACC augments initial expression, but prevents further escalation of psychomotor activity with repeated cocaine administration, in contrast to previous studies showing PT inhibition reducing expression, but enhancing escalating psychomotor activity. We also show that IT and PT neurons do not produce preference or aversion in the absence of drug treatments, but that PT neurons are involved in effort-based cocaine consumption with self-administration. In Chapter 5, we explore how cortical subtype activity in the OFC may be involved in behavioral outputs under normal conditions, examining decision-making and behavioral flexibility in the absence of drug use. Here, we find that IT inhibition increases behavioral flexibility and PT inhibition produces outcome-dependent alterations to reversal learning, whereas neither population impacts probabilistic decision-making.

## Table of Contents

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# Chapter 1: Introduction

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## 1.1 Overview of substance use disorders (SUDs)

Drug addiction is a heterogeneous disorder characterized by cyclic periods of drug use, withdrawal and abstinence, and drug-craving and recurrence of use (Koob & Volkow, 2016). Addiction is highly prevalent in our society, with an estimated 35 million people world- wide and 19.3 million people in the United States (US) currently meeting diagnostic criteria for a substance use disorder (SUD) (Substance Abuse and Mental Health Services Administration, 2019; United Nations Office on Drugs and Crime, 2019). Additionally, epidemiological surveys suggest that, in a person's lifetime, there is a ~10% prevalence of a SUD (Grant et al., 2016; Substance Abuse and Mental Health Services Administration, 2019). Drug addiction is also one of the largest public health problems in the US, with an annual financial burden of $740 billion in costs related to treatment, lost work productivity, healthcare, and crime (National Institute on Drug Abuse, 2020). These numbers are likely to increase as illicit drug use is rising, with a quarter of a billion people worldwide reporting use in the past year (United Nations Office on Drugs and Crime, 2019). Within the US, over 17 million people aged 12 and above are estimated to initiate drug use annually.

## 1.2 Polysubstance use with psychostimulants: public health and preclinical

Drug addiction is both pervasive and deadly, with an estimated 585,000 drug use-related deaths occurring each year (United National Office on Drugs and Crime, 2019). Nonetheless, although drug addiction and its impacts are often centered around individual drugs, drug use is largely found to involve multiple substances (Gjersing et al., 2013; Roy et al., 2013; Substance Abuse and Mental Health Services Administration, 2016). Indeed, drug-dependent individuals report an average use of 3.5 substances (Onyeka et al., 2012), including both simultaneous and sequential polydrug use. In addition, the likelihood of developing comorbid substance dependencies is high in clinical populations (Leri et al., 2004; Lorvick et al., 2018). Although which substances are co-used can vary, primary drug dependencies are typically found for alcohol, opiates, amphetamine, and methamphetamine, while cannabis and cocaine are more often reported as secondary- or tertiary-used substances (Substance Abuse and Mental Health Services Administration, 2016). The high prevalence of polysubstance use is particularly concerning given the impact that this can have on both SUD severity and treatment outcomes. For example, a polysubstance history is associated with greater unmet physical and mental health care needs, increased risk behavior, violence, and increased overdose and mortality risk

compared to single substance use (Pennings et al., 2002; Gilmore et al., 2018; Lorvick et al., 2018).

Polysubstance use is common among stimulant users with both concurrent and sequential drug consumption patterns. For instance, simultaneous use of psychostimulants and opioids is seen with both cocaine (“speedball”) and methamphetamine (“bombita”). Sequential use of psychostimulants and opioids is also common, including the use of cocaine or amphetamine to avoid opioid-related somatic withdrawal symptoms (Hunt et al., 1984; Ellis et al., 2018) and the use of opioids to reduce overexcitation following cocaine use (Kreek, 1997). Worldwide prevalence of psychostimulant use has remained relatively stable from 1990-2017, with 7.38 million reported to have an amphetamine use disorder and 5.02 million reported to have a cocaine use disorder (Degenhardt et al., 2018). However, the role of psychostimulants in SUD severity in the US is significant, with a 2.6 fold increase in the cocaine overdose death rate and 3.6 fold increase in the methamphetamine overdose death rate from 2000 to 2017 (Degenhardt et al., 2018), suggesting a broader role of psychostimulants in drug-related overdoses. Of note, cocaine and amphetamine users are predominantly polysubstance users, with one study reporting 74% and 80% incidence of polysubstance history, respectively (Kedia et al., 2007). Specifically, cocaine use and developing a cocaine use disorder is associated with concurrent heroin, cannabis, tobacco, and alcohol use Though these studies did not specify the patterns of polydrug use, a meta-analysis of reports on concurrent versus simultaneous cocaine use found a range of 24-98% of simultaneous cocaine and alcohol use and 12-76% incidence of simultaneous cannabis use (Liu et al., 2018). Rates of concurrent use were 37-96% for cocaine and alcohol use, 43-94% for cannabis use (Liu et al., 2018), 70-80% for cocaine and nicotine use (Budney et al., 1993; Weinberger & Sofuoglu, 2009), and 85-95% for amphetamine and nicotine use (Brecht et al., 2007; Grant et al., 2007). The high variability in reported frequencies highlights the complexity in identifying drug use patterns, which can vary across demographics, study periods, study structure, and definitions of concurrent and simultaneous use.

Polydrug use involving psychostimulants poses significant public health risks. For example, one study of amphetamine users has found them to be 21 times more likely to have a concurrent cannabis use disorder and 7 times more likely to have past-year concurrent cocaine use compared to those with no prior history of amphetamine use (Massaro et al., 2017). In addition, nearly one-third of overdose deaths involved both psychostimulants and opioids, such as heroin and fentanyl (Kariisa et al., 2019). The hazards of psychostimulant co-use also extend to other substances, with cocaine and cannabis use resulting in higher standardized death rates in

emergency department (ED) visits, suggesting elevated mortality risks with this combination (Gilmore et al., 2018), and cocaine and alcohol use increasing risk for cardiotoxicity compared to either drug alone (Pennings et al., 2002).

### 1.3 Behavioral Models of Substance Use

Behavioral models of drug addiction are used to examine the neurobiological underpinnings of the development, maintenance and relapse to drug use. The most commonly used models are locomotor sensitization (a progressive and persistent increase in locomotor responses to the same dose of a drug), conditioned place preference (CPP; a test of drug reward measured as an increase in time spent in a drug-paired chamber) and drug self-administration (response-contingent intake of drug) (see Panlilio and Goldberg, 2007; Spanagel, 2017; Kuhn et al., 2019 for review). Experimental designs using these models vary across a number of pharmacological and non-pharmacological parameters including contingency of drug use, amount of access to drug, the context associated with drug use and routes of administration. Here, we will describe select assays used and discussed in this thesis, noting that this is a small subset of behaviors examined in substance use models.

#### *Locomotor sensitization*

The sensitizing properties of psychoactive substances have classically been used as a proxy for measuring progression of addiction pathology, including compulsive drug-seeking and psychosis. Paradigms typically involve conditioning/training sessions, whereby animals repeatedly receive an injection of drug and their locomotor response is monitored. Following conditioning and a period of drug abstinence, animals are then given the drug in a “challenge” session to determine long-term adaptations to the sensitizing properties of drug reward. In sensitization paradigms, the increased response, typically measured through cage crossings, locomotor activity, and stereotyped movements (“stereotypies”), are found with repeated exposure to an unconditioned stimulus drug reward (Anagnostaras & Robinson, 1996). However, whether this increased responding is due to neurobiological adaptations resulting from drug exposure, an associative learning of drug-taking environments, or a combination of the two remains a point of debate (Anagnostaras & Robinson, 1996).

#### *Conditioned place preference*

Conditioned place preference (CPP) paradigms involve the repeated pairing of contextually unique chambers with different rewards, such that individuals develop an association between a

drug and the context it is paired with. Prior to drug pairing, baseline time spent within each context is analyzed with preconditioning sessions. Pairings are repeated for multiple sessions (conditioning phase), followed by a subsequent choice test where animals are given free access to explore all contexts in the absence of the reward. Time spent within each region is then compared to preconditioned baseline sessions (Bardo & Bevins, 2000). CPP is a popular method of studying drug reward due to its ease of implementation, inexpensiveness, and interpretability.

*Conditioned taste aversion*

Conditioned taste aversions (CTAs) consist of learned associations between a consumed reward and noxious stimuli which reduce subsequent seeking and consumption of that reward (Lin et al., 2017). CTA has been used to examine the aversive facets of drug use, with various classes of drugs producing CTAs to saccharin and other food rewards (Busse et al., 2005; D'Mello et al., 1981; Leblanc & Cappell, 1975; Mayer & Park, 1993; Riley et al., 1978). A common variant of CTA paradigms is pairing of *ip* or *sc* injections of a drug following sessions where animals have *ad libitum* access to palatable sucrose solutions or other flavored solutions (D'Mello et al., 1981; Mayer & Park, 1993), though this is also modeled with operant responding on FR schedules (D'Mello et al., 1981). The development of aversion to these rewards is typically assessed by comparing intake prior to pairings with the noxious stimuli to sessions following introduction of noxious stimuli with rewards (D'Mello et al., 1981).

*Self-administration*

Contingent administration of drug rewards is a widely used model for assessing various dimensions of drug dependency and addiction. Self-administration paradigms typically involve learning an action-outcome pairing of a drug reward delivered after completion of a response contingency (e.g. nose poke, lever press). Classic self-administration involves short-access (ShA) sessions of continuous drug access for the length of a session (~1-3 hours). These sessions typically require a single response, referred to as a fixed ratio one (FR1) schedule of reinforcement, for reward delivery (Spealman & Goldberg, 1978). More recent studies have modified classic ShA models to increase session duration (long-access, LgA), or intersperse periods of drug-availability with drug-unavailability within a single session (intermittent-access, IntA), or increase training to at least 30+ SA sessions (extend-access, ExtA). Unlike ShA self-administration, LgA and IntA, and ExtA models more easily produce variability in the development and expression of addiction severity, allowing researchers to selectively study

drug-induced alterations in vulnerable versus resilient groups (O'Neal et al., 2019; Zimmer et al., 2012 Oleson & Roberts, 2009) and model DSM criteria (Yager et al., 2019; Deroche-Gamonet et al., 2004). In addition to modifying individual session length, extended access (ExtA) self-administration has been used to study the development of addictive behaviors. ExtA self-administration has been used to model DSM criteria for substance use disorder (Yager et al., 2019; Deroche-Gamonet et al., 2004). In addition to the time course of self-administration, the number of responses for a single infusion can be increased (e.g., FR3, FR5), producing drug-specific alterations in intake patterns. Additionally, reinforcement can occur on a variable ratio (VR) schedule, in which the number of responses required for an infusion varies around an average value (e.g., VR10, VR15). A common method of assessing the motivational properties of a drug reward is through increasing the number of responses required for sequential drug delivery (Oleson & Roberts, 2009). Such progressive ratio (PR) schedules of reinforcement result in a "breakpoint" measure: the schedule in which an individual is no longer willing to work for a drug reward (Spealman & Goldberg, 1978).

*Extinction, reinstatement, and incubation of craving*

A significant impediment to long-term rehabilitation from SUDs is heightened sensitivity to drug-associated cues and contexts that prompt drug-craving and relapse. In preclinical models, this sensitivity is assessed using extinction/reinstatement paradigms. Following acquisition and self-administration sessions, animals undergo extinction sessions in which responses (e.g., nose poke, lever press) no longer result in drug delivery or cue presentation. After action-outcome responding has been extinguished, responding is reinstated by exposing animals to previously drug-paired cues (cue-induced), drug-paired contexts (context-induced), a stressful stimulus (stress-primed), or a challenge injection of drug (drug-primed). Extinction/reinstatement sessions have also been employed in CPP models (Venniro et al., 2016), allowing researchers to examine mechanisms of drug-craving and relapse following contingent or non-contingent drug administration. Finally, a variation of extinction/reinstatement termed "incubation of craving" (Grimm et al., 2001; Lu et al., 2004) has been developed to examine the role of forced abstinence, rather than extended extinction, in driving relapse, which involve minimal extinction training (sometimes a single day), followed by extended periods (weeks to months) where animals remain in their home cage, undergoing forced abstinence. After a period of abstinence, subjects are reintroduced to the drug-paired environment and undergo reinstatement, as described above.

*Behavioral economics*

Alternative measures and analyses of motivation have utilized the fundamentals of behavioral economics for investigation of intake and demand for various types of reward. Rather than altering required effort for drug acquisition through number of responses, behavioral economic paradigms examine intake and responding at various “prices” for drug consumption through changes in unit dose per infusion or infusion time (Oleson & Roberts, 2009). These paradigms are especially advantageous for the ability to extract normalized measures for comparison of demand and price sensitivity across rewards and is argued to be a powerful tool for understanding reinforcing properties of a substance, its demand implications for developing public policy, the efficacy of interventions, and for understanding interactions across various drugs and other rewards (Hursh & Winger, 1995; Mello & Negus, 2007). Additionally, demand curve analyses for alternative approaches from breakpoint measures remove the potential confounds of reward delay on reward cost and dissociation of cost related to effort versus opportunity costs presented by reward delay (Schelp et al., 2017).

More recently, studies are comparing single versus polysubstance self-administration to determine the effect of drug history on drug-induced molecular and circuit alterations (Briggs et al., 2018; Stennett et al., 2020; Zhu et al., 2020). Additionally, paradigms based on behavioral economic principles can determine the preferred level of drug intake (i.e., no-cost intake; $Q_0$), as well as the amount of effort an animal is willing to exert to defend $Q_0$ before consumption and responding begins to decline (i.e., price; $P_{max}$) (Oleson and Roberts, 2009). These paradigms are especially powerful in that they can use $Q_0$ and $P_{max}$ to generate normalized measures of value (i.e., essential value; α) and price ($_nP_{max}$), which have been used to compare price sensitivity, effort, and value across different drug and non-drug rewards in polysubstance models in several species (e.g., in rats, rhesus monkeys, and human participants) (Petry & Bickel, 1998; Ward et al., 2006; Wade-Galuska et al., 2007, 2011; Crummy et al., 2020). In particular, these studies permit examination of the relative reinforcing properties of different doses and classes of drugs (Wade-Galuska et al., 2007; Cooper et al., 2010; Huskinson et al., 2015), as well as alterations in cost valuation for a drug following pre-exposure to another drug (Cooper et al., 2010; Hofford et al., 2016; Morris et al., 2018). Direct quantification of the assigned value of these drugs across different polysubstance histories and drug doses is very useful for assessing the impact of polysubstance history on relative reinforcer value. Furthermore, these measures can be used to compare how polysubstance users value drug rewards across different experiment parameters (e.g., differences in priming dose of one drug, environmental context, pattern of drug use). Finally, clinical studies are using questionnaires or controlled laboratory environments to investigate the behavioral effects of a polysubstance

history. Specifically, these studies are using monetary choice procedures that compare assigned value of drugs at different doses (Greenwald et al., 2010), how assigned value changes for one drug with a change in price of another (Petry & Bickel, 1998; Petry, 2001; Sumnall et al., 2004; Chalmers et al., 2010), or how relative value of one drug changes with perceived change in quality of another available drug (Cole et al., 2008). Additionally, progressive ratio tests for a single drug or drug combinations to study motivation (Greenwald et al., 2010), and delay-discounting rates for money and drug rewards to study decision-making (Strickland et al., 2019) have also been performed. These studies permit comparison of perceived value across multiple drugs in participants with history of single or polysubstance use (for further review, see Heinz et al., 2012).

*Risk-based decision-making paradigms*

Many neuropsychiatric disorders are characterized by maladaptive decision-making, including, but not limited to, depression, anxiety disorders, obsessive-compulsive disorder, gambling disorders, and SUDs (Clark et al., 2013; Fettes et al., 2017; Hartley & Phelps, 2012; Schultz, 2011). Particular decision-making phenotypes altered in these disorders include motor impulsivity, delay discounting, and risk evaluation. Risk-based decision making is modeled in humans using paradigms such as the IOWA Gambling Task (IGT) where smaller-value, lower-penalty rewards are optimal over bigger immediate rewards with high risk of losses, and the Balloon Analogue Risk Task, where riskiness is rewarded to a certain extent before resulting in detrimental outcomes (Lejuez et. al, 2002; Winstanley, 2011). Rodent models of risky decision making include a variant of the IGT and probabilistic discounting, where rats have to choose between safe, certain options and options associated with larger rewards, but which probabilistically deliver lower-value or no rewards (Heilbronner, 2017; Winstanley, 2011). Alternative models examine decision-making under conditions pairing an aversive stimulus with a larger reward, increasing the probability of receiving an undesirable consequence when selecting ‘riskier’ options (Simon et al. 2009). The tendency of individuals to opt for risky choices in these disorders reflects high valuation of reinforcers despite adverse consequences, predisposing individuals to pursue rewards with potential punishment. Studies on risky decision-making in humans and animal models have found high-risk behaviors to be correlated with drug relapse, compulsive behaviors, self-injury and suicidal ideation (Clark et. al, 2013; Jollant et. al, 2010; Oldershaw et. al, 2009; Schoenbaum et al., 2006). Cocaine users exhibit altered decision-making, including greater impulsivity in discounting delayed rewards (Coffey et al., 2003; Johnson et al., 2015), and increased punishment sensitivity with probabilistic rewards (Simon et al., 2009).

Furthermore, punishment resistance to drug-seeking has been reported in animal models of compulsive drug-seeking during extended self-administration sessions (Pelloux et al., 2007), and escalated cocaine administration not only impairs optimal decision-making in rats, but correlates with susceptibility to reinstated cocaine-seeking (Cocker et al., 2020).

*Reversal Learning*

Impairments in response flexibility are notable in substance misuse and modeled in preclinical studies with reversal learning paradigms (Bechard et al., 2019 Verharen et al., 2018; Zhukovsky et al., 2019). These models consist of training subjects to differentiate between two options, one producing a rewarding outcome, while the other does not. After learning action-outcome contingencies, these pairings are switched, requiring subjects to switch behavioral strategies to respond on the previously unrewarded lever (Izquierdo et al., 2017). This paradigm is utilized in various forms for many models, including humans (Patzelt et al., 2014), primates (Jentsch et al., 2002), and rodents (Verharen et al., 2018).

1.4 Psychostimulant impacts on behavior in polysubstance models

One of the more commonly studied polydrug combinations has been administration of cocaine with other drugs (Francesco et al., 2003; Leri et al., 2003b; Substance Abuse and Mental Health Services Administration, 2016). However, in contrast to human reports (Heil et al., 2001; Williamson et al., 2006; Staiger et al., 2013; Preston et al., 2016; Lorvick et al., 2018; Kariisa et al., 2019), an increase in addiction-like severity has not been seen in preclinical studies, suggesting a need for models with greater translational relevance that can capture the enhanced severity seen in human polysubstance users. Specifically, animal studies of sequential cocaine and alcohol or cocaine and heroin use have not found differences in drug intake or reinstatement of drug-seeking as a function of single versus polydrug use (Pattison et al., 2014; Fredriksson et al., 2017; Crummy et al., 2020; Stennett et al., 2020). These effects were observed despite the fact that the studies varied in their use of contingent and non-contingent drug administration, drug doses, and species, including rats (Crummy et al., 2020; Stennett et al., 2020) and rhesus monkeys (Aspen and Winger, 1997). Consistent with this work, intermittent alcohol exposure has not been shown to affect cocaine self-administration (Aspen and Winger, 1997; Fredriksson et al., 2017) or the reinforcing properties of cocaine measured via demand curves in rhesus monkeys (Winger et al., 2007). In addition, it also has not been shown to effect progressive ratio tests of motivation for cocaine (Mateos-García et al., 2015), or the long-term reconsolidation of preference for cocaine in drug-paired contexts (Zhu et

al., 2020) in rats. In contrast, adolescent alcohol exposure has been shown to have long-lasting effects on cocaine self-administration and reward, suggesting that this population is particularly susceptible to the effects of polysubstance use. For example, adolescent alcohol exposure increases motivation for cocaine (Mateos-García et al., 2015), the development of a cocaine CPP in both mice (Molet et al., 2013) and rats (Hutchison and Riley, 2012; Mateos-García et al., 2015), and weakens cocaine-induced taste aversion (Busse et al., 2005). In addition, simultaneous heroin and psychostimulant administration increases the motivation to self-administer cocaine (Ward et al., 2006) and methamphetamine (Ranaldi and Wise, 2000), and both simultaneous and sequential administration of morphine and methamphetamine have been shown to be rewarding, as measured by the development of a CPP (Briggs et al., 2018). Pretreatment with an opioid also enhances methamphetamine-induced psychomotor sensitization (Liang et al., 2006). These findings suggest that opioids can enhance the rewarding and motivational properties of psychostimulants, particularly when administered simultaneously.

### 1.5 Overview of striatal anatomy, function, and role in substance use

The striatum's connectivity and integration of inputs from cortical, midbrain, and thalamic regions, forming the cortical-basal ganglia-thalamic (CBGT) circuitry, has garnered extensive interest in its role in motivational and affective processing. Many studies and reviews have investigated and discussed the regional, cell-type specific, and circuit contributions of striatal activity to the propensity, manifestation, and severity of drug use and misuse (select reviews include Kalivas & Volkow, 2005; Koob & Volkow, 2010; Lobo & Nestler, 2011; Yager et al., 2015; Volkow & Morales, 2015). Briefly, the basal ganglia is involved in the planning and initiation of motivated actions (Haber, 2003; Mink, 1996), and is comprised of the caudate, putatmen, globus pallidus, the substantia nigra (SNr), the ventral tegmental area (VTA) and the subthalamic nucleus (STN) (Haber, 2003). The caudate and putamen comprise the striatum, which is broadly divided into dorsal (DS), consisting of most of the caudate and putamen, and ventral (VS) components which are comprised of the NAc and rostro-ventral caudate and putamen (Haber, 2016). The DS is involved in mediating action outputs, whereas the NAc and ventral regions are the limbic-processing centers of striatum and are typically associated with reward (Gerfen & Surmeier, 2011; Haber & McFarland,1999). The DS is further subdivided into medial (DMS) and lateral (DLS) regions which respectively regulate goal-directed and habitual behaviors (Balleine et al., 2007), while the NAc is subdivided into the core (NAcC) and shell (NAcS) with the NAcC observed to mediate cue-outcome learning and reward prediction,

whereas the NAcS is more involved with hedonic value (Corbit & Balleine, 2011; Maldonado-Irizarry et al., 1995; Saddoris et al., 2013; West & Carelli, 2016). This region is composed of mostly GABAergic medium spiny projection neurons (MSNs; 95%), which are the main projection population with the remaining 5-10% made up of four types of aspiny interneurons - cholinergic, parvalbumin GABA-expressing, calretinin-expressing, and neuropeptide Y/somatostatin/nitric oxide-expressing interneurons (Gerfen & Surmeier, 2011; Kemp & Powell, 1971). Different types of MSN populations have been identified based on their projection pathway, receptor expression, and neuropeptide expression (Besson et al., 1990; Gerfen et al., 1990; Surmeier et al.; 1996). One class of MSNs makes up the direct pathway in the DS, monosynpatically projecting to the SNr and globus pallidus internal (GPi), express D1 receptors, substance P and dynorphin, while another class expressing D2 receptors and enkephalin, form the DS indirect pathway, with intermediate projections to the globus pallidus external (GPe) which subsequently sends glutamatergic projections to the STN (Besson et al., 1990; Gerfen & Surmeier, 2011; Yager et al., 2015). Direct and indirect pathway projections in the NAc are classified based on projections to the VTA (direct pathway) and ventral pallidum (VP; indirect pathway) (Haber & McFarland, 1999; Yager et al., 2015).

Many studies have focused on examining the impact of substance use on the functional role of striatum and accumbens in region-specific and cell-specific manners. There is substantial work, in particular, on the opponent roles of the direct and indirect pathways in the DS (Freeze et al., 2013; Ferguson et al., 2011; Hikida et al., 2010; Kravitz et al., 2012; Vicente et al., 2016). However, this dichotomy is less clear in the NAc (Kupchik et al., 2015; Smith et al., 2013), though studies have found opponent effects of D1 and D2 modulation on drug-related behaviors (Lobo et al., 2010; O'Neal et al., 2019). Generally, however, heightening of D1 or direct pathway activity or blunting of D2 or indirect pathway activity has been shown to increase locomotor sensitization (Dobbs et al., 2019; Ferguson et al., 2011). Direct pathway and D1 cell types are involved in processing of the rewarding properties of drugs, promoting drug preference, consumption, seeking (Cheng et al., 2017; Lobo et al., 2010; O'Neal et al., 2019) with loss of D1 activity attenuating cocaine and alcohol consumption (Caine et al., 2007; El-Ghundi et al., 1998). Activating D2 receptors, however, reduces the rewarding properties of drugs and self-administration and prevents reinstatement to drug cues (Lobo et al., 2010; O'Neal et al., 2019; Thanos et al., 2008). Taken together, there is strong evidence suggesting pathway and cell-type specific bidirectional modulation of learning and motivated behaviors impacted by substance use which must be considered when assessing the role of regional activity in behavioral outputs.

1.6 Overview of cortical anatomy, function, and role in substance use

The PFC is a critical regulator of top-down executive control, with diverse roles in sensory processing, reward learning, decision-making, outcome valuation, and behavioral initiation (Elliot et al., 2000). The high heterogeneity of the PFC adds to the complexity of understanding how it regulates these various cognitive functions, as well as how its dysregulation contributes to SUDs. Broadly, the more dorsal cortical regions (e.g., sensorimotor cortices) are involved in sensory and motor processing, and project to the dorsal striatum. dmPFC is also thought to be a hub for comparator signals based on attention (Padoa-Schioppa & Conen, 2017) and to promote action outputs with coupling to motor cortices (Hare et al., 2011; Padoa-Schioppa & Conen, 2017). dlPFC, functionally connected with vmPFC, is important for cognitive control, reinforcement learning, and action selection (Hiser & Koenigs, 2017; Rowe et. al, 2000).

Conversely, the medial prefrontal cortex (mPFC) is involved in affective-based behaviors, such as fear behaviors (Jhang et al., 2018). mPFC in rats includes anterior cingulate (ACC), prelimbic (PL), and infralimbic (IL) cortices, with ACC typically defined as dorsal mPFC (dmPFC) and PL and IL comprising ventral mPFC (VMPFC) in rats (Hoover & Vertes, 2007; Öngür & Price, 2000). mPFC is centrally involved in affective and motivated behavioral processing and regulation through glutamatergic projections which topographically innervate the VS: ACC and PL cortices project to the NAcC, while IL projects to the NAcS. Ventral mPFC is involved in subjective encoding and context-dependent processing (Apps & Ramnani, 2017).

The orbitofrontal cortex (OFC) is broadly divided into the medial OFC (mOFC) which has similar functional roles to the mPFC, and the lateral OFC, involved in reward, outcome, and probability valuation. OFC afferents project to the DS, NAcC, and NAcS (Haber, 2016). Additionally, both ACC and OFC have dense interconnectivity with the basalateral amygdala (BLA), and dopaminergic input from VTA (Volkow & Fowler, 2000), and send efferents to the VTA (Kalivas, 2004). Ventral medial prefrontal cortex (vmPFC) and OFC have close functional associations and contributions to decision-making. Damage to vmPFC results in deficits to value updating for reward and punishment contingencies and emotional deficits (Bechara et .al, 1994).

It is hypothesized that in SUDs result in a general hypofrontality of cortical activity, resulting in a loss of inhibitory control in regulating drug craving and seeking (Du et al., 2020; Kalivas et al., 2005; Koob & Volkow, 2010; Volkow et al., 2019) and reduced cortico-cortico and cortico-striatal functional connectivity (Motzkin et al., 2014; Tomasi & Volkow, 2013). Chronic alcohol and cocaine substance users exhibit reduced cortical metabolic activity (Volkow et al., 1992a;

Volkow et al., 1992b) and neuromodulator levels (Heidbreder et al., 1999). Conversely, hyperactivity following re-exposure to previously paired drug stimuli is reported in imaging studies of chronic cocaine users which have increased activation of OFC, DLPFC, and cingulate cortices following cocaine-cue exposure and reported periods of craving and altered connectivity to ventral and dorsal striatum (Volkow et al., 2006; Wilcox et al., 2011). Initial hypoactivity may be associated with psychostimulant- increased dopamine transmission, which reduces calcium-dependent presynaptic glutamate release and AMPA and NMDA-dependent postsynaptic activation (Bisagno et al., 2016). Cocaine augments catecholamine effects (dopamine and noradrenaline) and serotonin through blocked synaptic reuptake, with its effects largely resulting from altered dopamine transmission and functioning (Cunha-Oliveira et al., 2008; Bozarth & Wise, 1985). Cocaine also increases concentrations of extracellular glutamate in prefrontal cortex, accumbens, striatum, and VTA (Reid & Berger, 1996; Shin et al., 2016; You et al., 2007) . Preclinical studies in rats have additionally implicated alterations in GluR1 and GluR2 AMPA receptor subunit expression in NAc with increased surface receptor expression during cocaine withdrawal in sensitized rats, but decreased expression with cocaine or saline challenge treatments, suggesting receptor internalization and recycling due to increased glutamate levels (Boudreau et al., 2007). CB1 receptor activity is also thought to mediate LTD in GABAergic VTA neurons with chronic cocaine exposure, disrupting inhibitory control of VTA dopamine neurons in concert with glutamate receptor 1 and D2 receptor activation (Sidhpura & Parsons, 2011). Dissecting the contributions of cortical regions in substance use to behaviors, as well as specific connectivity to other regions implicated in the susceptibility and progression of substance dependencies, is of significant interest. Here, we will briefly discuss the role of select cortical regions, as well as how they are impacted by substance use.

*Prelimbic*

PL has analogous connectivity and functional roles as the dlPFC in primates (Vertes, 2004). Antergrade tracing studies show topographic PL innervation to prefrontal cortices, cingulate cortex, insula, NAcC, DMS, the VTA, the periaqueductal gray, lateral hypothalamus, dorsal and ventral raphe nuclei, the central nucleus of the amygdala, and the BLA (Heilbronner et al., 2016; Sesack et al., 1989; Vertes, 2004), while receiving input from regions including mOFC, insula, hippocampus, amygdala, and midline thalamic nuclei (Hoover & Vertes, 2007). It is thought to be necessary for acquisition of goal-directed behavior and maintenance of action-outcome encoding (Corbit & Balleine, 2003; Balleine & O'Doherty, 2010) and exhibits reduced activity as actions become habitual (Smith & Graybiel, 2013). Its importance for acquisition of learned

behaviors extends to initial development and expression of fear (Giustino & Maren, 2015). Studies of PL typically contrast its role to that of IL, suggesting opponent roles in regulating actions (Gourley & Taylor, 2016; Hayen et al., 2014; Pereira & Morrell, 2020), but comparative studies challenge this hypothesis (Caballero et al, 2019; Moorman et al., 2015).

The PL has been frequently studied in substance use models and is implicated in regulation of drug-seeking behaviors through its connectivity to the NAcC (Dalley et al., 2008; Everitt & Robbins, 2016; James et al., 2018;  McFarland et al., 2004; McGlinchy et al., 2016) and ventral pallidum (VP) (McFarland & Kalivas, 2001). PL inactivation does not affect motivated drug consumption (Caballero et al., 2019; Riaz et al., 2019), but impairs initial acquisition of self-administration (Di Ciano et al., 2007) and exhibits higher c-fos mRNA expression in rapid intake IntA models which produce greater self-administration and PR responding (Minogianis & Samaha, 2020) . In preclinical models, enhanced accumbal glutamate is correlated with greater cocaine-primed reinstatement to cocaine-seeking, with inactivation of PL and ACC reversing these effects (McFarland et al., 2003; McFarland et al., 2004), suggesting that PL activity is necessary for relapse to drug seeking. PL regulation of seeking and taking appears to be context-dependent, narrowing the role of PL activity to contextual regulation of drug use (Di Pietro et al., 2006). Hypoactivity of PL is necessary for footshock-induced compulsive drug seeking (Chen et al., 2013; Hu et al., 2019) with PL stimulant attenuating compulsive seeking, suggesting PL functioning is necessary for top-down inhibition of actions with aversive consequences.

*Infralimbic*

IL, in contrast to PL, is thought to be analogous to orbitomedial cortices in primates (Vertes, 2004) and is homologous in emotional processing across species (Heilbronner et al., 2016) with connections to limbic processing regions like the BLA and PAG, as well as contralateral cortex (Price, 2007) and exhibits similar inputs as PL (Giustino & Maren, 2015) . IL activity is important for habitual responding and the transition to more autonomic actions with extensive training (Shipman et al., 2019; Smith & Graybiel, 2013), as well as facilitates extinction learning and conditioned fear suppression (Giustino & Maren, 2015), and top-down inhibition of expression of positive and negative emotional states (Richard & Berridge, 2013).

IL activity does not appear to be involved in motivated drug consumption in progressive ratio (PR) drug administration (Caballero et al., 2019; Riaz et al., 2019), but is associated with extinction learning of drug associations, as IL inhibition induces cocaine seeking via slower

extinction acquisition and enhanced reinstatement for cocaine (Caballero et al., 2019; LaLumiere et al., 2010; Peters et al., 2008), specifically in cue-contextual reinstatement (Augur et al., 2016). Importantly, this observation is specific to drug seeking following extinction learning and does not extend to drug abstinence (Augur et al., 2016), reinforcing the significance of IL activity in extinction learning. These effects likely involve IL→NAcS projections projections, which when activated, reduces cocaine seeking (Peters et al., 2008). Additionally, IL inactivation increases cocaine preference over nondrug social rewards in CPP (Pereira & Morrell, 2020), but conversely, persistently reduces self-administration responding (Di Ciano et al., 2007), implicating disruption of habitual actions.

*Anterior cingulate*

The ACC is involved in processing of motor, emotional, and cognitive information, interfacing with limbic regions (i.e. amygdala, insula, accumbens, mPFC and OFC), sensorimotor (i.e. motor cortex, somatosensory cortex, visual cortex, and spinal cord), and executive regions regulating cognitive processes (i.e. dorsal PFC), with additional projections to DLS, and pontine nucleus (Coizet et al., 2017; Heilbronner & Hayden, 2016; Rolls 2018; Sesack et al., 1989). It receives reciprocal input from many cortical regions, including sensorimotor cortices and associated thalamic nuclei (Hoover & Vertes, 2007), as well as limbic regions such as the amygdala (Kolb, 1984), and interfaces with hippocampal regions (Rolls, 2018).

Functionally, the ACC is involved in effort-based processing (Engström et al., 2013; Hauber & Sommer, 2009; Walton et al., 2009), cost-outcome valuation (Floresco & Ghods-Sharifi, 2007), pain processing (Thompson & Neugebaur, 2019), tracking of expected rewards (Ha Baeg et al., 2009; Knuston et al., 2005), and in error detection and appraisal (Gehring et al., 1993; Hester et al., 2009; Simões-Franklin et al., 2010). Activity in ACC is associated with increased conflict in probabilistic decision-making (Rogers et al., 1999a) and aversive learning (Giustino & Maren, 2015). Its location in cognitive and limbic circuits and its function is part of a ‘saliency network’ (Engström et al., 2013).

The ACC is involved in processing of drug cues, showing activity with heroin cues (Liu et al., 2011), and cocaine cues (Ha Baeg et al., 2009). Drug use is associated with impairments to error detection related to ACC hypoactivity with substance users, including in cannabis users (Hester et al., 2009) and cocaine users (Kaufman et al., 2003). Evidence suggest changes in activity from cocaine use can be sex-specific; imaging analysis of stress responding and cocaine use have found heightened arousal correlated with activity in the ACC in females

compared to males despite similar reports of craving (Li et al., 2005). Impairments to effort-based processing is associated with ACC-NAcC connectivity, as disconnecting lesions reduce preference for larger, but greater effort-expenditure rewards in rats (Hauber & Sommer, 2009). Hyperconnectivity between cognitive processing centers (i.e. DLPFC) with the ACC in cocaine-dependent individuals is also reported during delay discounting and reversal learning tasks (Camchong et al., 2011). These data suggest that ACC activity is necessary for effort-based motivation for drug consumption, as well as a significant role in context-based drug seeking.

*Orbitofrontal*

Researchers have proposed a role for OFC as a cognitive map and integrator of sensory and emotional information, using this information to signal expectant outcomes to help guide subsequent actions (Schoenbaum et al., 1999, 2009; Schuck et al., 2016; Wilson et al., 2014). Indeed, studies demonstrate OFC activity is involved processing of environmental cues (Lopatina et al., 2015), response inhibition (Meyer & Bucci, 2016), processing of unexpected rewards (Volkow et al., 2004), and integration of information for decision-making (Padoa-Schioppa & Assad, 2006; Padoa-Schioppa & Conen, 2017; Rogers et al., 1999a), particularly with initial establishment of subjective preference in economic decision-making (Gardner et al., 2020). Its connectivity with limbic and sensorimotor regions makes it well positioned for information integration. Afferents from olfactory cortex, gustatory cortex, visual association cortices, somatosensory cortices, and temporal cortices is interconnected with OFC, particularly in lOFC (Elliot et al., 2000; Price, 2007). Additionally, OFC receives afferents from the amygdala and medial thalamus and projects to the enterohinal cortex, perihinal cortex, ACC, hypothalamus, VTA, pons, and striatum (Elliot et al., 2000; Kolb, 1984; Price, 2007). OFC is divided into the lateral OFC, medial OFC, ventral OFC, ventrolateral OFC, dorsolateral OFC, and agranular insula (Izquierdo, 2017). Anatomy divisions between these regions include projection targets. Broadly, mOFC is more homologous to mPFC, while vOFC and lOFC correspond to orbital cortices in primates (Price, 2007) with mOFC projections overlapping PL connections (Sesack et al., 1989) including to DMS, vOFC having central striatal projections, and lOFC exhibiting stronger DLS connectivity (Heilbronner et al., 2016). However, vOFC and lOFC do have projections to NAc (Heilbronner et al., 2016; Kolb, 1984).

The regional role of OFC in reversal learning, strategy shifting, discounting, outcome prediction, reward devaluation and other behaviors have notable overlap, but have distinct impacts even within regions based on targeting coordinates (Izquierdo, 2017). Lesions to orbital cortices produce deficits in decision-making capability (Rogers et al., 1999b). However, mOFC activity in

humans is greater with rewarding outcomes, while lOFC activity is associated with punished outcomes (O'Doherty et al., 2001), an observation extending to rodent models (Hu et al., 2019; Pascoli et al., 2015; Richard & Berridge, 2013). OFC is also thought to differentially encode risk, with positive correlations between risk aversion and BOLD activity in the lateral OFC and positive correlations for risk seeking and medial OFC BOLD activity (Tobler et. al, 2007), though this processing may be more related to saliency encoding (Ogawa et al., 2013). Opponent roles of mOFC and lOFC have also been shown in reversal learning (Hervig et al., 2020), suggesting significant functional heterogeneity within OFC. Lateral OFC is also thought to gate goal and habit-directed behaviors via direct and indirect modulation of dorsal striatum (Gremel et al., 2016; Gremel & Costa, 2013), serving as a decision-making integrator of value and outcome contingencies (Schoenbaum et al., 1999). Evidence also supports roles for vOFC and vlOFC in delay discounting, risk learning, and context-dependent outcomes (Izquierdo, 2017). However, there are many discrepancies on the role of OFC in mediating complex behaviors which depend on region and task structure, making a comprehensive understanding of the function of the OFC difficult to elucidate (Fuchs et al., 2004; Hervig et al., 2020; Stalnaker et al., 2015).

OFC activity mediates compulsive drug seeking (Pascoli et al., 2015). Recordings in OFC demonstrate increased encoding of cocaine preference versus nondrug rewards (Guillem & Ahmed, 2018), and greater activity with discriminative drug cues (Ha Baeg et al., 2009), and OFC activity is associated with disruptions in cue selectivity (Stalnaker et al., 2006). In addition, deficits in OFC activity are associated with learning outcomes, outcome valuation, and impaired behavioral flexibility with chronic cocaine administration (Stalnaker et al., 2006). Drug-induced alterations to impulsive choice are impacted by OFC lesions, which reduce time to cocaine acquisition and disrupt patterened self-administration (Huchteson & Everitt, 2003, but see Lasseter et al., 2009), but impairs cue-based cocaine seeking (Fuchs et al., 2004; Huchteson & Everitt, 2003; Lasseter et al., 2009) and alcohol-seeking (Arinze & Moorman, 2020). Similar to amphetamine users, OFC lesions also increase tendencies for selection of less likely outcomes, an observation seen in the decision-making of amphetamine users and mimicked with tryptophan depletion, suggesting a serotonergic-based mechanism of OFC in decision-making processes (Rogers et al., 1999b). Furthermore, increased ΔJunD in OFC is associated with larger reward preference in delay discouting following cocaine administration (Winstanley et al., 2007), but impaired behavioral flexibility and diminished OFC activity with cocaine administration, particularly when signaling expected aversive outcomes (Stalnaker et al., 2006). These deficits are linked to altered coupling of limbic regions, such as shifts in coherence between OFC and BLA, and OFC and NAc (McCracken & Grace, 2013). Taken together, OFC

activity is necessary for the development and integration of choice valuation and outcome and in regulating response strategies, with deficits in this regulation with substance use. In particular, its substance use produces deficits in OFC regulation of cognitive flexibility, decision-making, compulsive drug seeking, and cue-mediated reinstatement of drug seeking.

*Cortical cells types*

The PFC is organized into six distinct layers comprised of unique connectivity patterns and distinct cell types, including large pyramidal cells (~75-80% of total PFC neurons) (Beaulieu, 1993; Santana & Artigas, 2017). Cortical neurons in layers II/III project to other neurons within the PFC to regulate local cortico-cortical network activity (Gabbott et al., 2005; Shepherd, 2013) with biased feed-forward connectivity in layers I-III (Harris et al., 2018). Conversely, cortical neurons in layers V-VI send excitatory glutamatergic projections throughout the C-BG-T, including to the striatum, midbrain, amygdala, hippocampus, and thalamus (Beaulieu, 1993.; Gabbott et al., 2005; Haber, 2011; Santana & Artigas, 2017) with reciprocal connectivity to input regions (Harris et al., 2018). These output neurons include intratelencephalic (IT) cells, pyramidal tract (PT) cells, and corticothalamic (CT) cells, each of which has distinct connectivity patterns and electrophysiological properties (Baker et al., 2018; Shepherd, 2013). IT cells are located within layers II-VI and project bilaterally within the telencephalon, particularly to other cortical cells (layers II/III) and the striatum, with sparser projections to the BLA and claustrum. These connections tend to have multiple targets with collaterals to striatum and across the corpus callosum (Winnubst et al., 2019). PT cells (also known as corticofugal or extratelencephalic neurons) are located within layers Vb/VI and project to the striatum, midbrain, pons, and spinal cord, with individual PT cells innervating multiple sites via collaterals (Guo et al., 2018; Kita & Kita, 2012). Notably, PT neurons project ipsilaterally and have limited cortico-cortical projections (Shepherd, 2013). Finally, CT cells are in layer VI and project bilaterally and exclusively to the thalamus (Winnubst et al., 2019). While there is growing interest in the anatomical, electrophysiological, and molecular profiling of these excitatory populations, limited studies have focused on their contributions to behavioral outputs, though it has been suggested that altering their activity will have differential behavioral effects (Kalmbach et al., 2015; Pasquereau & Turner, 2011; Shepherd, 2013). The PFC also includes various populations of interneurons, which heavily modulates the output of the pyramidal projection neurons. These interneurons are distributed throughout the various cortical layers, and include parvalbumin-expressing (PV), somatostatin-expressing (SST), and fast-spiking serotonin 3a receptor-expressing (5-$HT_{3a}$) subtypes (Batista-Brito et al., 2017). SST interneurons (~30% of total

interneurons), localized within layers II/III and layer V, modulate the activity of other interneurons in layers I/IV and are implicated in gain and contextual feedback control (Batista-Brito et al., 2017; Van Versendaal & Levelt, 2016). 5-$HT_{3a}$ interneurons (~30% of total interneurons) have strong local connectivity across cortical layers and exert significant inhibitory control (Batista-Brito et al., 2017; Hestrin & Armstrong, 1996). $5HT_{3a}$ neurons can be further subclassified into VIP-expressing and reelin-expressing interneurons; VIP-subtypes, found throughout cortex but densely located in layers II/III, are usually bipolar cells which inhibit other interneurons, predominantly SSTs, and modulated by cholinergic and serotonergic innervation. Layer I is mainly comprised of neurogliaform $5HT_{3a}$ GABAergic cells expressing reelin, which inhibit activity via volume transmission of GABA (Van Versendaal & Levelt, 2016). PV interneurons (basket cells), comprising the largest group of cortical interneurons (~40% of total interneurons), are found mainly in layers IV/V and modulate cortical output via somatodendritic connections with glutamatergic neurons (Batista-Brito et al., 2017; Van Versendaal & Levelt, 2016).

1.7 Tools for dissecting circuit connectivity

Transient manipulations of circuit projections permit parsing of circuits and their role in mediating behaviors. The utility of these measures extends to their transient activity, allowing for selective, repeatable manipulations, as well as their utility for projection and cell-type specificity via targeting by genetic markers, projection patterns, and activity (Luo et al., 2018). Initial viral tools included opsins which could drive spike activity with millisecond-timescale precision via light-activated opening of cation channels, starting with channelrhodopsin (Boyden et al., 2005), and other classes of opsins which inhibit neuronal activity via conductance of chloride pumps, such as halorphodopsin (Gradinaru et al., 2008). Optogenetic tools have been vastly expanded in recent years with improved variants of excitatory and inhibitory opsins, as well as opsins which modulate intracellular G-protein coupled receptor cascades (Fenno et al., 2011), expanding the temporal precision for manipulations and targeting approaches for dissecting cell and circuit-level activity. DREADDs are a chemogenetic tool with derived from mutated endogenous human muscarinic G-protein coupled receptors to generate synthetic receptors expressed on targeted neuronal populations with minimal to no constitutive activity (Armbruster et al., 2007; Roth, 2016; Vardy et al., 2015). These receptors are selectively activated by otherwise inert compounds, including clozapine-N-oxide (CNO) (Armbruster et al., 2007) and compound 21 (Vardy et al., 2015). One of the commonly used inhibitory DREADDs is $Hm_4D_i$, a human muscarinic receptor which results in reduced cAMP signaling and activation of inward rectifying potassium channels.

$Hm_3D_q$ is another variant which increases activity through intracellular calcium (Roth, 2016) and is one of the most widely used for activation of targeted cells. While predominantly used for activating or inactivating targeted cell populations, DREADDs mediate effects through activation of intracellular G-protein signaling cascades which may have differential impacts depending on cell type. While powerful, concerns with DREADDs arise from potential off-target effects with ligands like CNO (Gomez et al., 2017), as well as receptor downregulation, and unknown effects on downstream pathways in altering beta-arrestin signaling (Roth, 2016). Newer DREADDs variants utilized mutated kappa-opioid receptors for inhibition targeted cells with the pharmacologically inert compound Salvnorin B (Vardy et al., 2015). The monitoring of cell-type activity was revolutionized with the development of genetically encoded calcium indicators (GECIS) which exhibit superior signal-to-noise ratio and preserved spectral bandwidth for optical stimulation and multiple-indicator imaging (Broussard et al., 2014). GECIs measure changes in intracellular calcium and serve as an indirect measure of neuronal activity. These indicators are comprised of a circularly-permuted green fluorescent protein chromophore, M13, and CaM, which, when bound to calcium, results in water-mediated interactions between CaM and cpGFP which increase fluorescence in the emission range (Girven & Sparta, 2017). Commonly used GECIs are in the GCaMP family, which utilize single-fluorophore sensors; the more recent variant, the GCaMP6 family, has high sensitivities and kinetics approaching discrimination of individual action potentials. Furthermore, GECIs - like GCaMP6s - have been stably expressed on timescales ranging from weeks to months, providing a means of *in vivo* monitoring of neuronal activity correlated with behavior (Cui et. al, 2014; Grienberger & Konnerth, 2012). Two-photon microscopy can reliably image structures at depths up to 1 mm and leave tissue intact and permitted imaging of deeper structures *in vivo*. The reduction in light scattering in two-photon imaging utilizes nonlinear photon absorption at excitation wavelengths near the near-infrared range, reducing light scattering and photobleaching for high-resolution imaging. However, the utility of this technique is limited by susceptibility to motion artifacts, requiring head fixation, and slow frame rates relative to activity (Broussard, et. al, 2014; Grienberger & Konnerth, 2012; Helmchen & Denk, 2005). Micro-endoscopes utilizing gradient refractive index lenses permit free movement of awake, behaving animals, but are limited by the necessary removal of cortical tissue for lens implantation (Grienberger & Konnerth, 2012). Mitigating limitations in head fixation and invasiveness of 2-photon or microendoscope imaging, fiber photometry utilizes a single, multi-mode optical fiber chronically implanted for long-term monitoring of population activity. At a diameters up to 400μm, the fibers minimally disrupt surface tissue; connection of the fiber to a lightweight, flexible patch cord allows for recordings of GCaMP correlated with behaviors in freely

moving animals to assess cellular activity in response to rewards, novelty, and social behaviors, among others (Gunaydin et al., 2014). Newer systems, such as frame-projected independent fiber photometry, expands on this technique by permitting imaging of multiple cell populations, imaging multiple brain regions simultaneously, and imaging of presynaptic terminals. To achieve projection or cell-type specific targeting, tyrosine site-specific recombinases, including CRE-loxP and FLP-FRT systems, are frequently utilized through viral injections or in transgenic animal models. CRE-lox systems, derived from P1 bacteriophages, utilize the protein, Cre recombinase, which recognizes 34 base pair (bp) loxP sites, which can be oriented for inducible activation, deletion, and translocation of genes; similarly, FLP, derived from the yeast species *Saccharomyces cerevisae* binds to the 34 bp Flp recognition sequence (FRT sites), can be used for various recombination strategies (Meinke et al., 2016). These recombinases can be packaged in vectors with retrograde and anterograde promoters to achieve projection-specific targeting paired with CRE or FLP inducible opsins, GECIs, and DREADDs.

## 1.8 Thesis overview

The aim of this thesis is to examine impacts of drug history in a preclinical model of substance use, as well as to dissect contributions of cortical circuits to psychostimulant-mediated behaviors and complex motivated behaviors. Chapter 2 addresses the first question through a rat self-administration model of polysubstance use of cocaine and heroin compared to single substance models, comparing their behavior in paradigms examining drug consumption, effort to maintain drug consumption, and cue-induced relapse to drug seeking. To achieve the aims of the second question, chemogenetic and optogenetic tools were utilized for inhibition and stimulation, respectively, of distinct cortical projection populations during various behavioral assays. Chapter 3 investigates the impact of chemogenetic inhibition of IT neurons in the ACC on the cocaine-induced locomotor activity and sensitization, while Chapter 4 uses chemogenetic inhibition of IT and PT neurons and optical stimulation of PT neurons to investigate the impact of IT and PT modulation on sucrose preference, place preference, and cocaine self-administration. Chapter 5 extends the investigation the role of IT and PT neurons in the OFC to decision-making in a probabilistic decision-task, and in behavioral flexibility through a reversal learning task by selective chemogenetic inhibition and concurrent inhibition of these populations. Finally, Chapter 6 will discuss these findings, as well as outstanding questions and future directions.

**Chapter 2:**

**The Impact of drug history on addiction-related phenotypes**

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# The impact of cocaine and heroin drug history on motivation and cue sensitivity in a rat model of polydrug abuse

Elizabeth A. Crummy, Elizabeth A. Donckels, Britahny M. Baskin, Brandon S. Bentzley, and Susan M. Ferguson

**Author Information**

*Neuroscience Graduate Program, University of Washington, Seattle, WA, USA*

Elizabeth A. Crummy, Britahny M. Baskin, and Susan M. Ferguson

*Center for Integrative Brain Research, Seattle Children's Research Institute, Seattle, WA, USA*

Elizabeth A. Crummy, Elizabeth A. Donckels, Britahny M. Baskin, and Susan M. Ferguson

*Department of Psychiatry and Behavioral Sciences, Stanford University, Palo Alto, CA, USA*

Brandon S. Bentzley

*Department of Psychiatry and Behavioral Sciences, University of Washington, Seattle, WA, USA*

Susan M. Ferguson

**Corresponding Author**

Correspondence to Susan M. Ferguson

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## Author Contributions

E.A.C, E.A.D, and S.M.F designed the experiments. E.A.C, E.A.D, and B.M.B performed the experiments. E.A.C analyzed the data; B.S.B and S.M.F assisted with analysis and interpretation. E.A.C and S.M.F wrote the manuscript with input from the other authors.

**Abstract**

*Rationale*

Comorbid use of heroin and cocaine is highly prevalent among drug users and can greatly increase addiction risk. Nonetheless, little is known regarding how a multi-drug history impacts motivation and cue responsivity to individual drugs.

*Objective*

We used behavioral-economic procedures to examine motivation to maintain drug consumption and tests of drug-seeking to drug-associated cues to assess sensitivity to heroin and cocaine-associated cues in rats that had a self-administration history of heroin, cocaine, or both drugs.

*Results*

Unexpectedly, we found that groups with a polydrug history of heroin and cocaine did not have higher levels of motivation or cue-induced reinstatement of drug-seeking for either cocaine or heroin compared to single drug groups. Nonetheless, we did find drug-specific differences in both economic price and cue sensitivity. Specifically, demand elasticity was lower for cocaine compared to heroin in animals with a single drug history, but not with polydrug groups. In addition, cocaine demand was predictive of the degree of cue-induced reinstatement of drug-seeking for cocaine following extinction, whereas heroin demand was predictive of the degree of reactivity to a heroin-associated cue. Furthermore, although cue reactivity following the initial self-administration phase did not differ across cues and drug history, reactivity to both heroin and cocaine cues was greater during subsequent heroin use compared to cocaine use, and this enhanced reactivity to heroin cues persisted during forced abstinence.

*Conclusions*

These results indicate that there is a greater motivation to maintain cocaine consumption, but higher sensitivity to drug-associated cues with a history of heroin use, suggesting that cocaine and heroin may drive continued drug use through different behavioral processes.

## Introduction

Comorbid use of heroin and cocaine is frequently reported in clinical research of various populations with substance use disorders (Leri et al. 2003, 2004; Williamson et al. 2007; Wang et al. 2017). In fact, studies have found that up to 80% of people with a heroin use disorder also use cocaine (Leri et al. 2003), and are 15 times more likely to develop a cocaine use disorder than people that only use cocaine (National Survey on Drug Use and Health 2011-2013). Notably, involvement of opioids in cocaine overdoses has increased from 29% to 63% from 2000 to 2015 (Mcall Jones et al. 2017). Thus, cocaine and heroin co-use is not only widespread, but it is also associated with unmet physical and mental healthcare needs, poorer treatment outcomes, and greater addiction severity, including persistence of drug use and risk of overdose (Evans et al. 2017; Hartel et al. 1995; Kerr et al. 2005; Kinner et al. 2012; Lorvick et al. 2018). It is critical, therefore, to have a better systematic understanding of the neurobiological alterations resulting from polysubstance use, as well as how drug history impacts addiction severity. To date, the majority of animal studies investigating these questions have examined simultaneous use of these drugs, such as with “speedball” administration of heroin and cocaine (Pattison et al, 2014), but have largely ignored sequential use, although this pattern of multi-drug taking is reportedly more ubiquitous (Leri et al. 2004; Hayden et al. 2014).

The purpose of this study was to begin to address this gap in the research by investigating the effects of drug history on two measures of addiction severity. Accordingly, we established a paradigm whereby rats were trained to self-administer single (cocaine or heroin) or multiple (alternating sessions of cocaine and heroin) drugs before undergoing assessment on several tests of addiction-like behavior, including motivation to maintain drug consumption and sensitivity to drug-associated cues. Motivation was examined using a behavioral economics paradigm which permits quantitative comparison of demand for different rewards across normalized measures (Hursh 1991). This method is advantageous over the more commonly used progressive ratio (PR) schedules of reinforcement because price is increased by decreasing the unit dose of a drug rather than increasing effort, thereby avoiding confounds of delay on cost and ceiling effects of limited consumption that are inherent to PR schedules (Bickel et al. 1990, Schelp et al. 2017). Drug-seeking was assessed by both cue-induced reinstatement following extinction and by cue-reactivity tests administered at multiple timepoints after forced abstinence from rewards. Given reports that heroin users show inelasticity to heroin and cocaine price, but cocaine users show elastic demand for cocaine (Jofre-Bonet and Petry 2008), we hypothesized that a polydrug history would exacerbate responding and demand for drugs of abuse, and predicted that a polydrug

history would result in greater demand for cocaine and heroin compared to single drug histories, with all groups showing inelastic demand for heroin consumption. In addition, we predicted that polydrug groups would show greater cue-induced responding for both cocaine and heroin compared to single drug groups.

## Methods

### Animals

All experiments were approved by the Seattle Children's Research Institute Institutional Animal Care and Use Committee and were conducted in accordance with National Institutes of Health guidelines. Male Sprague-Dawley rats (n=38 Envigo) weighing 250-274g upon arrival were pair-housed in a temperature- and humidity-controlled vivarium on a 12-hour light/dark cycle. Rats were acclimated for at least three days prior to any experimental manipulations. Food was provided *ad libitum* until the start of the experiment, after which rats were mildly food restricted and fed 40±1g of chow per day per cage. Water was available *ad libitum.* Prior to the start of self-administration, rats were divided into three groups pseudorandomly: Rats that self-administered a single drug daily (Single Drug High (SDH); cocaine, n=7 or heroin, n=6), rats that alternated between drug and flavored food pellet self-administration sessions (Single Drug Low (SDL); cocaine + flavored food pellets, n=6 or heroin + flavored food pellets, n=6), and rats that alternated administration of two drugs (Poly Drug (PD): cocaine + heroin, n=7 (Experiment 1) and n=6 (Experiment 2)). Experimenters were not blinded to group assignment.

### Drugs

Cocaine HCl and Diamorphine HCl were obtained from the National Institute on Drug Abuse and were dissolved in sterile 0.9% saline.

### Surgery

Rats were anesthetized with isoflurane (2-5% inhaled; Patterson Veterinary) during the procedure and received an injection of meloxicam (0.1 mg/kg SC; Patterson Veterinary) prior to surgery for analgesia. Rats were monitored for a minimum of three recovery days following surgery prior to the start of self-administration. Chronic indwelling catheters were placed into the right jugular vein and attached to a back-mounted port as previously described (Crombag et al., 2000). Following

surgery, catheters were flushed daily with 0.1 mL of sterile saline containing 10 mg/mL gentamicin sulfate (Patterson Veterinary) to prevent occlusions and minimize risk of infection. Catheter patency was tested prior to the first self-administration session and following completion of each experimental phase by IV injection of up to 0.3 mL of methohexital sodium (Brevital sodium: 10 mg/mL in sterile water; Patterson Veterinary). Rats that became ataxic within five seconds were considered to have patent catheters. If catheter patency was lost during any phase of the paradigm, the rat was removed from subsequent analysis.

## Behavior

### *Self-administration chambers*

Behavioral testing occurred in 20 standard operant self-administration chambers equipped with retractable levers, stimulus lights, a house light, a feeder, and a metal grid floor (Med Associates ENV-007CT). The front wall housed two white stimulus lights, one located above each lever, with an additional green stimulus light above the white stimulus light on one side. The back wall contained a white house light. A syringe pump (Med Associates PHM-100VS), located outside on top of the chamber, delivered either cocaine or heroin via tubing attached to the catheter backport. All tubing was attached to a suspended swivel (Instech 375/22) to allow rats to move freely within the chambers.

### *Self-administration training*

A timeline of the full behavioral paradigm is shown in Figure 1a. Depending on group assignment and session, a response on the lever resulted in presentation of a cue light and either a 0.4 mg/kg infusion of cocaine, a 0.15 mg/kg infusion of heroin (each delivered in 50 µL over 2.8 seconds), or administration of four food pellets (banana- or chocolate-flavored), followed by a 20 second timeout period during which the cue light above the lever was illuminated. Sessions occurred seven days per week during the light cycle and alternated every other day between administration of cocaine (Experiment 1) or heroin (Experiment 2) + presentation of a white cue light on one lever (lever A) and administration of cocaine, heroin or flavored food pellets + presentation of a green cue light on the alternate lever (lever B). This design resulted in the following groups: Rats that self-administered a single drug daily (Single Drug High (SDH); cocaine or heroin), rats that alternated between drug and flavored food pellet self-administration sessions (Single Drug Low

(SDL); cocaine + flavored food pellets or heroin + flavored food pellets), and rats that alternated administration of two drugs (Poly Drug (PD): cocaine + heroin). Once a reinforcer was assigned to lever A and lever B, it was not changed for the duration of the experiment. No inactive lever was presented. Sessions lasted two hours on a fixed ratio (FR1) schedule to establish modest drug intake without escalation so that potential group differences in *ad libitum* consummatory behavior could be observed while avoiding confounds from differences in appetitive seeking that are inherent to higher-order ratio schedules (Roberts et al. 2013). The doses of heroin was selected because it resulted in higher intake during self-administration, and the cocaine dose was selected because we have previously found that it produces robust self-administration; both doses are on descending limbs of the dose-response curve, which provides greater intake (Kerstetter et al. 2016; Leri et al 2001; Yager et al. 2018).

*Cue reactivity tests*

Following the end of the initial self-administration phase, rats underwent three, two-hour sessions of cue responding on the lever where the reinforcer was previously paired with the green stimulus light (i.e., lever B). For these tests, rats were connected to the infusion line, placed in the chamber and each session began with extension of lever B. Responses on a FR1 schedule resulted in illumination of the green cue light above the lever for 20 seconds, but no reward administration. Tests occurred at three timepoints: 24 hours following the final self-administration session, 24 hours following the final day of the threshold procedure, and two days after cue-induced reinstatement on lever A (i.e., at 1, 13, and 29 days following the end of initial self-administration training). Unlike the cue-induced reinstatement test on lever A, no extinction sessions were performed for lever B prior to the cue reactivity sessions.

*Behavioral economics*

Twenty-four hours after the first cue reactivity test, rats underwent a between-session threshold procedure. In this paradigm, the unit price (responses/mg of drug) of cocaine or heroin was increased each session by reducing the drug concentration (mg/kg) that was infused for each lever press by a quarter-log scale (0.421, 0.237, 0.133, 0.075, 0.041, 0.024, 0.013, 0.075, 0.0041, 0.0024, 0.0013 mg/kg per infusion) for cocaine, or a third-log scale ( 0.533, 0.247, 0.115, 0.053, 0.025, 0.012, 0.005, 0.002, 0.001, mg/kg per infusion) for heroin. Rats had access to drug for the

entirety of each two-hour self-administration session, with no timeout periods between infusion. Because drug concentration was changed between sessions, the flow rate, volume, and infusion time were kept constant across sessions, unlike previous studies which decreased infusion time to change dose (Oleson and Roberts, 2009). Infusions were administered on an FR1 schedule and were paired with illumination of the white cue light during each infusion (50 µL in 2.8 seconds).

*Demand curve analysis*

Demand curve analysis was performed by graphically extracting parameters ($Q_o$, $P_{max}$, $O_{max}$) for each animal as previously described (Oleson & Roberts, 2009), and representative demand curves for cocaine and heroin are provided to illustrate derivation of these parameters (Figure 2). $Q_0$ was determined from the average responding at the highest unit dose of drug (average intake during first three threshold sessions) when drug price is minimal. $P_{max}$ was graphically determined as the inverse of the unit dose eliciting maximal levels of responding prior to reduction in responding, and decreased intake from $Q_0$ (apex of the response curve; derivative of intake curve at -1). $O_{max}$ was defined as the number of responses at $P_{max}$. Alpha (α) is a measure of consumption that is sensitive to price, with higher values corresponding to faster declines in the demand curve, indicating higher price sensitivity and elasticity (Schelp et al. 2017). This value was calculated using the exponential demand equation $log\ Q\ =\ log\ Q_0\ +\ k(e^{-\alpha Q_0 C} - 1)$(Hursh and Silberberg, 2008).

*Extinction*

Twenty-four hours after the second cue reactivity test on lever B, rats underwent three additional days of self-administration of cocaine or heroin on lever A (depending on their initial drug assignment to that lever) followed by extinction training on that lever. Extinction sessions lasted for two hours and consisted of extension of lever A (i.e., the lever that had been paired with the white cue light). Responses on the lever had no programmed consequence (i.e, no drug infusion or stimulus light illumination). Extinction training lasted for ten days.

*Cue-induced Reinstatement*

Following the final extinction session, rats underwent a two-hour cue-induced reinstatement test for cocaine or heroin on lever A (depending on their initial drug assignment to that lever). The session began with extension of lever A into the chamber. Responses on the lever resulted in illumination of the white cue light above the lever but no drug infusion.

*Statistical analysis*

Analyses were performed using GraphPad Prism 8. FR1 sessions on lever A between groups were analyzed using two-way repeated measures (RM) analyses of variance (ANOVA)s. FR1 sessions were analyzed for each lever using one-way RM ANOVAs, with comparisons against the first self-administration session. Total intake across groups was analyzed using one-way ANOVA. Paired, two-tailed t-tests were performed for comparing total infusions on levers A and B. Group comparisons of demand curves were made with two-way (Drug History x Price) ANOVAs for response and intake curves, and group comparisons of behavioral economic parameters were made using ordinary one-way ANOVAs. Responding during extinction and reinstatement was analyzed with RM two-way (drug history x time) ANOVAs. Cue reactivity was compared for each reinforcer across experiments using a two-way (Experiment x Drug Cued) ANOVA, and cue tests using RM two-way (Session x Experiment) ANOVA. Linear regressions were performed to determine the relationships between $\alpha$ and $Q_0$ values and extinction day one, reinstatement, cue test two, and cue test three. RM ANOVAs with over two factors were adjusted for unequal variability of differences using Geisser-Greenhouse sphericity corrections. All post hoc analyses were Bonferroni corrected for multiple comparisons, unless otherwise stated.

**Results**

*No group differences in the acquisition of cocaine or heroin self-administration*

A summary of the experimental design and timeline is shown in Figure 1a. Briefly, rats were divided into three groups prior to beginning each experiment: Rats that self-administered a single drug daily (Single Drug High (SDH)), rats that alternated between self-administration of drug and flavored food pellets (Single Drug Low (SDL)), and rats that alternated between self-administration of cocaine and heroin (Poly Drug (PD)). A two-way ANOVA of lever A sessions for each drug history group revealed no differences in responding for cocaine across groups (Figure 1b: Experiment 1, no main effect of Drug History: $F_{(2,17)}=1.08$, *n.s.*), nor for heroin (Figure 1f: Experiment 2, no main effect of Drug History: $F_{(2,15)}=1.96$, *n.s.*), but did reveal that all groups

learned to self-administer the lever A reward across ten sessions (Main effect of session: Experiment 1 (Cocaine Lever A): $F_{(1.88, 31.97)}$=*24.69, p<0.0001;* Experiment 2 (Heroin Lever A): $F_{(2.66, 39.90)}$=*12.00, p<0.0001)*. For lever A cocaine administration, there was a significant interaction of Session x Drug History, suggesting there were differences in learning rates across groups for the lever A reward (Session x Drug History Cocaine (Lever A): $F_{(18,153)}$=*2.40, p=0.002*). Post-hoc analyses show greater cocaine intake on the final lever A session in SDH vs. PD groups (Bonferroni-corrected, *p<0.05*). RM one-way ANOVAs for self-administration across sessions for lever B rewards revealed that, although the number of responses varied by reinforcer, all groups increased their responses for each reinforcer over sessions in Experiment 1 (Fig. 1b-c; Experiment 1 (Cocaine); SDL-Cocaine A: $F_{(3.04, 15.19)}$=8.93, $p$< 0.005; SDL-Food: $F_{(3.56, 17.79)}$ = 8.96, $p$=0.0005; SDH-Cocaine A: $F_{(2.85, 17.07)}$ = 11.34, $p$<0.0005; SDH-Cocaine B: $F_{(2.31, 13.88)}$ = 27.03, $p$<0.0001; PD-Cocaine: $F_{(2.34, 14.02)}$ = 7.57, $p$<0.005; PD-Heroin: $F_{(1.98, 11.86)}$ = 3.88, $p$=0.051) and in Experiment 2 (Fig. 1d-e; Experiment 2 (Heroin); SDL-Heroin: $F_{(1.56, 7.77)}$= 3.61, , $p$= 0.08 ; SDL-Food: $F_{(2.68, 13.39)}$ = 14.15, $p$<0.0005; SDH-Heroin A: $F_{(1.55, 7.77)}$ = 5.39, $p$<0.05; SDH-Heroin B: $F_{(1.37, 6.84)}$ =4.22, $p$=0.07; PD-Heroin: $F_{(2.19, 10.96)}$=4.86, $p$<0.05; PD-Cocaine: $F_{(2.35, 11.74)}$=10.49, $p$<0.005). As expected, since SDH groups have 20 self-administration sessions of cocaine or heroin compared to the 10 sessions of the other groups, ordinary, one-way ANOVAs for drug history revealed that the SDH groups had significantly higher levels of total drug intake of cocaine (Fig. 1f; $F_{(2, 17)}$ =30.26, $p$<0.0001) and heroin (Fig. 1g; $F_{(2, 15)}$ =19.73, $p$<0.0001). However, Bonferroni-corrected post-hoc analyses showed no differences in intake levels between the SDL and PD groups for either cocaine (Fig. 1f)) or heroin (Fig. 1g)). In addition, responses on lever A and B did not significantly differ in SDH groups for cocaine or heroin, indicating that responding was not biased towards a particular lever (Paired t-test for infusions on levers A and B; Cocaine (Fig. 1l) or Heroin (Fig. 1p)). To determine if the order of heroin and cocaine self-administration impacted total intake in the PD group, intake of this group was compared across experiments. Two-way ANOVA analysis revealed no significant main effect of experiment (Fig. 1h-l; $F_{(1,11)}$=*0.81, n.s.*) and no significant interaction of Drug x Experiment ($F_{(1,11)}$=*1.29, n.s.*), suggesting that intake of both drugs was not influenced by which drug was initially self-administered. Nonetheless, as noted with other analyses, differences in total intake were found between cocaine and heroin, (Main effect of drug: $F_{(1,11)}$=*38.79, p<0.0001*).

*Behavioral Economics*

Across-session threshold procedures were used to assess motivation as a function of drug history. Multiple demand parameters were extrapolated from the threshold procedures, and

include: $Q_o$, which is the initial intake of drug at minimal to no cost of consumption; $P_{max}$, which is the maximal price individuals are willing to pay to maintain initial intake levels (i.e., to defend $Q_o$); $O_{max}$, which is the level of responding at $P_{max}$, and alpha (α), which is a normalized measure that is inversely related to $P_{max}$ normalized to $Q_o$, and is representative of price elasticity. Relative to cocaine, heroin demand curves were noticeably rightward shifted to higher price points for all animals, with some rats persistently increasing responses even at the lowest price points (Figure 2a-b).

*Demand for cocaine is not dependent on cocaine drug history.*

All groups in Experiment 1 exhibited a sensitivity to changes in price for cocaine, as indicated by a decrease in responding at lower doses in the threshold procedure (Figure 2a; two-way RM ANOVA main effect of Price on responding: $F_{(2.33, 32.65,)}=25.63, p<0.0001;$ main effect of Price on intake: $F_{(2.13,29.76)}=75.92, p<0.0001$). This sensitivity did significantly differ by drug history responding (Figure 2a main effect of Drug History on responding: $F_{(2,14)}=5.56, p<0.05;$ interaction of Price x Drug History: $F_{(20,140)}=2.01, p=0.01$), but not by intake (Figure 2a main effect of Drug History on intake: $F_{(2,14)}=1.45, n.s.;$ interaction of Price x Drug History: $F_{(20,140)}=1.04, n.s.)$ ; post-hoc Bonferroni-corrected analyses indicate that differences in response at each price occurred between SDH and PD groups ($p<0.05$). One-way ANOVA found no significant group differences in $Q_o$ (Figure 3d; $F_{(2,13)}$ = 0.15, n.s.), which averaged between 14-16 mg/kg, or $P_{max}$ (Figure 2e; $F_{(2,13)}$=0.75, n.s.). However, $O_{max}$ was significantly higher in the SDH group compared to the PD group, consistent with response vs. price analyses (Figure 2f; $F_{(2,13)}$=4.47, $p$<0.05; Bonferroni-corrected post hoc comparisons: SDH versus PD, $p$<0.05; SDH versus SDL, $p$=0.09). Taken together, these results suggest that a higher level of cocaine intake during training elicits greater overall responding for cocaine, but drug history does not alter baseline consumption or motivation to self-administer cocaine.

*Demand for heroin is not dependent on heroin drug history.*

As with cocaine, all groups in Experiment 2 exhibited a sensitivity to changes in price for heroin (Figure 2b. main effect of Price on responding: $F_{(2.05,26.64)}=29.67, p<0.0001;$ main effect of Price on intake: $F_{(1.65, 21.41)}=22.99, p<0.0001$); however, there were no significant differences in sensitivity between groups, though there was a trend for differences in responding (Figure 2b; no main effect of Drug history on responding: $F_{(2, 13)}=3.70, n.s.;$ no main effect of Drug history on intake: $F_{(2,13)}=0.70, n.s.$). An interaction was found for Price x Drug history on responding ($F_{(16,104)}=2.51, p<0.01$), but not on intake ($F_{(16, 104)}=1.29, n.s.)$. Bonferroni-corrected post-hoc

analyses, however, did not reveal significant differences in responding between groups at any price . In addition, there were no significant differences in $Q_0$ (Figure 2g; one-way ANOVA: $F_{(2,14)}$ =1.45, n.s.), $P_{max}$ (Figure 2h; $F_{(2,14)}$=1.16, n.s.), or $O_{max}$ (Figure 2i; $F_{(2,14)}$ = 0.60, n.s.) as a function of drug history. These results indicate that neither the degree of heroin intake nor combining heroin use with cocaine use altered the motivation to self-administer heroin.

*Price sensitivity is lower for cocaine than heroin*

Alpha ($\alpha$) provides a normalized measure of elasticity to changes in price, which permits comparison of price sensitivities across different reinforcers (Hursh and Winger, 1995). Thus, we can use $\alpha$ to assess and compare the relative demand elasticity of heroin and cocaine across drug histories. We found drug-specific differences in $\alpha$ values, with significantly higher heroin values compared to cocaine values (Figure 2c, two-way ANOVA: Main effect of Drug $\alpha$: $F_{(1,27)}$=23.31, p<0.0001; no Main effect of Drug history: $F_{(2,27)}$= 0.78, n.s.; no Interaction of Drug x Drug history: $F_{(2,27)}$*=2.09, n.s.*). Interestingly, this difference was only in groups with single drug histories, as $\alpha$ values for heroin and cocaine were not significantly different in the PD groups (Figure 2c, Bonferroni-corrected post-hoc tests: SDL cocaine vs. heroin: $p$<0.01; SDH cocaine vs. heroin: $p$<0.001, PD cocaine vs. heroin, *n.s.*). Together, these data suggest that cocaine has a greater motivational value than heroin, but that this distinction is eliminated with a history of self-administering both drugs.

*Motivation for cocaine but not heroin is predictive of cue-induced reinstatement of drug-seeking*

We next examined whether drug history affects reinstatement of drug-seeking by a drug-associated cue. Following the threshold procedure and three additional days of cocaine (Experiment 1) or heroin (Experiment 2) self-administration, rats underwent ten sessions of extinction training on lever A. As expected, all groups significantly decreased responding across extinction sessions (Figure 3a, two-way ANOVA; Cocaine: Main effect of session, $F_{(2.73, 35.52)}$=13.18, $p$<0.0001; Figure 3b, two-way ANOVA Heroin: Main effect of session, $F_{(2.87,37.28)}$=7.58, $p$=0.0005), but extinction responding did not differ across drug histories (Cocaine: no Main effect of Drug History, $F_{(2,13)}$*=0.51, n.s.*; Heroin: no Main effect of Drug History, $F_{(2,13)}$*=0.03, n.s.*). There was a Session x Drug History interaction for heroin extinction (Heroin: $F_{(18,117)}$*=1.73, p< 0.05*) though post hoc analyses did not report differences in responding across any groups for any extinction session . In addition, all groups in Experiment 1 made significantly more lever responses during the cocaine cue-induced reinstatement test compared to the last 3 days of extinction (Figure 3c, Main effect of reinstatement, $F_{(1,13)}$*=24.27, p<0.001*). However, there were

no significant differences in lever responses across groups (Figure 3c, Drug history: $F_{(2,13)}$= 0.51, *n.s.*). For Experiment 2, there was a main effect of responding between extinction and reinstatement (Figure 3d, Heroin: Main effect of reinstatement, $F_{(1,13)}$=*53.67, p<0.0001*). As with cocaine reinstatement, there were no significant differences in lever responses between groups for the cue associated with heroin (Figure 3d, Drug history: $F_{(2,13)}$=0.14, n.s.). Together, these data suggest that neither the total amount of drug intake nor combining drug use with alternate reinforcers impacted the degree of drug-seeking for a cue associated with cocaine or heroin use.

To determine if no-cost drug intake or price elasticity were predictive of cue-induced drug-seeking following extinction, regressions for $Q_o$ and $\alpha$ versus responding during the reinstatement tests were performed, as in previous studies (Bentzley et al., 2014; Cox et al., 2018). Groups were collapsed for regression analyses. Price elasticity (i.e., $\alpha$) was predictive of reinstatement to cues associated with cocaine (Figure 3e: $F_{(1,14)}$=9.12, $p$<0.01, $R^2$=0.39) but not heroin (Figure 3e: $F_{(1,14)}$=1.56, n.s.), with lower $\alpha$ values associated with higher levels of cue-induced responding. In contrast, no-cost intake (i.e., $Q_o$) was not predictive of cue-induced drug-seeking for either drug reward (Figure 3f: Cocaine: $Q_o$: $F_{(1,14)}$=1.57, n.s.; Heroin: $F_{(1,14)}$=0.19, n.s.). Neither $\alpha$ nor intake were predictive of responding on the initial extinction session (Figure 3g,h) n.s. . These data suggest that the degree of motivation for cocaine, but not heroin, is predictive of the degree of cue-induced reinstatement of drug-seeking.

*Heroin and cocaine use differentially affect cue reactivity*

During the self-administration phase, groups that underwent cocaine (Experiment 1) or heroin (Experiment 2) self-administration on lever A also underwent self-administration for either cocaine, heroin or food on lever B. Thus, this paradigm allows us to examine how drug history and continued use of one drug affects drug-seeking of another reinforcer after forced abstinence. Cue-reactivity tests were administered on lever B at three time points throughout the paradigm, with no extinction sessions on this lever prior to these cue tests: 24 h after the last self-administration session, 13 days after the last self-administration session (which was 24 h following the final threshold session), and 29 days after the last self-administration session (which was 12 days after cessation of all drug self-administration) (Figure 4a). There were no differences in cue-induced responding on Test 1 across groups or across experiments (Table 1: two-way ANOVA; No main effect of cue ($F_{(2,26)}$=0.72, n.s.), No main effect of experiment ($F_{(1,26)}$=0.50, n.s.), and No Cue x Experiment interaction ($F_{(2,26)}$=0.69, n.s.)), indicating that baseline cue reactivity was the same for all cues, and was not influenced by drug history. In contrast, groups that were subsequently maintained on heroin (i.e. the Experiment 2 SDL, SDH, and PD groups) showed

significantly greater responding for all reward-associated cues on Test 2 compared to groups that were subsequently maintained on cocaine (i.e. the Experiment 1 SDL, SDH, and PD groups) (Table 1; Main effect of experiment $F_{(1,29)}$=32.19, $p$<0.0001). In addition, although responding was not different across cues associated with heroin, cocaine or food in groups being maintained on cocaine, there was a significant difference in responding across these cues in groups being maintained on heroin (i.e. differences between SDL, SDH, and PD groups in green cue responding from Experiment 2) (Table 1; Main effect of cue $F_{(2,29)}$=3.53, $p$<0.05). Similarly, following a period of complete drug abstinence, animals that had a history of heroin use showed significantly greater responding for cues associated with all rewards on Test 3 compared to animals that had a history of cocaine (i.e. greater responding across all groups from Experiment 2 vs. groups in Experiment 1) (Figure 4d; Main effect of experiment $F_{(1,28)}$=10.56, $p$<0.01). These results suggest that cue reactivity is more persistent during continued heroin use and continues into abstinence.

All animals underwent all three cue tests; therefore, this paradigm can also be used to determine how continued drug use and abstinence affect reactivity to the same cue over time. For both food- and cocaine-associated cues, there was a significant decrease in responding following a period of cocaine or heroin maintenance compared to initial responding (i.e. differences between SDL groups in Experiments 1 and 2 for food cues and differences between the SDH group from Experiment 1 and the PD group from Experiment 2 for cocaine cues) (Figure 4b,c; Food cue: Main effect of session: $F_{(1.18,10.58)}$=13.39, $p$<0.01, no Main effect of Experiment: $F_{(1,9)}$=*0.65, n.s.* ; Cocaine cue: Main effect of session: $F_{(1.53,12.23)}$=14.76, $p$=0.001, no Main effect of experiment: $F_{(1,8)}$=*0.87, n.s.*). In contrast, responses to a cue associated with heroin decreased following cocaine maintenance (i.e. the Experiment 1 PD group) (Figure 4d, Main effect of session, $F_{(1.35,12.10)}$=5.52, $p$<0.05; post hoc Bonferroni corrected for row effect: test 2 $p<0.05$), and this effect persisted following abstinence, whereas responding to a heroin-associated cue did not change across tests in animals with a history of heroin use (i.e. the Experiment 2 SDH group) (Fig. 4d, Main effect of experiment: $F_{(1,9)}=10.03$, $p<0.05$). However, no interaction was found for Session x Experiment. Together, these results suggest that cued seeking is blunted for all rewards during ongoing cocaine use but only for non-heroin rewards during on-going heroin use. In addition, this cued seeking begins to rebound during forced abstinence from cocaine but not heroin.

Linear regressions were calculated to determine the predictive validity of $\alpha$ and $Q_0$ to drug-seeking following threshold testing for cocaine (experiment 1) or heroin (experiment 2). We found

that $\alpha$ for heroin was significantly correlated with cue reactivity (Figure 4e; $\alpha$: $F_{(1,14)}$=5.38, $p$<0.05, $R^2$=0.28;) and trended towards significance for cocaine elasticity and cue reactivity (Figure 4e; $\alpha$: $F_{(1,14)}$=3.48, $p$=0.08, $R^2$=0.20) when cue responsivity was tested 24h after the threshold procedure. However, $\alpha$ did not correlate with cue responsivity following periods of total drug abstinence (i.e., during the third cue test) (Figure 4g, Cue test 3). In contrast, $Q_0$ for cocaine nor heroin was correlated with reactivity to reward-associated cues when tested either at 24h post-thresholding (Figure 4f, Cue test 2) or following complete drug abstinence (Figure 4h, Cue test 3*)*. These data suggest that the degree of motivation for heroin, but not cocaine, is predictive of the degree of reactivity to reward-associated cues.

**Discussion**

In order to examine the effects of cocaine and heroin poly-drug use on measures of addiction severity (economic demand parameters, cue-induced reinstatement following extinction, cue reactivity), we compared responses of rats that had a self-administration history of heroin, cocaine, or sequential use of both drugs. We found that groups with a polydrug history did not have higher levels of motivation or cue-induced reinstatement of drug-seeking for either cocaine or heroin when compared to single drug groups. This was surprising as previous work has found that extended periods of heroin self-administration enhance the motivating properties of cocaine, as demonstrated by increased breakpoints under a progressive ratio schedule of reinforcement (Ward et al. 2005). Nonetheless, given that studies have shown positive correlations between non-fatal overdoses and heroin/cocaine polydrug users (Kerr et al. 2007), as well as increased susceptibility to pulmonary disease and mechanical ventilation with cocaine use in heroin users (de Wit et al. 2008; Levine et al. 2005), understanding how the concurrent use of these drugs impacts other aspects of use, including the aversive consequences of withdrawal and overdose, would improve our understanding of potential risks and is essential for developing effective treatment options for patients with multi-drug dependencies.

Polydrug self-administering groups were compared to groups that self-administered either equivalent or larger amounts of cocaine or heroin, which allowed us to assess how total amount of drug intake, as well as multi-drug use, affects economic demand. Interestingly, we found that the total amount of drug consumed had no effect on demand elasticity for either cocaine or heroin. However, demand elasticity was predictive of the degree of cue-induced reinstatement of drug-seeking for cocaine, but not heroin. Previous studies have found that intermediate training doses of cocaine (0.5 and 1 mg/kg per infusion) and heroin (50 and 100 µg/kg per infusion) produce higher breakpoints on a progressive ratio schedule (Arnold and Roberts 1997) and greater

persistence in responding to drug-associated levers during extinction (Leri and Stewart 2001) compared to lower or higher training doses. These inverted U-shaped dose-response curves are consistent with our findings for cocaine, with demand curves exhibiting a similar pattern and $P_{max}$ coinciding with a unit dose around 41 µg/kg/infusion. However, the demand curves for heroin were markedly different, as they continued to rise up to prices that are indicative of doses as low as 1 µg/kg/infusion, whereas previous work with multiple training doses found peak progressive ratio responding at a 50 µg/kg/infusion training dose (Arnold and Roberts 1997).

The present work utilized a two-hour continuous access schedule of sequential polydrug administration of cocaine and heroin; future studies utilizing self-administration paradigms that consistently produce escalation of drug intake, such as six-hour or intermittent access, may help to reveal whether demand elasticity of heroin can be modulated by drug consumption patterns, as is the case with cocaine. Intermittent access schedules, which produce cocaine intake levels that are comparable to short access schedules but under a different temporal pattern, lead to higher $P_{max}$ values and greater responding ($O_{max}$) during within-threshold procedures than either long- or short-access trained rats (Zimmer, Oleson and Roberts 2012), suggesting pattern can supersede impact of total intake. Furthermore, environmental factors can also impact drug self-administration preference and affective valence in both human polydrug users and rodent polydrug self-administration models, with cocaine consumption preferred in non-resident environments and, conversely, preference for heroin in home environments (Caprioli et al. 2009, De Luca et al. 2009, De Pirro et al. 2018). In addition, polydrug users report differential cravings for cocaine and heroin throughout the day (Phillips et al., 2013). Thus, modulating the temporal and environmental conditions in our sequential use model may impact demand elasticity as well as sensitivity to drug-associated cues not currently captured in our paradigm.

This study did not examine whether polydrug use produces distinct or over-lapping molecular and circuit alterations, or if the effects are synergistic from those induced by exposure to a single drug. However, in support of over-lapping alterations, behavioral studies have found cross-locomotor sensitization between cocaine and heroin, as well as enhanced cocaine self-administration in rats undergoing protracted heroin withdrawal (Leri et al., 2003). Additionally, using a sequential heroin and cocaine self-administration paradigm, similar levels of glutamatergic neuronal activation were found following exposure to heroin- or cocaine-associated cues (Rubio et al. 2018) and methadone maintenance reduces cocaine-induced increases in mu-opioid receptor expression in the nucleus accumbens (Leri et al 2006). In support of synergistic effects,

sequential application of heroin and cocaine directly to cortical neurons induces greater neurotoxicity compared to exposure to either drug alone (Cunha-Oliveira et al. 2010).

Demand elasticity provides a normative measure of motivation across rewards; therefore, we can use this measure to directly compare motivation to cocaine and heroin in comorbid users. It was surprising that group differences were not found between the single- and poly-drug groups as human studies have found differences in demand curves for drug purchases as a function of drug history, with cocaine purchases being inelastic in heroin users but elastic in cocaine users (Petry and Bickel, 1998). Nonetheless, there was a stark contrast in demand curve patterns between cocaine and heroin. In particular, demand curves were shifted upward for cocaine and more sigmoidal in shape, indicating that cocaine produced a larger range of responses across the threshold procedure. In addition, responding for heroin was maintained by negligible doses per infusion, suggesting that heroin elicits perseverative responding. Of note, $\alpha$ values, which adjust for different no-cost intake levels, were lower for cocaine than heroin, indicating that cocaine demand is more price inelastic and has a greater motivational value than heroin. These findings are consistent with human studies of price elasticity, which found that polysubstance users make more cocaine purchases compared to heroin purchases across prices and that demand for heroin is largely inelastic across drug history, but are inconsistent with reports of elastic demand in single-substance cocaine users (Petry and Bickel 1998; Jofre-Bonet and Petry 2008). These results are also in-line with studies that found that rats will almost exclusively choose cocaine when given access to different combinations of cocaine or heroin (Ward, Morgan and Roberts 2005) as well as show greater responding on a cocaine-associated lever when both cocaine- and heroin-associated levers are presented during extinction following a poly-drug history (Leri and Stewart 2001).

Interestingly, we found that a polydrug history appears to remove the differences in price elasticity observed with single drug histories, which suggests that multi-substance use changes price valuation across drugs. Consistent with this, cross-price elasticity in human polydrug users has been reported to be drug specific, with increases in cocaine price reducing both cocaine and heroin consumption, while increases in heroin price augment cocaine consumption (Jofre-Bonet and Petry 2008). In addition, a study of heroin price changes in heroin users found that demand was equally inelastic for heroin and cocaine (Petry and Bickel 1998). Similarly, studies in rhesus monkeys comparing dose-dependent preferences for remifentanil and cocaine found minimal bias shifts at comparable doses, similar demand elasticity, and similar $P_{max}$ values (Koffarnus and Woods 2008; Wade-Galuska, Winger, and Woods 2007). Together, these data suggest that

heroin and cocaine are highly substitutable, which in turn may explain the similarities in demand elasticity between cocaine and heroin that we observed in the PD group.

In order to examine sensitivity to the same cues over time, we used a forced abstinence model. Unlike traditional extinction and reinstatement paradigms, this paradigm does not extinguish action-response and environmental contingencies prior to drug-seeking tests where lever responding elicits delivery of cues previously associated with rewards. This model is thought to mimic scenarios in clinical populations where drug users undergo periods of voluntary or involuntary removal from drug access (See, Elliot, and Feltenstein 2007; Reichel and Bevins 2009). Following the end of the initial self-administration phase, we tested cue responsivity at baseline (i.e., 24 h later), as well as cue responsivity following continued use of cocaine or heroin (which occurred on a different lever and was associated with a different cue), and following a period of complete drug abstinence. Of note, baseline cue reactivity was not different across cues, nor was it affected by self-administration history. However, unlike with cues associated with food or cocaine, responsivity to cues associated with heroin was maintained across all three cue tests, but only in animals that continued to self-administer heroin between the first and second cue tests (i.e., during the threshold procedure). These results suggest a persistent sensitivity to heroin cues that was driven by ongoing heroin use. The reductions in responsivity to cues associated with cocaine over multiple tests are consistent with a previous study that found reduced responding on subsequent relapse tests for methamphetamine (Reichel and Bevins 2009). We also found that, unlike the relationship between demand elasticity and cue-induced reinstatement following extinction, demand elasticity was predictive of the degree of responsivity to heroin-associated cues, but not cocaine-associated cues. The heightened and persistent reactivity to heroin-associated cues is particularly concerning as reports indicate that the increases in putative relapse triggers (i.e., mood changes and exposure to drug-associated cues) that normally only precede cocaine use in single drug users are increased prior to the initiation of heroin craving in comorbid users (Epstein et al. 2009).

In summary, the present work demonstrates that compared to single drug use, polydrug use reduces differences in price elasticity to cocaine or heroin without altering motivation or cue-induced reinstatement. In addition, this work reveals that cocaine elicits higher motivation for maintaining drug use than heroin, but cue sensitivity is heightened to heroin-associated cues compared to cocaine-associated cues. Taken together, these data suggest that cocaine and heroin may drive continued drug use through different behavioral processes. Thus, further elucidation of the mechanisms that underlie cocaine and heroin use in polysubstance users is

essential for addressing the increasing prevalence of polysubstance use, as well as to develop effective treatments to mitigate its effects. Finally, it is worth noting that although sex-specific differences were not examined in the present work, recent studies have reported a higher frequency of polysubstance use in females compared to males (Lorvick et al. 2018; Cropsey et al. 2015). Therefore, future work examining sex-specific effects on the measures studied here may reveal further distinctions in poly- versus single-drug history.

**Figures**

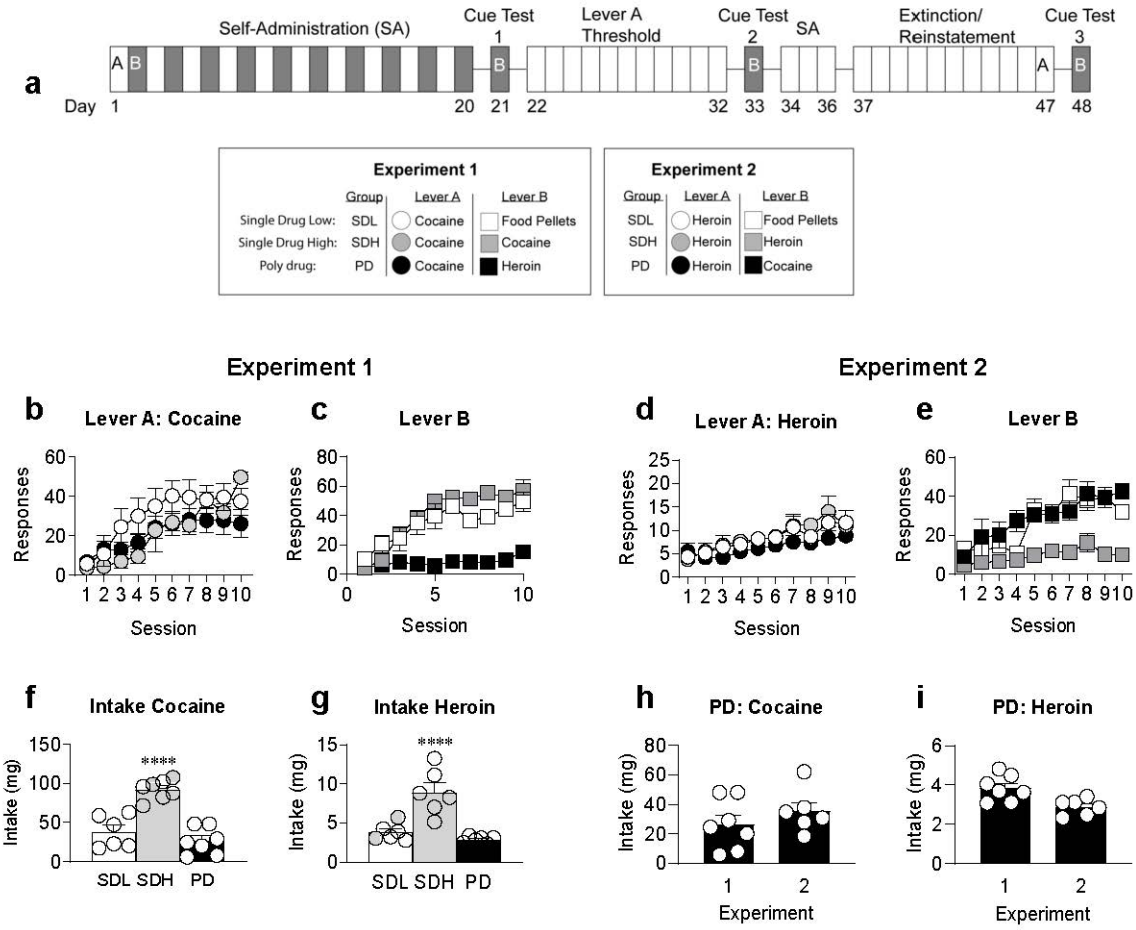



Fig 1**. Self-administration paradigm. a.** Timeline of experimental paradigm. Lever A (white boxes) was associated either with an infusion of cocaine (Experiment 1) or heroin (Experiment 2) across all groups. Lever B (gray boxes) was paired with either: flavored food pellets (SDL), cocaine (Experiment 1: SDH; Experiment 2: PD) or heroin (Experiment 1: PD, Experiment 2: SDH). **b.** Experiment 1 SDL (white circles), SDH (gray circles), and PD (black circles) groups acquire cocaine self-administration across ten total sessions on lever A. **c.** Experiment 1 SDL (white squares), SDH (gray squares), and PD (black squares) groups acquire food pellet,

cocaine, and heroin administration across ten sessions on lever B, respectively. **d.** Experiment 2 SDL, SDH, and PD groups acquire heroin self-administration across ten sessions on lever A. **e.** Experiment 2 SDL (white squares), SDH (gray squares), and PD (black squares) groups acquired food, heroin, and cocaine self-administration across ten sessions on lever B, respectively. **f.** Intake for cocaine is greater for SDH history than SDL or PD history across all self-administration sessions. **g.** SDH intake for heroin was higher than SDL and PD groups. **h.** Heroin intake was not biased to lever A or B in SDH rats. **h.** Total intake of cocaine in PD rats across ten sessions did not differ between Experiments 1 and 2. **i.** Total intake of heroin in PD rats across ten sessions did not differ between Experiments 1 and 2. ****p<0.0001, ***p<0.005 significance between drug history groups. Data are presented as mean ± SEM. Experiment 1: SDL n=6, SDH n=7, PD n=7; Experiment 2: SDL n=6, SDH n=6, PD n=6.

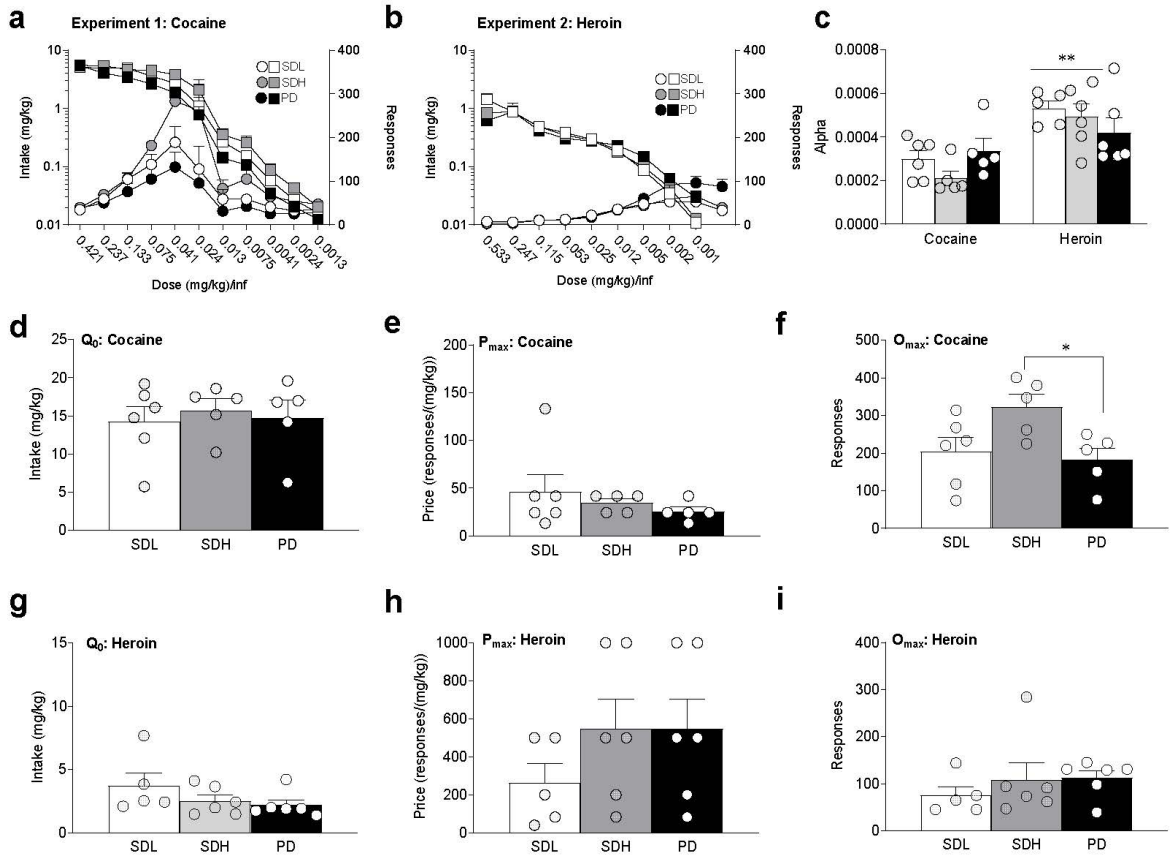


Fig 2. **Cocaine and heroin demand as a function of drug history a.** Intake (square) and response (circle) curves for cocaine and **b.** heroin. The number of presses (responses) and total mg infused (intake) at each price point are shown for SDL (white), SDH (gray), and PD (black) groups. Price is increased with decreasing doses of cocaine (Experiment 1) or heroin (Experiment 2) per infusion. **c.** Price elasticity (α) for SDL, SDH, and PD groups for cocaine and heroin threshold. Cocaine α was significantly lower for SDL and SDH compared to heroin α, but not for PD groups **d.** No-cost cocaine intake ($Q_o$) is independent of drug history. **e.** Maximal price to maintain initial intake ($P_{max}$) for cocaine is independent of drug history. **f.** Responding at $P_{max}$ for cocaine is larger with greater consumption history than polydrug history (SDH vs. PD). **g.** No-cost heroin intake ($Q_o$) is independent of drug history. **h.** Heroin $P_{max}$ is independent of drug history. **i.** Responding at $P_{max}$ does not depend on drug history. **p<0.01, across drugs. Data are presented as mean ± SEM. Cocaine (Experiment 1): SDL n=6, SDH n=5, PD n=5; Heroin (Experiment 2): SDL n=5, SDH n=6, PD n=6.

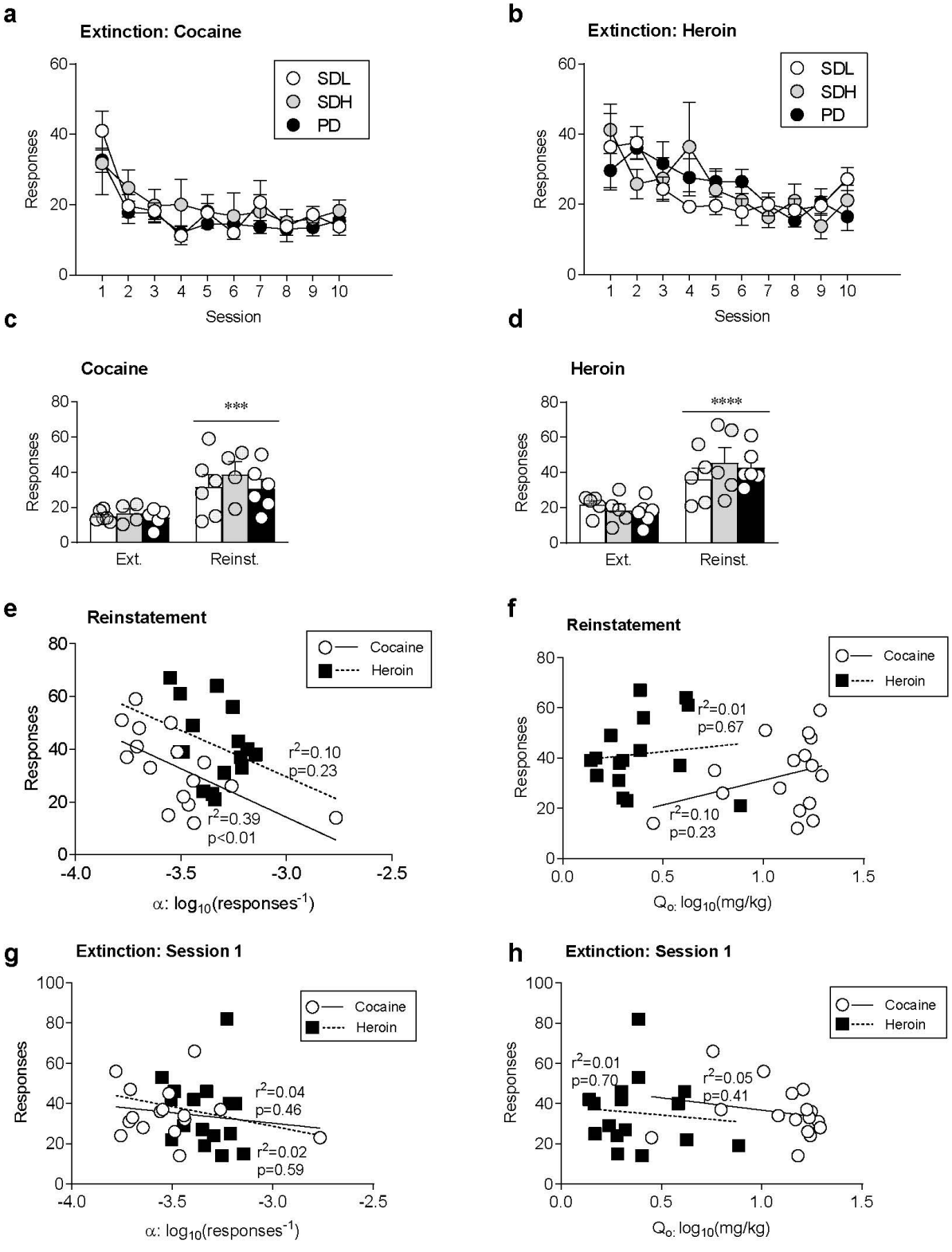

a
Extinction: Cocaine
SDL
SDH
PD
Responses
Session
b
Extinction: Heroin
SDL
SDH
PD
Responses
Session
c
Cocaine
***
Responses
Ext.
Reinst.
d
Heroin
****
Responses
Ext.
Reinst.
e
Reinstatement
Cocaine
Heroin
r2=0.10
p=0.23
r2=0.39
p<0.01
Responses
α: log10(responses-1)
f
Reinstatement
Cocaine
Heroin
r2=0.01
p=0.67
r2=0.10
p=0.23
Responses
Qo: log10(mg/kg)
g
Extinction: Session 1
Cocaine
Heroin
r2=0.04
p=0.46
r2=0.02
p=0.59
Responses
α: log10(responses-1)
h
Extinction: Session 1
Cocaine
Heroin
r2=0.01
p=0.70
r2=0.05
p=0.41
Responses
Qo: log10(mg/kg)

Fig 3. **Cue-induced reinstatement as a function of drug history.** All groups decrease lever A responding for **a.** Cocaine or **b.** Heroin after removal of drug and cue pairing for ten sessions. **c.** All groups reinstatement similarly to cocaine following extinction. **d.** Groups with a history of daily drug administration (SDH and PD) increase responding when presented with heroin cues, but not with intermittent drug access (SDL). Level of responding is similar on average across groups for cocaine and heroin cues. **e.** Price elasticity ($\log_{10}(\boldsymbol{\alpha})$) is predictive of degree of reinstatement of seeking to cocaine (White circles, solid regression line), but not heroin (black squares, dashed regression line), cues. **f.** No-cost drug intake ($\log_{10}(Q0)$) is not predictive of reinstatement of drug-seeking to cocaine or heroin cues. **g.** $\boldsymbol{\alpha}$ is not predictive of level of responding during the initial extinction session for cocaine or heroin. **h.** $Q_0$ is not predictive of reinstatement for cocaine or heroin cues. Drug history groups: SDL (White circles, bars), SDH (grey circles, bars), PD (black circles, bars).***$p<0.001$, ****$p<0.0001$ . Data are presented as mean ± SEM. Extinction vs. Reinstatement testing was performed using the average of the last three days of extinction. Cocaine: SDL n=6, SDH n=4, PD n=6; Heroin: SDL n=5, SDH n=5, PD n=6. Linear regressions for cocaine and heroin were from Reinstatement and Extinction Day 1 results collapsed for all groups.

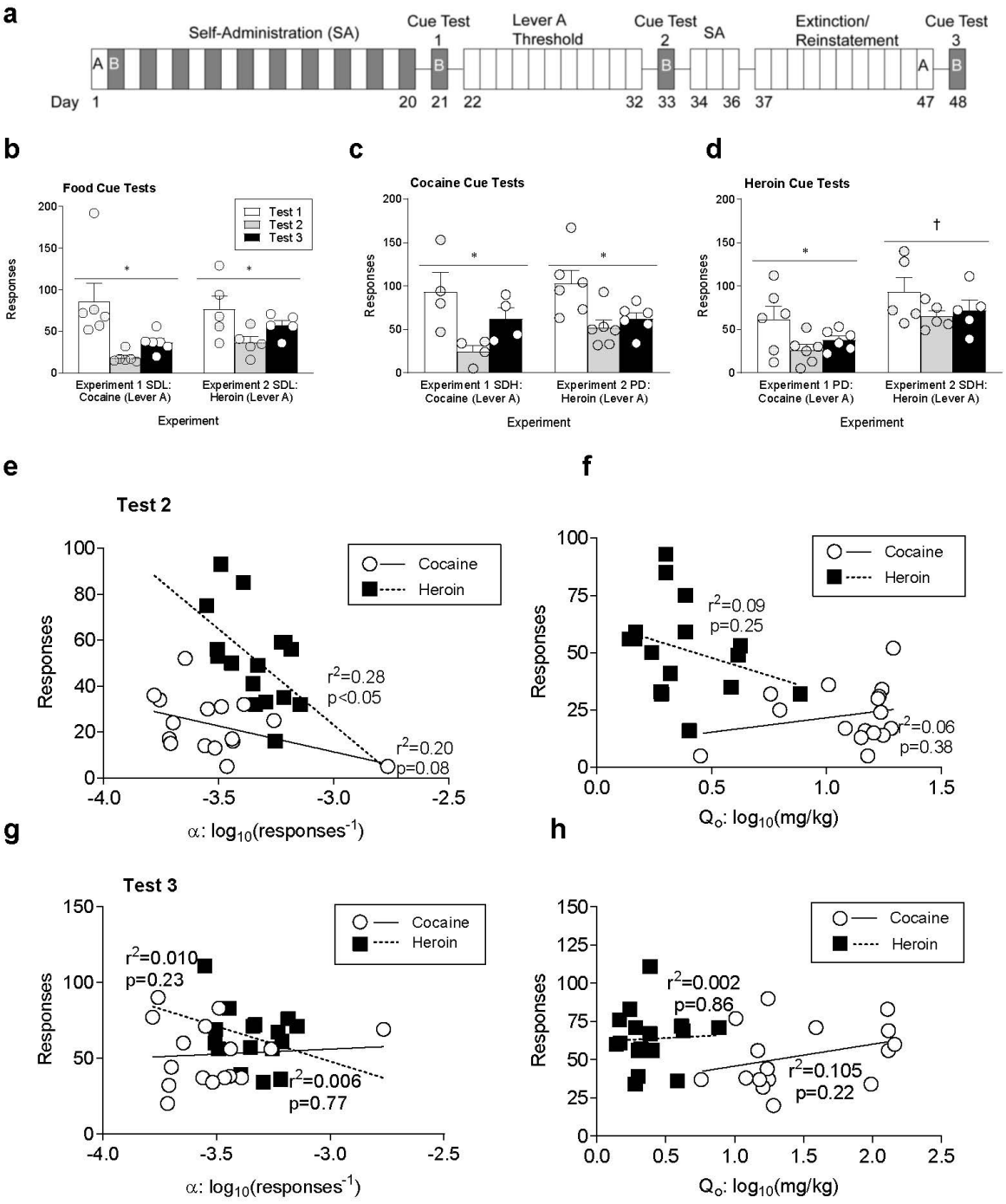



Fig 4. **Cue reactivity as a function of drug history. a.** Lever B Cue tests were administered: 1) 24-hours after the final self-administration session on lever B (Test 1, white bars) 2) 13 days after self-administration (Test 2, gray bars), and 3) 29 days after self-administration (Test 3, black bars). **b.** With cocaine administration (Experiment 1), responding to food cues (SDL group) decreases

between the first and second test, and begins to rebound between tests two and three. A history of heroin administration (Experiment 2) decreases food cue reactivity (SDL group) between the first and second tests. **c.** Cocaine cue reactivity decreases during the period of cocaine thresholding (Experiment 1, SDH group Test 2), and rebounds during complete drug abstinence (Test 3). Heroin administration (Experiment 2, PD group) results in maintained reduction in cue reactivity to cocaine (Test 2), even after complete drug abstinence (Test 3). **d.** Responding to heroin cues decreases with cocaine administration (Experiment 1, PD group, Test 2) between cue tests, while maintenance on heroin (Experiment 2, SDH group) produces persistent heroin cue reactivity. **e.** Price elasticity ($\log_{10}(\boldsymbol{\alpha})$) is predictive of cue reactivity following heroin (black squares, dashed regression line), but not cocaine (white circles, solid regression line) thresholding (Cue Test 2). **f.** No-cost intake ($\log_{10}(Q0)$) of cocaine or heroin is not predictive of cue reactivity following thresholding (Cue Test 2). **g.** $\boldsymbol{\alpha}$ is not predictive of reactivity to reward cues following abstinence from lever A rewards (Experiment 1: cocaine, Experiment 2: heroin; Cue Test 3). **h.** Q0 is not predictive of reactivity to reward cues following abstinence from lever A rewards (Experiment 1: cocaine, Experiment 2: heroin; Cue Test 3) Linear regressions for Experiment 1 cocaine lever A (white circles, solid regression line) and Experiment 2 heroin lever B (black squares, dotted regression line) cue reactivity responding with SDL, SDH, and PD groups collapsed for each experiment against behavioral economic measures α and $Q_0$. *p<0.05, significance between cue tests, †p<0.05, significance between experiments. Data are presented as mean± SEM. Cocaine: SDL n=6, SDH n=4, PD n=6; Heroin: SDL n=5, SDH n=5, PD n=6. Linear regressions for Experiment 1: Cocaine and Experiment 2: Heroin cue reactivity Tests 2 and 3 responding were collapsed for all groups.

**Table 1: Cue Reactivity Tests of Lever B rewards after forced abstinence.** Calculated least squares mean for both Experiment 1 (Cocaine Lever A) and Experiment 2 (Heroin Lever A), and Two-Way ANOVA of Drug Cued on Lever B x Experiment 1 vs. 2 for each Cue Test. Data are presented as Mean ± Standard Error of the mean (SEM).

**p<0.05,** p<0.01,**** p<0.0001* with respect to differences in responding between experiments or the reward corresponding to the lever B cue (i.e. responding for Lever B rewards for SDL, SDH, and PD groups).

| | Least Squares Mean of Experiment 1: Cocaine (Lever A) | Least Squares Mean of Experiment 2: Heroin (Lever A) | Mean Difference (SEM of Difference) | Two-Way ANOVA: Experiment | Two-Way ANOVA: Reward Cued (Lever B) | Two-Way ANOVA: Interaction (Experiment x Reward Cued) |
|---|---|---|---|---|---|---|
| Cue Test 1 (Day 1) | 80.28 | 90.70 | -10.42 (14.77) | F (1, 26) = 0.4979 | F (2, 26) = 0.7171 | F (2, 26) = 0.6866 |
| Cue Test 2 (Day 13) | 21.65 | 51.02 | -29.37 (5.177) | F (1, 29) = 32.19**** | F (2, 29) = 3.525* | F (2, 29) = 1.531 |
| Cue Test 3 (Day 29) | 44.35 | 63.79 | -19.44 (5.982) | F (1, 28) = 10.56** | F (2, 28) = 1.774 | F (2, 28) = 2.549 |

## Chapter 3:

## Characterizing the role of IT projections neurons in cocaine sensitization

Parts of this chapter were included in a publication in Nature Communications. The full citation is as follows:

Garcia, A.F., Crummy, E.A., Webb, I.G., Nooney, M, & Ferguson, S.M. (In press, 2020). Distinct populations of cortical pyramidal neurons mediate drug reward and aversion. *Nature Communications.*

The manuscript sections included here were written by EAC, with edits from SMF.

- This data is in part prepared for a publication with Aaron F. Garcia, Zackari Murphy, Isah Webb, Reiley Durre, and Susan M. Ferguson as co-authors

### Abstract

Dysregulation in activity and connectivity within the cortico-basal ganglia circuit, including cortical hypoactivity, are known to underly substance use disorder progression and pathology. A well-established phenomenon is the escalation of motor effects of stimulants. The manifestation and persistence of these psychomotor effects in part involve enhancement medial prefrontal cortical activity and of glutamatergic transmission to the VTA and nucleus accumbens. Little is known, however, about the role of specific cell types in mediating these effects. Intratelencephalic (IT) and Pyramidal Tract (PT) are two classes of glutamatergic cortical projection neurons that exhibit unique morphology, firing patterns, and connectivity. However, little is known about their functional role in mediating behavioral outputs. Of interest is their common projection to the striatum, which is frequently implicated in processing and coordination of reward, aversion, learning, and motivated behaviors. Furthermore, the cortico-basal ganglia circuit comprising cortex and striatum is also known to be dysregulated in substance use disorders. Data in our lab suggests PT inactivation increases the rewarding value of cocaine and transiently alters cocaine sensitization. Nonetheless, how IT neurons influence the development of locomotor sensitization to cocaine has yet to be elucidated. In order to address these questions, IT neurons were chemogenetically manipulated during cocaine sensitization and changes in ambulatory activity

were tracked during conditioning and cocaine treatment, as well as with subthreshold doses of cocaine following prolonged withdrawal (i.e., during a cocaine challenge). Surprisingly, unlike PT neurons, IT inhibition initially enhances cocaine-induced locomotor activity, but prevents subsequent escalation of activity with repeated treatment. Nonetheless, these effects do not persist following withdrawal. These findings demonstrate opponent regulation of IT and PT activity on cocaine-induced locomotor activity, and emphasize unique cortical subtype-specific contributions to behavioral outputs.

**Introduction**

Repeated drug exposure produces alterations in cortico-basal-ganglia (CBG) circuitry which are heavily implicated in the development and progression of stimulant use disorders (Nestler, 2001). One consequence of chronic psychostimulant use is the enhancement of stimulant effects on behavior (i.e. “sensitization”), including psychomotor effects such as increased stereotypies and locomotor activity (Robinson & Berridge, 2001). The behavioral and neurobiological impacts of repeated drug use can remain for months (Paulson et al., 1991), including long-term adaptations to the CBG circuit involving changes in accumbal and prefrontal dendritic morphology (PFC; Nestler 2001; Robinson & Kolb, 1997), connectivity (Robinson & Kolb, 1997), and increased sensitivity of dopamine neurons in the VTA to glutamate (Almodóvar-Fábregas et al., 2006; White et al., 1995). Furthermore, the neurobiological underpinnings of sensitization are thought to be homologous with those underlying reward (Wise & Bozarth, 1987) and craving (Kalivas et al., 1998; Steketee, 2003), making it a useful measure for studying CBG-activity in relation to drug use and addiction.

Activity of the medial PFC (mPFC), comprised of prelimbic, infralimbic, and dorsal and anterior cingulate cortices, is altered during sensitization (Pierce & Kalivas, 1997; Steketee, 2003). In particular, enhanced glutamate transmission is found in the VTA, and increased extracellular dopamine is found in the nucleus accumbens (Kalivas, 2000; Pierce & Kalivas, 1997). These effects are thought to be partially mediated by reduced dopamine transmission in the PFC, increasing excitatory amino acid transmission to the VTA and accumbens (Goldstein & Deutch, 1992; Kalivas, 2000; Kroener & Lavin, 2010; Pierce & Kalivas, 1997) and contributing to the pathological progression of substance dependence. The impact of altered corticostriatal activity, however, while heavily investigated, is not fully understood.

Several populations of excitatory projection neurons exist within cortex. Broadly, these consist of: 1. Bilaterally-projecting populations which are restricted to the telencephalon (intratelencephalic,

or IT neurons), 2. extra-telencephically-projecting populations with ipsilateral tracts along the brainstem, projecting to targets outside of the telencephelon, including the pons, brainstem, and midbrain structures (pyramidal tract, or PT neurons), and 3. extra-telencephalically projecting populations innervating the thalamus (cortico-thalamic neurons; Baker et al., 2018; Guo et al., 2017). Importantly, both IT and PT neurons send projections to the striatum (Kincaid et al., 1998). While studies have found that these populations differ both structurally and electrophysiologically (Dembrow et al., 2011; Deng et al., 2015), limited work has examined the role of these subtypes in behavior. Therefore, we sought to investigate the contributions of IT and PT neurons in the development of cocaine-induced locomotor sensitization.

**Methods**

*Animals*

All experiments were approved by the Seattle Children's Research Institute Institutional Animal Care and Use Committee and were conducted in accordance with National Institutes of Health guidelines. Male Sprague-Dawley rats (n=46, Envigo) weighing 250-274g upon arrival were pair-housed in a temperature- and humidity-controlled vivarium on a 12-hour light/dark cycle. Rats were acclimated for at least three days prior to any experimental manipulations. Food and water were provided *ad libitum*.

*Drugs*

Cocaine HCl (obtained from the National Institute of Drug Abuse) was dissolved in 0.9% saline and administered *ip* at a concentration of 15mg/kg during the treatment phase, and 10mg/kg during the challenge challenge phase of the sensitization experiments. Clozapine-*N*-Oxide (CNO) was obtained from the National Institute of Health through the Rapid Access to Investigate Drug Program and prepared by dissolving in dimethyl sulfoxide (DMSO; Sigma Aldrich, D650) in a hot water bath, then further diluted in sterile water to a final concentration of 5% DMSO. CNO was prepared prior to test sessions at a concentration of 5 mg/kg CNO and administered at 1 mg/kg *ip*. Vehicle injections were 5% DMSO in sterile water administered at a volume of 1 mL/kg.

*Surgery*

Rats were anesthetized with isofluorane (2-5% inhalation, Patterson Veterinary). Rats were administered meloxicam (0.2mg/kg *sc*, Patterson Veterinary) prior to surgery as an analgesic. Rats underwent a minimum of three days of post-procedure monitoring following surgeries.

*Viral strategy*

To achieve selective expression in IT neurons, rats received unilateral infusions of $AAV_{rg}$-pgk-CRE (Addgene #24593); and $AAV_{rg}$-EF1α-FLPO (Addgene #55637) into different hemispheres of dorsomedial striatum (DMS: A/P: +0.2, M/L: ±2.0, D/V: -4.1 mm relative to bregma), while the complementary CRE- and Flp- dependent AAV-$hM_4D_i$ was injected into the contralateral ACC (A/P +2.5, M/L ± 0.7, D/V -1.9 mm relative to bregma). Virus was allowed a minimum of two weeks to express prior to the start of behavioral test sessions.

*CTB Tracing*

Rats (n=3) received an infusion of 1% w/v in phosphate buffer (Conte et al., 2009) cholera toxin subunit B (CTB) conjugated to AlexaFluor 488 (CTB-488; C-34775, Thermofisher) unilaterally into the pons (350 nl, 70 nl/min), and an infusion of CTB conjugated to AlexaFluor 647 (CTB-647, C-34778, Thermofisher) unilaterally into the contralateral DMS (350 nl/ 140nl/min). Twenty-one days later, rats were anesthetized with Beuthanasia-D (Patterson Veterinary) and transcardially perfused with PBS, followed by 4% PFA. Brains were extracted, fixed overnight in 4% PFA, and post-fixed in 30% sucrose. Brains were sectioned at 40 µm with a vibrating microtome. Z-stacks of the rostro-caudal axis of the ACC were collected with confocal microscopy (x20; Zeiss LSM 710) and localization of CTB-488 and CTB-647 were quantified using ImageJ (v1.52s; NIH).

*Sensitization*

Cocaine-induced psychomotor effects were measured using locomotor activity boxes (San Diego Instruments). A minimum of two weeks following surgery, rats were pseudorandomly divided into one of four groups: pretreatment with CNO (5 mg/kg, *ip*) or vehicle (5% Dimethyl sulfoxide (DMSO) in sterile water, *ip*) 30 minutes prior to treatment with either cocaine (15 mg/kg, *ip*; Vehicle + Cocaine: n=11, CNO + Cocaine: n=14) or saline (0.9%, *ip*; Vehicle + Saline: n=10, CNO + Saline: n=11) for seven sessions across 14 days (one sessions every other day). Ambulations resulting in two consecutive beam breaks were recorded in locomotor activity boxes (San Diego Instruments) over 60 minutes. Two weeks following the final treatment session, rats underwent a challenge session where they all received an injection of vehicle 30 minutes prior to treatment with a subthreshold dose of cocaine (10 mg/kg *ip*) in the absence of CNO treatment. To determine if IT inhibition alters the expression of sensitization, a second challenge was administered to a subset of subjects, where groups received their respective pretreatments (5% DMSO vehicle or 5 mg/kg CNO; Vehicle + Cocaine: n=9, CNO + Cocaine: n=11, Vehicle + Saline: n=8, CNO + Saline: n=10) 30 minutes prior to treatment with 10 mg/kg of cocaine.

*Immunohistochemistry*

Following completion of behavioral paradigms or a minimum of two weeks to ensure viral expression in tracing experiments, rats were anesthetized with Beuthanasia-D and transcardially perfused with 1x phosphate-buffered saline (PBS; pH = 7.4 on ice), followed by 4% paraformaldehyde (PFA) in PBS. Brains were extracted and fixed overnight in PFA and subsequently stored in 30% sucrose (in PBS) prior to being coronally sectioned on a vibrating microtome (40 µm thickness). To confirm DREADD expression, tissue was washed in 1x PBS (3 x 10 minutes) prior to incubating in blocking buffer consisting of normal goat serum (5% NGS), Triton-X (0.25%), and PBS (120 minutes). Tissue was then transferred from blocking solution to primary antibody solution (2.5% NGS, 0.25% Triton-X, 1:1000 rabbit polyclonal anti-GFP, ThermoFisher # A-11122, 1:400 mouse monoclonal anti-mcherry, Clontech # 632543, 24 hours) before being washed in PBS (4 x 10 minutes) and subsequently incubated in secondary antibody solution (2.5% NGS, 0.25% Triton-X, 1:500 Alexa Fluor 488 goat anti-rabbit ThermoFisher # A-11034, 1:400 Alexa Fluor 568 goat anti-mouse ThermoFisher # A-11004, 120 minutes). Finally, sections were washed in PBS (3x10 minutes), mounted on slides, and cover slipped with Vectashield mounting medium with DAPI (Vector Laboratories H-1500). Slides were imaged with a Zeiss LSM 710 confocal microscope and processed using Image J (NIH) and Adobe Photoshop software.

*Statistical analysis*

Analyses were performed using GraphPad Prism 8. Cage crossings during the first conditioning session were analyzed using a two-way RM ANOVA. Habituation, pretreatment, and treatment total ambulations across conditioning sessions were compared using a three-way RM ANOVA of Session x Pretreatment (CNO vs. Vehicle) x Treatment (Cocaine vs. Saline) with Geisser-Greenhouse corrections for sphericity were performed to assess changes in locomotor behavior with repeated drug exposure and IT cell-type inhibition. Differences in total ambulations during challenge in habituation, pretreatment, and treatment phases were determined with a two-way ANOVA. Within-session changes in ambulations across groups were assessed with a three-way RM ANOVA of Time x Pretreatment (CNO vs. Vehicle) x Treatment (Cocaine vs. Saline) for both conditioning sessions and challenge. For locomotor activity differences for IT vs. PT, CNO groups were normalized as percent of vehicle ambulations and data were analyzed across the session for the IT and PT groups using two-way repeated-measures analysis of variance (ANOVA) (Group x Time).

**Results**

Previous studies in our lab have demonstrated that chemogenetic inactivation of PT neurons decreases movement following acute cocaine treatment, but increases locomotor activity with repeated inactivation and cocaine treatment. To assess if chemogenetic reduction of IT activity differentially impacted sensitization, IT neurons where targeted using a quad-viral approach, whereby CRE- and Flp-dependent AAV-$HM_4Di$ viruses were infused into opposite hemispheres of the ACC, while the corresponding retrograde $AAV_{rg}$-CRE or $AAV_{rg}$-Flp viruses were infused into contralateral DMS. After allowing a minimum of two weeks for DREADD expression, rats were pseudorandomly divided into groups receiving either a control vehicle pretreatment or 5 mg/kg of CNO, followed by a treatment of either 0.9% saline or 15 mg/kg cocaine, which were repeated over seven sessions occurring every other day (Figure 1).

All rats exhibited similar locomotor activity when initially introduced to locomotor chambers (Figure 2a. two-way ANOVA Main effect of Treatment: $F_{(1,42)}$=0.3950, p=0.53; Main effect of Pretreatment: $F_{(1,42)}$=0.2279, p=0.64; interaction of Treatment x Pretreatment: $F_{(1,42)}$=0.0171, p=0.90) and following administration of DMSO control and CNO (Figure 2b. two-way ANOVA Main effect of Treatment: $F_{(1,42)}$=0.0097, p=0.92; Main effect of Pretreatment: $F_{(1,42)}$=2.891, p=0.10, interaction of Treatment x Pretreatment: $F_{(1,42)}$=0.3093, p=0.58). Initial exposure to cocaine treatments produced psychomotor effects as cage crossings were significantly higher compared to saline (Figure 2c. two-way ANOVA Main effect of Treatment: $F_{(1, 42)} = 38.10$, p<0.0001), additionally, inhibition of IT neurons did not generally alter motor activity, but did significantly augment activity in cocaine-treated rats (Figure 2c. No main effect of Pretreatment: $F_{(1, 42)} = 3.728$, p=0.06; interaction of Pretreatment x Treatment: $F_{(1, 42)} = 6.979$, p<0.05). Post-hoc analyses confirm this effect was specific to pairings with cocaine (Bonferroni post-hoc: Saline differences in Pretreatment, p>0.99; Cocaine differences in pretreatment, p<0.01). Together together, acute IT inhibition enhanced the expression of psychomotor effects from cocaine administration.

Throughout induction of psychomotor sensitization, rats exhibited similar baseline activity during chamber habituation (Figure 3a: RM three-way ANOVA Main effect of Treatment: $F_{(1, 42)} = 0.01048$, p=0.92; Main effect of Pretreatment: F (1, 42) = 0.9114, p=0.35), but, across sessions movement significantly decreased (Figure 3a: Main effect of Session: $F_{(4.489, 188.5)} = 91.45$, p<0.0001), indicating that exploration decreased as the context became less novel. When administering pretreatment injections, rats subsequently receiving cocaine had heightened anticipatory responding, indicated by significantly higher movement compared to saline controls (Figure 3b: Main effect of Treatment: $F_{(1, 42)} = 8.226$, p<0.01). When IT neurons

were inhibited with CNO, significant decreases in ambulations across all saline and cocaine - treated rats was present (Figure 3b: Main effect of Session: $F_{(3.738, 157.0)} = 24.68$, $p<0.0001$; Main effect of Pretreatment: $F_{(1, 42)} = 4.311$, $p<0.05$). However, these decrease in cocaine-induced activity did not bring activity to the same levels as saline treatments, and increased across sessions with repeated cocaine treatment (Figure 3c: Main Effect of Session: $F_{(4.237, 178.0)} = 10.66$, $p<0.001$; Main effect of Treatment: $F_{(1, 42)} = 153.2$, $p<0.0001$). Cocaine treatment resulted in continued increases in movement, while IT inhibited decreased cocaine-induced ambulations with repeated treatment (Figure 3c: Main Effect of Pretreatment: $F (1, 42) = 3.937$, $p=0.054$, Session x Pretreatment: $F_{(6, 252)} = 4.149$, $p<0.001$). This dampened activity did not occur with saline treatment, suggesting that these effects were specific to cocaine-induced activity, rather than general locomotor inhibition (Figure 3c: Session x Pretreatment x Treatment: $F (6, 252) = 3.822$; $p<0.05$). These shifts in activity were exemplified across different stages of sensitization. With early development of cocaine sensitization, IT inhibition increased cocaine-induced locomotion (Figure 3d: Treatment Day 2: Main Effect of Pretreatment: $F_{(1, 42)} = 6.538$, $p<0.05$), which did not persist to later sessions (Figure 3e Treatment Day 4: Main Effect of Pretreatment: $F_{(1, 42)} = 2.563$, $p=0.12$); Figure 3f: Treatment Day 7: Main Effect of Pretreatment: $F_{(1, 42)} = 1.320$, $p=0.26$). Together, these results suggest an increase in activity when IT activity is blocked during the first two sessions of cocaine treatments, but prevents further sensitization to cocaine with repeated cocaine treatment.

To determine if the effects of repeated chemogenetic inhibition of IT neurons during the development of cocaine sensitization had persistent effects, all rats were given a cocaine challenge where they received a low dose of cocaine in the absence of CNO. All groups exhibited similar levels of activity during initial habituation to the locomotor chambers regardless of previous vehicle or CNO pretreatment history and cocaine and saline treatment history (Figure 4a: Main effect of Pretreatment: $F_{(1, 42)} = 0.6157$, $p=0.44$; Main effect of Treatment: $F_{(1, 42)} = 1.754$, $p=0.19$;Pretreatment x Treatment: $F_{(1, 42)} = 0.03695$, $p=0.85$). Following vehicle pretreatment, cocaine groups exhibited higher activity (Figure 4b: Main effect of Treatment: $F_{(1, 42)} = 16.00$, $p<0.01$), but with no significant difference based on previous vehicle or CNO pretreatment (Figure 4b: Main effect of Pretreatment: $F_{(1, 42)} = 0.2108$, $p=0.65$; Pretreatment x Treatment: $F_{(1, 42)} = 2.163e\text{-}005$, $p=0.99$). A low dose of cocaine elicited a significantly higher number of ambulations in rats that had previously received cocaine compared to those that had received saline (Figure 3c: Main effect of Treatment: $F_{(1, 42)} = 50.33$, $p<0.0001$), suggesting that cocaine sensitization was persistent in these groups. However, there were no differences in ambulations between the cocaine groups, (Figure 4c: Main effect of Pretreatment: $F_{(1, 42)} =$

0.03150, p=0.86), suggesting that IT inhibition during cocaine treatment did not have lasting effects on the persistence of sensitization.

To determine whether IT inhibition alters the persistence of cocaine sensitization, all groups received a second cocaine challenge, but received CNO pretreatment prior to the administration of cocaine. No baseline differences in movement were found during habituation, nor differences following administered pretreatments, suggesting no anticipatory increases or deficits in movement. Though there was a trend in cocaine treatment increasing total crossing, this effect did not quite reach significance (Figure 4f: Main effect of Treatment: $F_{(1, 34)} = 3.343$, p=0.08) However, when examining within-session changes in activity following treatment, an interaction between Time x Treatment was found (Figure 4h: Time x Treatment interaction: $F_{(59, 2006)} = 2.138$; p<0.0001), suggesting that brief increases in movement were found with subjects that had a history of cocaine. However, no differences were found in persistence of sensitization with a previous history of CNO treatment pairings with cocaine compared to DMSO-treated controls (Figure 4f. Main effect of Pretreatment: $F_{(1, 34)} = 0.2303$, p=0.63), suggesting that IT neuronal activity is not necessary for the persistence of sensitized locomotion.

To determine if IT and PT neuronal populations in the ACC represent distinct, non-overlapping groups of cells, a retrograde cholera toxin subunit B (CTB) conjugated to AlexaFluor (AF) 488 (CTB-488) was infused unilaterally into the pons and a retrograde CTB conjugated to AF647 (CTB-647) was infused into the contralateral DMS (Figure 5A). We found that 38% (33 of 86) of ACC neurons were labeled as putative PT (CTB-647+) neurons, 51.6% (45 of 86) were labeled as putative IT (CTB-488+) neurons, and 10.4% (8 of 86) were co-labeled with both fluorophores (Figure 5a,b); suggesting that ~90% of IT and PT neurons comprise distinct neuronal populations. To determine if manipulation of the activity of these two populations is sufficient to drive distinct behavioral responses, we compared the effect of transiently decreasing IT or PT cortical activity on locomotor responses to cocaine during repeated cocaine. We found that the effect of inhibition on cocaine-induced locomotor activity significantly varied as a function of cell-type with IT inhibition increasing and PT inhibition decreasing the number of evoked ambulations (Figure 5c, 2-way RM ANOVA, group x time interaction: $F_{(29,667)} = 1.97$, $p < 0.002$). Together with the rabies and CTB tracing studies, these data provide evidence that IT and PT neurons can regulate behavioral activity through distinct cortical circuits.

**Discussion**

Brain cocaine concentration in the medial prefrontal cortex is correlated with degree of cocaine-

induced hyperlocomotion (Carey et al., 1994). Dopaminergic regulation of prefrontal activity is reduced with cocaine sensitization, while glutamatergic transmission is increased in accumbal and midbrain regions (Pierce & Kalivas, 1997), with AMPA receptor antagonism blocking the development of cocaine sensitization (Parikh et al., 2014). D2-receptor activity in the PFC is disrupted following cocaine sensitization with D2 reduction of GABA transmission seen following withdrawal from cocaine treatments (Kroener & Lavin, 2010). These data suggest that alterations in glutamatergic projections to accumbens activity is involved in the development of sensitization; however, evidence suggests that development of sensitization is projection specific, as reduced inhibitory drive to corticofugal projections enhances glutamatergic transmission to regions such as the VTA (Liu & Steketee, 2011; Liu & Steketee, 2016). Furthermore, the development and persistence of sensitization are thought to be regulated by different regions of the striatum (Todtenkopf et al., 2004), different receptor subtypes (Goldstein & Deutch, 1992; Yamamoto & Zahniser, 2012), and by regional differences in receptor activity (Marcott et al., 2018). As D1 and D2 MSNs in dorsal striatum are thought to regulate actions and reinforcement in an opponent manner (Gerfen & Surmeier, 2011; Kravitz et al., 2012), this regulation may extend to cell-type specific activity. Consistent with this idea, it has been shown that chemogenetic inhibition of D1 MSNs reduces amphetamine sensitization, while inhibition of D2 MSNs promotes amphetamine sensitization (Ferguson et al., 2011).

Rabies tracing of IT and PT cortical populations and the CTB tracing presented in this chapter demonstrate that IT and PT populations are largely distinct projection populations (Garcia et al., in press). Together with previous data on PT sensitization (Garcia, 2018) and with the results presented here, there is evidence for differential regulation of behavioral outputs following cocaine administration. Specifically, our studies demonstrate that transient inactivation of IT cortical projection neurons to DMS during during initial (i.e, two sessions) exposure to cocaine produces an enhancement of locomotor activity. However, with continued intermittent cocaine treatment, inactivation of IT neurons prevented the escalation of ambulations that normally occurs during cocaine treatment. The significance of these findings is notable, as previous studies from our lab demonstrated that inhibition of PT neurons had the opposite effect. That is, there was decreased movement during initial exposure to cocaine compared to controls, but PT inhibition ultimately led to a greater amount of sensitization to the locomotor effects of cocaine compared to controls (Garcia, 2018), consistent with hypotheses suggesting increased corticofugal projection activity (Goldstein & Deutch, 1992; Pierce & Kalivas, 1997). Nonetheless, neither of these manipulations effected the persistence of sensitization that can be seen following withdrawal, suggesting that

the alterations that occurred during cocaine treatment may have more of an effect on the development of cocaine sensitization. Indeed, acute inhibition of IT neurons following withdrawal support the idea that IT activity is not required for the expression of stimulant-motor effects, as no differences were seen in CNO paired with cocaine treatment compared to controls, consistent with the results of the initial challenge. The mechanisms of initial development and prolonged sensitization are known to be distinct, and dysregulated glutamatergic signaling is involved in the development of sensitization (Vanderschuren & Kalivas, 2000), suggesting that the role of IT and PT activity may be involved in the acquisition of cocaine sensitization, but not in its long-term expression.

IT and PT neurons have been shown to preferentially project to D1 and D2 MSNs, respectively (Lei et al., 2004; Shepherd, 2013, but see Wall et al., 2013). Manipulation of these neuronal populations, therefore, may differentially alter innervation of striatal MSN populations, resulting in distinct behavioral outputs following cocaine treatment. Previous work in our labs has demonstrated opposing roles of IT and PT manipulations on the alteration of reward aversion and preference with cocaine administration (Garcia et al., in press). This study extends the unique role of these populations to the psychomotor effects of cocaine. Specifically, IT and PT contributions to corticostriatal regulation of behavior may be thought of in a framework whereby reducing direct pathway activity via inhibition of IT neurons biases inhibition of outputs by D2 MSNS, whereas PT inhibition results in the opposite shift to D1 activity and augmented action initiation, particularly with repeated cocaine treatment shifting activity from NAc to dorsal striatum circuitry (Everitt & Robbins, 2013).

Given the unique regulation of IT and PT neurons by differential receptor expression, future studies understanding how neurotransmitter release in cortical regions, including dopamine, GABA, and serotonin, coupled with monitoring and manipulation of IT and PT activity, are warranted. Alterations in these transmitters are found in cortical and downstream mesolimbic targets with early treatments and later drug treatments which produce sensitization (Jayaram & Steketee, 2005, Heidreder et al., 1999; Zayara et al., 2011). Furthermore, the role of various transmitters, cortical regions, and downstream subcortical regions is inconsistent across different stages of locomotor sensitization induced by amphetime, cocaine, opioids, and other drugs (Pierce & Kalivas, 1997; Vanderschuren & Kalivas, 2000). Therefore, a complete understanding of the role of cortical projection subtypes in the initiation, development, expression, and persistence of locomotor sensitization will involve examining their activity in the context of multiple drugs, transmitters, and cortical regions.

## Figures

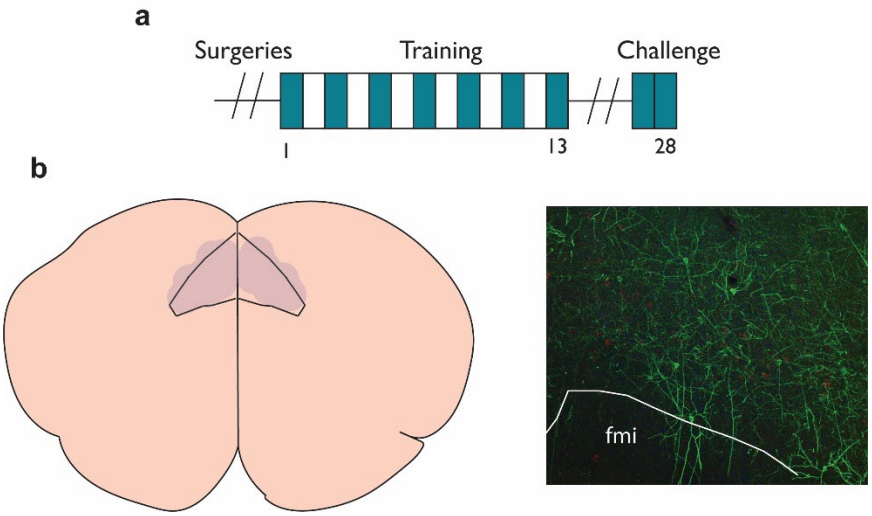



**Figure 1. Timeline of sensitization paradigm and IT targeting. a.** For a minimum of two weeks following viral surgeries, rats underwent training sessions every other day, for a total of seven sessions. Following a two-week withdrawal period, a challenge dose of cocaine was administered without pretreatment with CNO. The following day, a second challenge was performed with acute IT inhibition with CNO pretreatment in rats previously administered CNO. **b.** Targeting of IT neurons in ACC with representative image of flp-dependent DREADD (n=46).

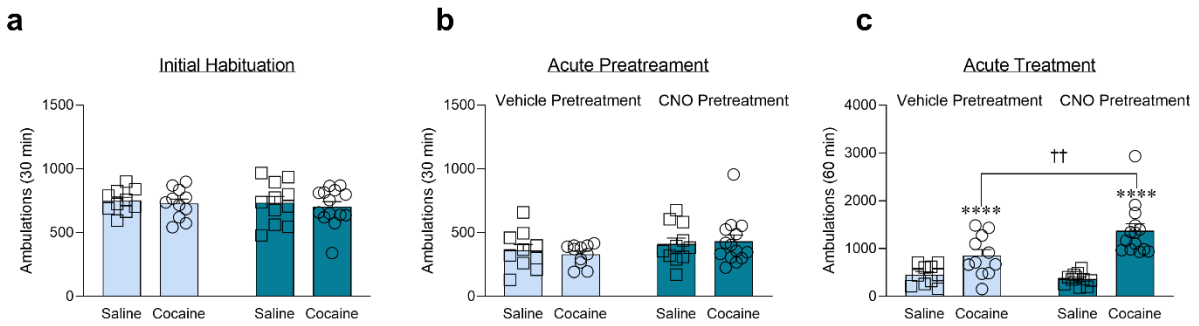


**Figure 2. Acute cocaine psychomotor stimulation is augmented with IT inhibition. a.** All rats exhibit similar baseline levels of activity in locomotor chambers. **b.** Initial CNO or vehicle treatments do not effect motor activity. **c.** Cocaine increases cage crossings, but is significantly increased when paired with IT inhibition. Error bars represent mean ± SEM. ****- p<0.0001 Saline vs. Cocaine ††- p<0.01 Pretreatment x Treatment (Bonferroni post-hoc corrected).

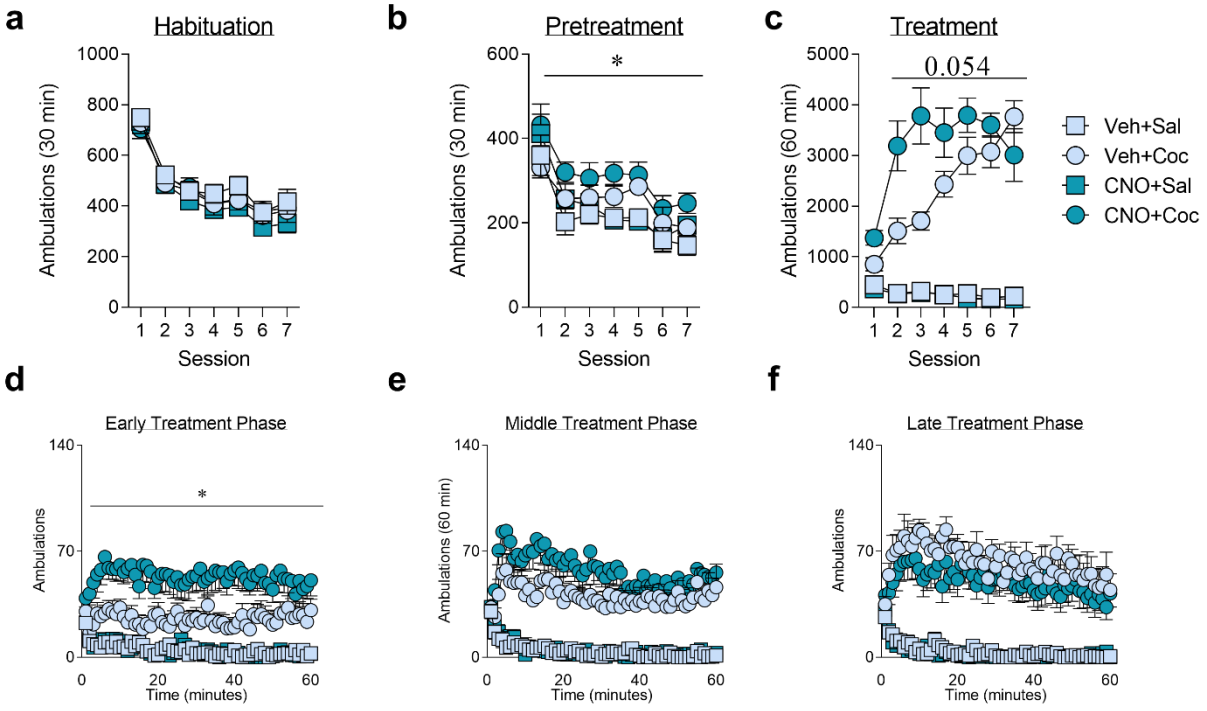



**Figure 3. IT inhibition transiently increases cocaine-induced ambulatory activity with early exposure. a-c.**Total ambulations in habituation sessions across initial conditioning sessions (a), pretreatent with CNO (dark blue) or vehicle (light blue) (b), and following treatment with cocaine (circles) or saline (squares) (c). **d-f.** Ambulations in one-minute intervals during treatment sessions in early sensitization sessions (d) were significantly higher with IT inactivation, but does not increase further by the middle of sensitization sessions (e), and ultimately results in higher ambulations in vehicle-pretreated subjects, though not significantly so (f). Error bars represent mean ± SEM. * p<0.05 CNO vs. Vehicle.

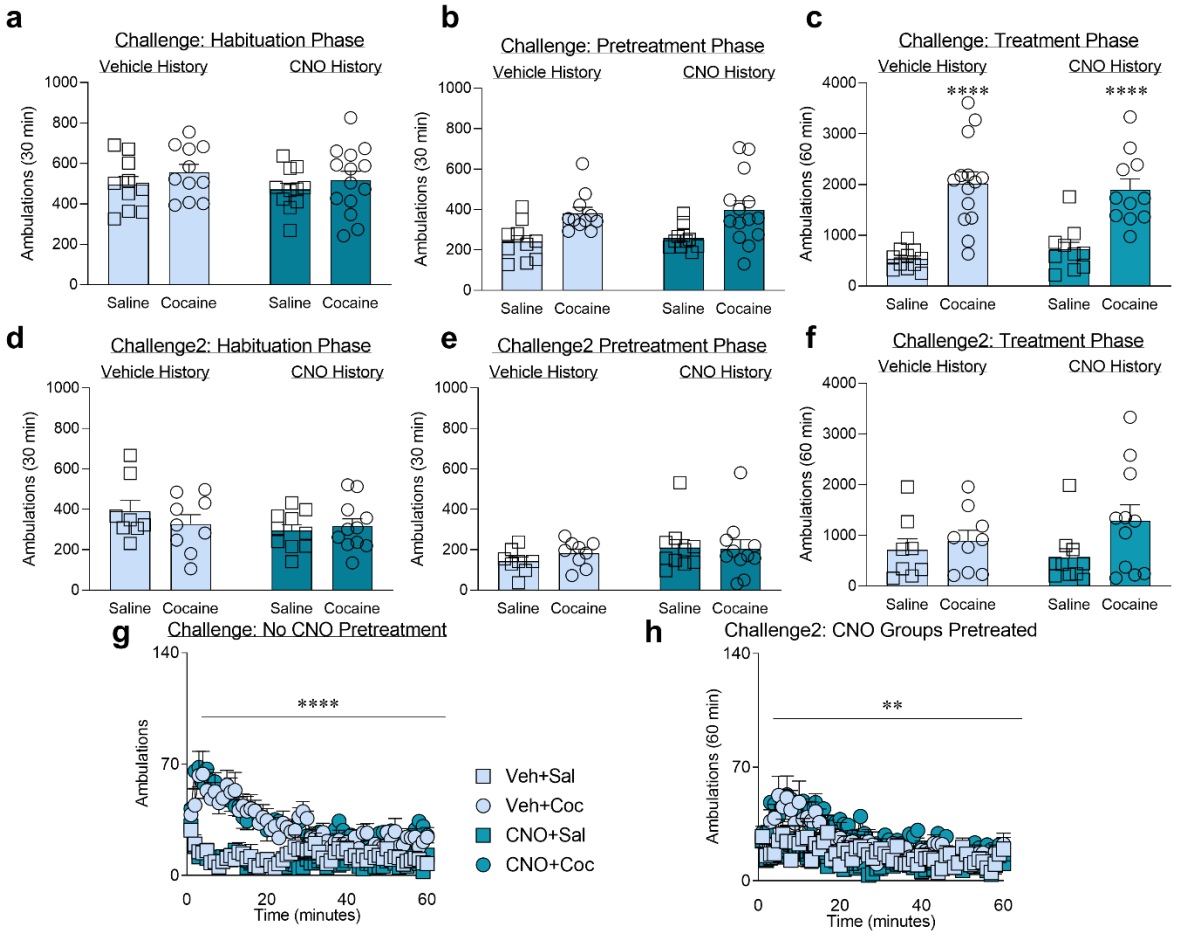



**Figure 4: Effects of inactivating IT neurons do not persist to challenge.** Two weeks following the final sensitization session, subjects were administered a subthreshold dose of cocaine with no CNO pretreatment. **a-c.** No differences in ambulations were found in habituation and pretreatment (a, b), while cocaine sensitized locomotor responding (c). **d-f.** To determine if acute inactivation explained the enhanced movement seen in early conditioning, a second challenge whereby CNO pretreatment groups did receive pretreatment was performed. No differences in ambulations were seen at any phase of the session. **g-h.** Timecourse of treatment sessions for the first (g) and second (h) challenges also do not suggest within-session differences in activity. Error bars represent mean ± SEM. **-main effect of Treatment $p<0.01$ **** - main effect of Treatment $p<0.0001$.

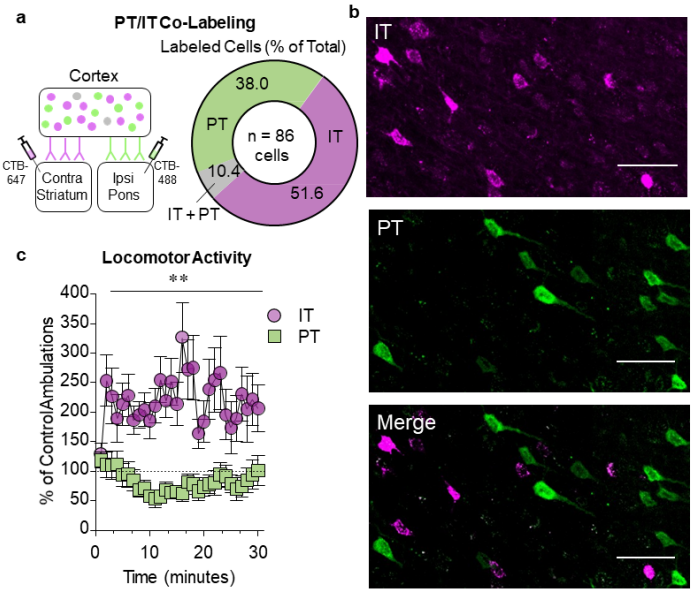



**Figure 5. PT and IT neurons in ACC are anatomically and functionally distinct. a.** Left: Viral strategy for anatomical tracing of IT and PT neurons with CTB (N=3). Right: Quantification of neuronal labeling. 38% of labeled cortical neurons are PT+ (Green), ~52% are IT+ (Magenta) and ~10 % are co-labeled from 86 total cells counted. **b**. Representative photomicrographs of neuronal labeling of IT cells (top), PT cells (middle) and a merged image (Bottom). Scale bar = 50 μm. **c.** Line graphs where data points represent the mean ± SEM percent of control (vehicle DREADD, b=11-12) ambulations induced by a compound stimulus (novelty + 15 mg/kg cocaine, *ip*). IT inhibition (magenta, N=14) significantly increased and PT inhibition (green, N=11) significantly decreased the percent of control activity, indicating that the cell types are functionally distinct. Statistical analysis: **p<0.001, group x time interaction, 2-way RM ANOVA.

## Chapter 4:

## Investigating IT and PT projection neuronal activity in reward and aversion

Parts of this chapter were included in a publication in Nature Communications. The full citation is as follows:

Garcia, A.F., Crummy, E.A., Webb, I.G., Nooney, M, & Ferguson, S.M. (in press, 2020). Distinct populations of cortical pyramidal neurons mediate drug reward and aversion. *Nature Communications.*

The manuscript sections included here were written by EAC, with edits from SMF.

### Abstract

We have recently found distinct roles of IT and PT neurons in anterior cingulate cortex (ACC) in the appetitive and aversive components of cocaine use, using conditioned place preference (CPP) and conditioned taste aversion (CTA) assays. Specifically, inactivation of IT neurons blunted a cocaine-induced CTA to sucrose whereas PT inactivation increased the rewarding value of cocaine. To extend this work and determine if these neuronal subtypes play more general roles in reward and aversion processing, chemogenetic inhibition of IT neurons and optogenetic stimulation of PT neurons was performed in CPP and CTA assays, respectively. Utilizing optogenetic stimulation during CPP and inhibitory DREADDs during conditioned taste aversion, PT and IT neurons were respectively stimulated or inhibited with saline treatment pairings. Under these conditions, no place preference or sucrose aversion was observed, suggesting that PT and IT alterations do not directly impact reward and aversion processing. In addition, in order to examine the role of these subtypes in volitional drug use and their necessity for motivated intake, PT neurons were chemogenetically inhibited throughout the cortex in rats with cocaine self-administration history in various behavior economic assays. Interestingly, inhibition of PT neurons impacted effort-based cocaine administration, particularly in measures related to motivated responding in the absence of aversive contexts. These findings provide evidence for a role of this cortical subtype in mediating the rewarding properties of cocaine consumption and drug-seeking.

## Introduction

Pathological drug seeking and consumption, and compulsivity to maintain drug use despite adverse consequences are well-established behavioral manifestations of substance use disorders (for reviews, see Koob & Volkow, 2010, 2016). Impairments in cortical activity are consistently found in drug users, including those that use cocaine (Kaufman et al., 2003; Volkow et al., 2009). These deficits have been linked to heightened drug craving and cue sensitivity (Meyer et al., 2016), suggesting a contributing role for loss of executive control and top-down inhibition of cortical activity in these behaviors. The anterior cingulate cortex (ACC), in particular, is involved in salience attribution to rewards (Alexander et al., 2015) as well as pain processing (Becerra et al., 2013), and exhibits hypoactivity following repeated cocaine use (Kaufman et al., 2003). Thus, the ACC is an interesting target for examining aversive and appetitive processing.

Within the cortex, there are several classes of pyramidal projection neurons that have been identified: Intratelencephalic (IT) neurons located in layers II, III, and Va, Pyramidal Tract (PT) neurons confined to layers Vb and 6, and Cortico-thalamic (CT) neurons in layer 6. Although all of these neuronal populations are glutamatergic, they vary in their projection targets and firing patterns (Anastasiades et al., 2019; Dembrow & Johnston., 2014; Leyrer-Jackson & Thomas, 2018). These unique features suggest that there may be subtype-specific differences in cortical processing and subsequent behavioral output. While research has begun to dissociate the role of these subpopulations in movement regulation (Soma et al., 2017), their contribution to motivated behaviors have not been explored.

Our lab has investigated the role of IT and PT neurons in the ACC in mediating the rewarding and aversive properties of cocaine using cocaine-induced place preference and conditioned sucrose taste aversion assays. However, the role of IT and PT neurons in driving preference and aversion in normal contexts is not known. These assays, while providing evidence for affective processing, are also limited by their noncontingent route of drug treatment and do not investigate the impact of these neurons on drive towards consumption and seeking of these rewards. To begin to investigate these important questions, I examined whether PT inactivation modulates reward preference following cocaine self-administration in tasks assessing reinforcement and effort for drug consumption. Specifically, the effect of chemogenetic inhibition of, PT neurons was examined during two types of behavioral economic tasks using within-session thresholding paradigms whereby the price of cocaine is increased by: 1. Decreasing the dose administered

per infusion in a single self-administration session (Oleson & Roberts, 2009; Oleson et al., 2011), and 2. Administering concurrent footshocks at increasing durations throughout a single self-administration session (Bentzley et al., 2013; Kawa et al., 2016). These paradigms permit investigation of several dimensions of cocaine use, including preferred consumption, effort, and resistance to punishment, allowing for investigation of how PT neurons impact different facets of drug misuse.

**Methods**

*Animals*

All experiments were approved by the Seattle Children's Research Institute Institutional Animal Care and Use Committee and were conducted in accordance with National Institutes of Health guidelines. Male Sprague-Dawley rats (Envigo) weighing 250-274g upon arrival were pair-housed in a temperature- and humidity-controlled vivarium on a 12-hour light/dark cycle. Rats were acclimated for at least three days prior to any experimental manipulations. Food and water were provided *ad libitum* for CPP and CTA experiments, while rats undergoing self-administration were mildly food restricted (40±1g per cage). For self-administration experiments, rats were removed from analyses due to loss of patency (N=3) and lack of viral expression (n=1).

*Drugs*

Cocaine HCl (obtained from the National Institute of Drug Abuse) was dissolved in 0.9% saline and prepared for a final concentration of 0.4 mg/kg/inf for self-administration experiments and 0.038 mg/kg/inf for within-session punishment to increase number of responses required to reach desired cocaine concentrations (Bentzley 2013; Kawa, 2016). Clozapine-*N*-Oxide (CNO) was obtained from the National Institute of Health through the Rapid Access to Investigate Drug Program and prepared by dissolving in dimethyl sulfoxide (DMSO; Sigma Aldrich, D650) in a hot water bath, then further diluted in sterile water to 5% DMSO. Clozapine was diluted in 6% DMSO (Sigma Aldrich; C6305).

*Viral targeting*

To selectively target IT neurons in the ACC, male Sprague Dawley rats received unilateral infusions of $AAV_{rg}$-pgk-CRE (Addgene #24593); and $AAV_{rg}$-EF1α-FLPO (Addgene #55637) into different hemispheres of dorsomedial striatum (DMS: A/P: +0.2, M/L: ±2.0, D/V: -4.1 mm relative to bregma; 1000nl/side, 200 nl/min), while the complementary CRE- and Flp- dependent AAV-

$hM_4D_i$ was injected into the contralateral ACC (A/P +2.5, M/L ± 0.7, D/V -1.9 mm relative to bregma; 500nlside, 100 nl/min). To target ACC PT neurons, rats received bilateral infusions of CAV-CRE into the caudal pontine nucleus (PNc: A/P: -9.2, M/L: ±0.5, D/V: -9.8 mm relative to bregma; 750 nl/side, 150 nl/min), while AAV9-EF1a-double floxed-hChR2(H134R)-EYFP-WPRE-HGHpA (Addgene #20298-AAV9; 500 nl/side, 100 nl/min) was bilaterally infused into the ACC. To nonspecifically target PT neurons throughout cortex, rats received bilateral injections of $AAV_{rg}$-hSyn-$hM_4D_i$-mCherry (Addgene #50475; 750 nl/side, 75 nl/min) in the pontine nucleus (A/P -9.2, M/L ± 0.2, D/V -9.4 mm from Bregma). All injector needles were held at the target site for 5 minutes to allow for virus to diffuse. Virus was allowed a minimum of two weeks to express prior to the start of behavioral test sessions.

*Catheter Implants*

Rats were anesthetized with isoflurane (2-5% inhaled; Patterson Veterinary) during the procedure and received an injection of meloxicam (0.1 mg/kg SC; Patterson Veterinary) prior to surgery for analgesia. Rats were monitored for a minimum of three recovery days following surgery prior to the start of self-administration. Chronic indwelling catheters were placed into the right jugular vein and attached to a back-mounted port. Following surgery, catheters were flushed daily with 0.1 mL of sterile saline containing 10 mg/mL gentamicin sulfate and heparin sulfate (30 usp/10 mL; Patterson Veterinary) to prevent occlusions and minimize risk of infection. Catheter patency was tested prior to the first self-administration session and following completion of each experimental phase by IV injection of up to 0.3 mL of methohexital sodium (Brevital sodium: 10 mg/mL in sterile water; Patterson Veterinary). Rats that became ataxic within five seconds were considered to have patent catheters.

*Conditioned Place Preference*

Rats (N=5) underwent conditioned place preference (CPP) testing using a two-chamber apparatus (24 x 12 x 12 in, with a divider in the middle). The two chambers differed in their wall patterns (horizontal vs vertical stripes). Animals were first allowed to freely explore the apparatus for 20 min. Over the following three days, animals underwent six 20 min conditioning sessions (one in the morning and one in the afternoon separated by at least 4h). During these conditioning sessions, the rats were administered a treatment consisting of 0.9% saline or 0.9% saline + stimulation via head-mounted blue LED (TBSI) at 20 Hz, 20 ms pulse duration for 60 sec with 30 sec of no stimulation and restricted to one side of the chamber. The order that they received the treatments was counterbalanced each day. The day after the last set of conditioning sessions,

animals were allowed to freely explore the chamber for 20 min. Time spent in each chamber during pre- and post-conditioning test sessions were scored from videos.

*Conditioned Taste Aversion*

Rats (N=15) underwent conditioned taste aversion (CTA) testing. One day before testing began, animals were allowed to explore and habituate to the cage for 30 min. During testing, animals were placed into a cage with access to 15% sucrose solution. At the end of the session, animals received either vehicle (5% DMSO/95% sterile water, *ip;* N=7) or CNO (5 mg/kg, CNO, *ip;* N=8) injections followed 15 min later by saline (0.9%, *sc*) injections. Animals underwent four 30 min conditioning sessions across 7 days. Time spent drinking was scored from videos by an experimenter blind to the conditions.

*Self-administration*

Behavioral testing occurred in 20 standard operant self-administration chambers equipped with retractable levers, stimulus lights, a house light, a feeder, and a metal grid floor (Med Associates ENV-007CT). The front wall housed two white stimulus lights, one located above each lever, with an additional green stimulus light above the white stimulus light on one side. The back wall contained a white house light. A syringe pump (Med Associates PHM-100VS), located outside on top of the chamber, delivered cocaine via tubing attached to the catheter backport. All tubing was attached to a suspended swivel (Instech 375/22) to allow rats to move freely within the chambers.

*Intermittent Access Paradigm*

Following at least three days of recovery from catheter surgeries, rats were trained to lever press for a 0.4 mg/kg infusion of cocaine (50 µL over 2.8 seconds) on a fixed-ratio 1 (FR1) schedule for five days. The session ended after rats received either 10 infusions or after 3 hours. Following training, rats were switched to an intermittent-access (IntA) FR1 schedule for cocaine self-administration for 15 days. IntA sessions consisted of 5 minute drug-available periods of access to cocaine whereby an active lever press resulted in presentation of the associated white cue light over the active lever for 4 seconds along with an infusion of cocaine. Additional lever presses during the cue light presentation were recorded, but did not result in additional infusions. Presses on the inactive lever had no programmed consequence. Drug available periods were followed by 25-minute periods of drug unavailability signaled by presentation of a white house light, where levers were extended and responses recorded, but presses had no programmed consequence.

A single session consisted of 6 drug available and 5 drug unavailable periods for a 155 minute duration.

*Behavioral Economics*

After completing self-administration, rats (N=13) were trained on a within-session behavioral economics paradigm (Bentzley et al., 2013; Kawa et al., 2016; Oleson et al., 2011). The unit price (responses/mg of drug) of cocaine was increased in 10-minute time bins throughout the session by reducing the pump infusion time for the final concentrations (0.3835, 0.2156, 0.1213, 0.0682, 0.0383, 0.0216, 0.0121, 0.0068, 0.0038, 0.0022, 0.0012 mg/kg per infusion). Rats had access to drug for the entirety of each two-hour self-administration session, with no timeout periods between infusion. Infusions were administered on an FR1 schedule and were paired with illumination of the white cue light during each infusion. Animals were run daily on this paradigm for six sessions to obtain stable responding prior to chemogenetic manipulations.

*Footshock Threshold*

Following the threshold procedure, rats (N=11) were re-baselined to a new self-administration cocaine dose (0.0383 mg/kg per infusion) corresponding to the midpoint of the threshold curve for three days. After these sessions, rats underwent a baseline footshock session, whereby a response on the active lever resulted in a 0.25 mA footshock. To increase the cost of cocaine administration, the duration of the footshock was increased across the session in 11 10-minute bins on a log scale (0.2, 0.25, 0.32, 0.4, 0.5 0.63, 0.8, 1, 1.26, 1.59, and 2.0 seconds). A maximum charge value was obtained by taking the product of responses x shock amplitude (mA) x shock duration (s).

*Demand curve analysis*

Demand curve analysis was performed by fitting demand curves with an exponential demand equation: $log\ Q\ =\ log\ Q_0\ +\ k(e^{-\alpha Q_0 C}-1)$ to obtain calculated parameters ($Q_o$, $P_{max}$, $O_{max}$, α) minimizing the residual sum of squares for each animal as previously described (Bentzley et al., 2013). For calculations, $k$ was set to 3.2 for all animals.

*Locomotor Assay*

Following completion of all self-administration and behavioral-economic paradigms, rats (N=14), were placed into locomotor chambers (San Diego Instruments) where beam break crossings could be recorded to measure movement activity. Rats were given an injection of either vehicle

(6% DMSO *ip*), CNO (5 mg/kg in 6%DMSO, *ip*) or clozapine (0.1 mg/kg, *ip*) in three separate sessions, with cage crossings recording for 60 minutes following treatment.

*Statistics*

Data was analyzed with Graphpad Prism 8.4.3. CPP scores and within-session measure s(Pmax, $Q_0$, Omax, and max charge) were analyzed with paired, two-tailed t-tests. CTA measures, self-administration, within-session, footshock threshold responding, and changes in within-Session measures classified by severity were analyzed with a repeated measures (RM) two-way ANOVA. RM One-Way ANOVA was used to compared cage crossings following treatments in locomotor assays. Bonferroni post-hoc corrections were applied.

**Results**

Studies in our lab have demonstrated a reduction in a cocaine conditioned place preference when activating PT neurons, and a reduced sucrose aversion when inhibiting IT neurons (Garcia et al., in press). To determine if there is an inherent shift in preference or aversion when manipulating these populations, a conditioned place preference (CPP) assay (Figure 1a) and conditioned taste aversion assay (CTA) (Figure 1b) were performed with either stimulation of PT neurons paired with saline treatments, or inhibition of IT neurons prior to saline treatments, respectively. Stimulation of PT neurons did not induce a preference or aversion in a CPP assay (Figure 1a. Pre-Post-test time: Paired, two-tailed t-test: t=0.9999, df=2, p=0.42). In addition, inhibition of IT neurons did not affect sucrose drinking compared to controls (Figure 1b. Two-way RM ANOVA: Number of drinking bouts: Main effect of Session $F_{(2.292, 29.80)} = 0.3370$, p=0.75; Main effect of Pretreatment $F_{(1, 13)} = 0.7865$, p=0.39; Session x Pretreatment $F_{(3, 39)} = 1.619$, p=0.20; total time drinking: Main effect of Session $F_{(2.064, 26.83)} = 0.3094$, p=0.74; Main effect of Pretreatment $F_{(1, 13)} = 0.3033$, p=0.59, Session x Pretreatment $F_{(3, 39)} = 1.358$, p=0.27). Together, these data suggest that inherent changes in ACC IT and PT activity do not modulate general place preference and taste aversion.

Because these initial studies used noncontingent cocaine administration, we next wanted to study their role in more complex motivated preference and aversion for drug rewards during volitional administration of cocaine. As the projections of PT neurons lend themselves to widespread, full cortical targeting, self-administration studies were conducted with pan-cortical PT manipulations. Following training to acquire cocaine administration on an FR1 schedule (5 sessions), rats underwent an intermittent access (IntA) self-administration procedure (15 sessions) prior to within-session thresholding and punishment thresholding paradigms, as outlined (Figure 2a.). For

nonselective inhibition of PT neurons, a retrograde, constitutively-expressing DREADD was injected into the pontine nucleus. Using this approach, labeling of cell bodies was found throughout cortex, including medial prefrontal regions (i.e. cingulate and prelimbic), orbitofrontal, motor, and sensorimotor cortices (Figure 2b.). During IntA, rats learned to associate responding on the active lever with cocaine infusions during drug available periods (Figure 2c. RM Two-Way ANOVA: Main effect of Session: $F_{(3.405, 44.27)} = 1.291$, $p=0.29$; Main effect of Active vs. Inactive Lever $F_{(1.000, 13.00)} = 37.02$, $p<0.0001$; Session x Active vs. Inactive Lever: $F_{(3.319, 43.15)} = 3.399$; $p<0.05$).

After establishing SA, rats were trained over six sessions on a within-session thresholding paradigm, where the price of cocaine was increased across the session by decreasing the unit-dose with each cocaine infusion. Following establishment of baseline responding, rats were administered clozapine-*N*-oxide (CNO in 5% DMSO) or vehicle (5% DMSO in sterile water) in a counterbalanced manner 30 minutes prior to the session, to allow for transient inhibition of PT neurons. Test sessions were separated by one threshold session without treatment, to allow for washout of CNO. Rats exhibited sensitivity to changes in price (Figure 3a: Main effect of Price: $F_{(1.439, 17.27)} = 11.86$, $p<0.01$), and reduced their overall level of responding when administered CNO (Main effect of Treatment: $F_{(1.000, 12.00)} = 9.801$, $p<0.01$). This reduction was notable at prices where level of effort to maintain no-cost consumption began to significantly increase (Price x Treatment: $F_{(2.756, 33.08)} = 5.582$, $p<0.01$). However, although inhibition of PT neurons did not alter baseline, no-cost consumption (3c. $Q_0$: $t=1.193$, $df=12$, $p=0.26$) or the maximal price to defend no-cost consumption (3b. Pmax: Paired two-tailed t-test $t=1.221$, $df=12$, 0.25), CNO inhibition did significantly reduce responding at Pmax (3d. Omax: $t=2.534$, $df=12$, $p<0.05$). Together, these results suggest that PT inactivation reduces overall level of effort exerted for cocaine administration, but does not impact consumption when minimal effort is required, nor the overall sensitivity to the price of cocaine consumption.

In order to assess how PT neurons regulate drug consumption in the face of negative consequences (i.e., footshock), rats were re-baselined to cocaine self-administration for three sessions prior to pairings of cocaine administration with a mild footshock (0.25mA). To increase the price of cocaine consumption with punishment, the duration of the footshock increased every ten minutes, while the strength of the shock and cocaine dose remained constant throughout the session. Baseline sessions, where no treatment was given prior to the footshock session, show a trend in sensitivity to the duration of the footshock, though this effect did not quite reach significance (Figure 4a. RM One-Way ANOVA: $F_{(2.731, 27.31)} = 2.905$, $p=0.06$).

However, PT inhibition did not significantly alter responding during punished thresholding compared to controls, though there was diminished overall responding with CNO treatment compared to vehicle controls, as found during unpunished thresholding (Figure 4a. RM Two-Way ANOVA: Main effect of Shock Duration: $F_{(1.833, 18.33)} = 2.926$, p=0.08 ; Main effect of Treatment: $F_{(1.907, 19.07)} = 3.632$, p=0.05.; Shock Duration x Treatment $F_{(3.627, 36.27)} = 2.258$, p=0.09). The value of cocaine paired with footshock punishment, assessed by taking a normalized value using footshock duration, amplitude, and number of responses to calculate a maximum charge accepted for cocaine administration, was also not affected when paired with PT inactivation (Figure 4b. paired two-tailed t-test t=0.2867, df=10, p=0.78). These data suggest that inhibiting PT activity does not affect sensitivity to punished-seeking and cost valuation for cocaine administration.

Intermittent access has been shown to produce a distribution of drug administration patterns (Garcia et al., 2020; O'Neal et al., 2020). Performance during intermittent access sessions can be compared based on z-scores from several metrics: Consistency of intake during drug availability, total intake across all self-administration sessions, and seeking during drug unavailable periods on the active lever (O'Neal et al., 2020). Using an average z-score, rats were classified into low-, medium, and high-risk responders to cocaine self-administration. To determine if performance during either motivation-thresholding or punishment-thresholding was related to responding during self-administration, Pmax, $Q_0$, Omax, and Max Charge measures were sub-classified by self-administration severity scores and compared between treatment sessions. PT inhibition produced a change in Pmax, regardless of severity score, though there was a trend for Pmax to be lower for low-risk rats compared to other groups, (Figure 5a. Main effect of Treatment $F_{(1, 10)} = 1.148$, p=0.31; Main effect of Severity $F_{(2, 10)} = 3.509$, p=0.07; Treatment x Severity $F_{(2, 10)} = 0.5173$, p=0.61). No effects were found for Q0 (Figure 5b. Main effect of Treatment $F_{(1, 10)} = 1.649$, p=0.23; Main effect of Severity: $F_{(2, 10)} = 1.155$, p=0.35; Treatment x Severity: $F_{(2, 10)} = 1.281$, p=0.32), but responding was significant based on treatment, though not by severity for Omax (Figure 5c. Main effect of Treatment $F_{(1,10)} = 5.874$, $p<0.05$; Main effect of Severity: $F_{(2, 10)} = 0.4817$, p=0.63; Treatment x Severity: $F_{(2, 10)} = 0.9780$, p=0.41), and no differences were found for Max Charge (Figure 5d. Main effect of Treatment: $F_{(1, 8)} = 0.01661$; p=0.90; Main effect of Severity: $F_{(2, 8)} = 0.5355$, p=0.61; Treatment x Severity: $F_{(2, 8)} = 0.5597$, p=0.59). Collectively, severity classification did not explain notable, or lack of, effects observed with PT inhibition.

It is important to note that our targeting strategy would inactivate PT neurons throughout cortex, including motor and sensorimotor regions. To determine whether the diminished responses observed with PT inhibition could be explained, in part, by impairments to motor activity, locomotor activity was assessed following treatment with a vehicle control or CNO, with the order of sessions counterbalanced. A final session with 0.1 mg/kg of clozapine was used to address the potential off-target effects observed with CNO. Surprisingly, total ambulations increased with administration of CNO compared to vehicle, but clozapine did not produce this effect (Figure 6a. RM One-way ANOVA $F_{(1.870, 26.18)} = 4.038$, $p<0.05$; Bonferroni post-hoc comparisons: Vehicle vs. CNO, $p<0.05$, Vehicle vs. Clozapine, n.s., Clozapine vs. CNO, n.s.). While movement decreased throughout the sessions as rats habituated to locomotor chambers (Figure 6b. RM two-way ANOVA Main effect of Time: $F_{(6.873, 75.60)} = 34.14$, $p<0.0001$), increased movement by CNO was notable throughout the session (Figure 6b. Main effect of Treatment: $F_{(1.773, 19.50)} = 4.054$, $p<0.05$) suggesting that the effects found on Omax were not resulting from loss of locomotor activity.

**Discussion**

Here, we demonstrate that altering activity of IT and PT neurons is not sufficient to affecting CPP or sucrose drinking, suggesting that these neurons do not regulate general reward and aversion processing. Previous work found that PT neurons modulate a CPP to cocaine, and IT neurons regulate a cocaine-induced sucrose taste aversion, these results suggest that the rewarding and aversive properties of cocaine are in part mediated by IT and PT neurons. However, given that these effects were found following noncontingent cocaine exposure, we extended these results by examining the effects of PT neuronal inhibition on reward and aversive processing during cocaine self-administration. These data suggest that PT neurons are involved in effort-based cocaine consumption, but not aversive valuation, consistent with CPP studies from our lab (Garcia et al., in press.). Nonetheless, it was surprising that these neurons did not impact measures of appetitive consumption ($Q_0$), and had the opposite effect on effort (Omax) than would be predicted based on CPP studies. These conflicting results may be due to the route of administration and regions targeted. Furthermore, these paradigms model different facets of appetitive reward value: Hedonic preference (CPP), and motivated and effort-based consumption (within-session cocaine thresholding), which model different phases of the addiction cycle (i.e. Binge/intoxication and Withdrawal/Negative Affect; Koob & Volkow, 2010).

The impact of PT inactivity was largely on responding during thresholding, particularly as the cost of consumption to achieve desired cocaine intake increased. This is notable, as baseline

consumption was not significantly altered. The absence of an effect on Pmax also suggests that deficits were not necessarily due to reduced demand for cocaine. Omax, essentially the work exerted at the maximal accepted price of consumption, is associated with intervention outcomes in alcohol users (MacKillop & Murphy, 2007), suggesting potential clinical relevance for predicting treatment outcomes. The robust decrease in work for cocaine consumption, therefore, warrants further study, particularly with PT manipulations on abstinence and relapse to drug seeking. Similar effects on breakpoint measures have been observed with pharmacological antagonism of dopamine (DA) D1 receptors in the prefrontal cortex, particularly when the price of cocaine is higher (i.e. lower unit doses), compared to effects at lower price points for cocaine consumption (Oleson & Duvauchelle, 2006). Given that PFC DA D1 receptor antagonism reduces preference for higher-effort rewards and progressive ratio breakpoints (McGregor & Robers, 1995; Oleson & Duvauchelle, 2006; Schweimer & Hauber, 2006), PFC computations for effort valuation and reinforcement of cocaine consumption may involve IT and PT modulation via D1 receptors. In particular, future work should examine the role of PT neurons specifically in medial prefrontal regions. Inactivation of prelimbic cortex, for instance, is known to promote drug-seeking (Limpens et al., 2015), while ACC and OFC are involved in valuation for and effort-based decision making (Azab & Hayden, 2018). Manipulations specific to these regions, therefore, may help clarify the role of regional PT activity during cocaine self-administration and its involvement in different phases of the addiction cycle.

Previous work with economic value of reinforcers in punishment conditions generated demand curves by increasing costs using greater shock magnitudes (Bentzley et al., 2013; Kawa et al., 2016). By effectively increasing the price to administer cocaine, a motivational “max charge” value is obtained by accounting for the number of infusions at a given shock magnitude, the magnitude of the shock, and dose. Here, we effectively increased costs through increased duration of the administered footshock punishments. PT neurons, however, do not appear to be involved in valuation and seeking under punished contexts, as max charge did not differ between control treatments and CNO. This is consistent with CTA experiments that found no significant effects of PT inhibition on the development of a cocaine-induced sucrose taste aversion (Garcia et al., in press.), nor on activity associated with restrained seeking with punished lever pressing (Kim et al., 2017). Caveats to this conclusion, however, come from the lack of significant differences in responding to differences in shock duration, though trends indicate some sensitivity across the session. Additionally, rats may have reduced responding across sessions due to shock sensitivity, rather than from treatments. Future work, therefore, could utilize alternative parameters to alter price, including differences in shock magnitude, or

other types of aversive stimuli (e.g. air puff, lithium chloride, tones). Given the effects of IT neurons on aversive processing of cocaine-induced aversion, focusing future work on IT subpopulations would also clarify the distinct roles of these subtypes in reward-related and aversion-related behaviors.

Given that only a subset of drug users progress to meeting DSM criteria for a substance use disorder (SUD), understanding individual variability in SUD risk and how this corresponds to differences in behavioral phenotypes is necessary for obtaining a complete understanding of the circuitry regulating drug-seeking and consumption. IntA access self-administration models lend themselves to investigating individual variability and have been shown to produce greater escalated drug intake and drug-seeking, and correlate with the development of psychomotor sensitization (Carr et al., 2020; Garcia et al., 2020.; Kawa et al., 2016; Zimmer et al., 2012). Using a composite z-score derived from performance metrics during IntA (O'Neal et al., 2020), rats were classified into three "risk" groups, and motivation measures under control and CNO conditions were compared. Surprisingly, desired consumption, effort, and motivated seeking were not impacted by self-administration severity and was not altered in a severity-dependent manner with PT-neuron inactivation. This may, however, be due to lack of power in these preliminary results and requires further studies as proposed above.

One potential confound of non-selective targeting of PT populations is the expression and manipulation of PT neurons in motor cortices. Indeed, CNO administration prior to cocaine treatments abolished the increase in ambulatory responding in cocaine-treated controls prior to cocaine treatment (Garcia, 2018). To address this, locomotor activity was recorded prior to and following treatment with either a vehicle control, CNO, or clozapine. Unexpectedly, CNO increased the number of cage crossings compared to controls, despite the fact that differences in clozapine and CNO treatments were not significantly different. Nonetheless, given that CNO-mediated inhibition did not dampen locomotor activity, these results indicate that the effects of PT inhibition were unlikely due to off-target effects (Gomez et al., 2017). Previous sensitization studies in PT neurons did however, show a dampening of cage crossings with initial development of cocaine sensitization, while repeated CNO and cocaine pairings resulted in enhanced development of sensitization after seven sessions (Garcia, 2018). It may be that initial PT inhibition and cocaine pairings, therefore, dampened activity, and may have produced differences in responding following repeated CNO pairings. However, these locomotor effects were in the absence of cocaine, which was not seen during CNO treatment with saline controls

during sensitization. Therefore, alterations to motor cortical activity do not explain the effects seen in this study.

Encoding of motivation involves changes in tonic dopamine levels (Volkow et al., 2004) and increased glutamatergic transmission within the nucleus accumbens (NAc) core (Kalivas, 2004). Cortical afferents send glutamatergic inputs to the dorsal striatum, NAc, amygdala, and VTA (Kalivas, 2004), which are sensitized with cocaine administration (Kalivas, 2004). Dopamine D1 receptor antagonism in medial prefrontal cortex (McGregor & Roberts, 1995), cortical lesions (Smith et al., 2008), as well as L-DOPA pretreatment (Antinori et al., 2017) decrease break-points under progressive ratio schedules of cocaine administration, which implicate altered glutamatergic transmission due to dopamine binding in the PFC. Furthermore, PFC projections enhance signal-to-noise for transmission at VTA targets (Almodóvar-Fabregas et al., 2002), drive NAc activity (Brady & O'Donnell, 2004), and are modulated by VTA inputs (Buchta et al., 2017). Differences in cortical subtypes extend to dopamine receptor expression. Activation of dopamine D1 receptors in cortical projection subtypes enhancing firing in IT and PT neurons (Anastasiades et al., 2019; Leyrer-Jackson & Thomas, 2019) and VIP interneurons (Anastasiades et al., 2019). However, subsets of PT neurons exhibiting persistent burst-firing following D1 agonism with fiber stimulation (Leyrer-Jackson & Thomas, 2019), and PT neurons are also reported to express dopamine D2 receptors, which produce after-depolarizations persisting for hundreds of milliseconds (Gee et al., 2012). Furthermore, a subset of IT neurons express and are dampened by dopamine D3 receptor activation (Clarkson et al., 2017). How extra-telencephalic projection activity impacts projection outputs, as well as their regulation by dopamine in the context of cocaine administration, could provide mechanistic explanations for the results seen in this study. The reciprocal connectivity of the VTA to PT neurons, and how this connectivity is changed with cocaine use, are additional directions for establishing PT-specific connectivity to the CBG and its effects on drug reinforcement.

Assessing the impact of PT manipulations on the acquisition and escalation of cocaine self-administration would address the role of these populations in the consummatory aspects of cocaine use in the absence of effort-based alterations, as well as help determine if the observed effects of PT manipulation on reward processing may be based on learning action-outcome contingencies rather than activity following established drug administration. Alternatively, comparing changes in motivation resulting from CNO treatments during self-administration compared to normal cocaine administration could clarify outstanding questions of the

significance of PT neurons on the initial learning, progression, and expression of behaviors related to escalated substance use, misuse, and relapse.

These data provide additional evidence for the role of IT and PT neurons in the processing of rewarding and aversive stimuli and provide preliminary evidence for the contributions of PT neurons in volitional cocaine use and its motivational properties. We find that manipulations to these populations in the absence of a rewarding stimulus (i.e. cocaine) do not produce changes to preference and aversion, but that PT inhibition reduces effort-based cocaine consumption, but not in aversive contexts. The specific circuits and mechanisms underlying this regulation, and the role of IT and PT neurons in behaviors examining the role of craving and relapse, will need to be elucidated.

## Figure Legends

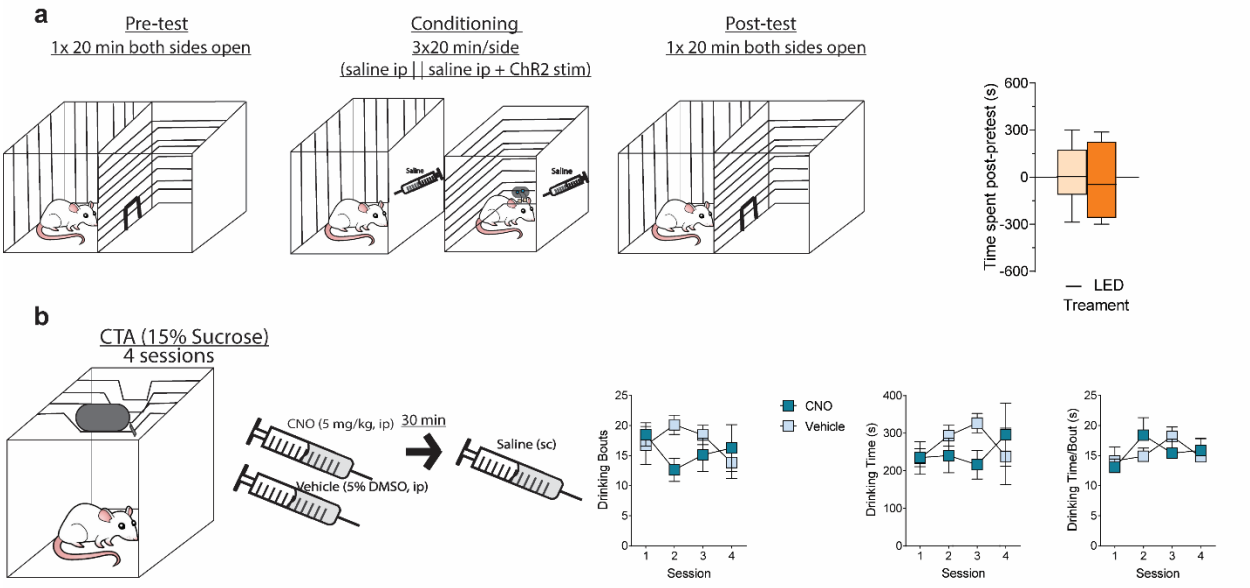



**Figure 1: Alterations of PT and IT activity under baseline conditions does not alter preference or aversion. a.** Rats (N=5) underwent an initial pretest session prior to three sessions each of saline in one side of the chamber and saline + optical stimulation of PT neurons expressing ChR2. Following the final conditioning session, rats were permitted to freely explore the CPP chamber with no treatments administered in a post-test session. Preference was determined by time in control vs. treatment regions (post-pretest). **b.** Rats (N=15) receiving either vehicle (N=7) or CNO (N=8) 30 minutes following sessions with freely available 15% sucrose did

not demonstrate any differences in number of drinking bouts across the four sessions, the total drinking time, or the the time per bout. Data is reported as mean ± SEM.

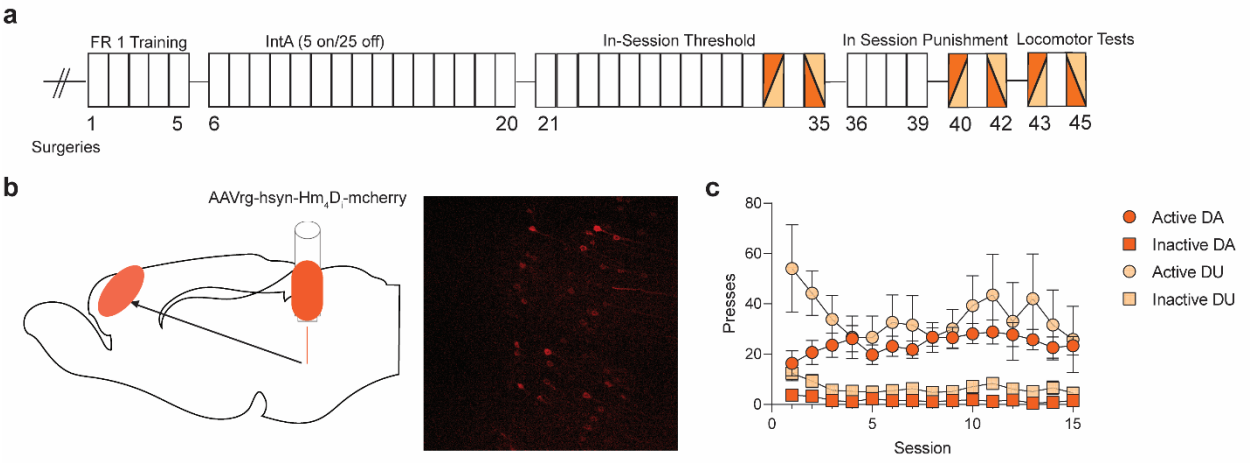



Figure 2: **Overview of general PT inhibition self-administration paradigm. a.** Timeline of self-administration, within-session threshold, within-session punishment, and locomotor assays. **b.** Targeting strategy for PT cortical neuronal inhibition by injecting a nonselective retrograde AAV-$Hm_4D_i$ into the pontine nucleus with a representative image of DREADD expression in medial prefrontal cortex (N=15). **c.** Acquisition of intermittent access over 15 self-administration sessions following FR 1 training for cocaine.

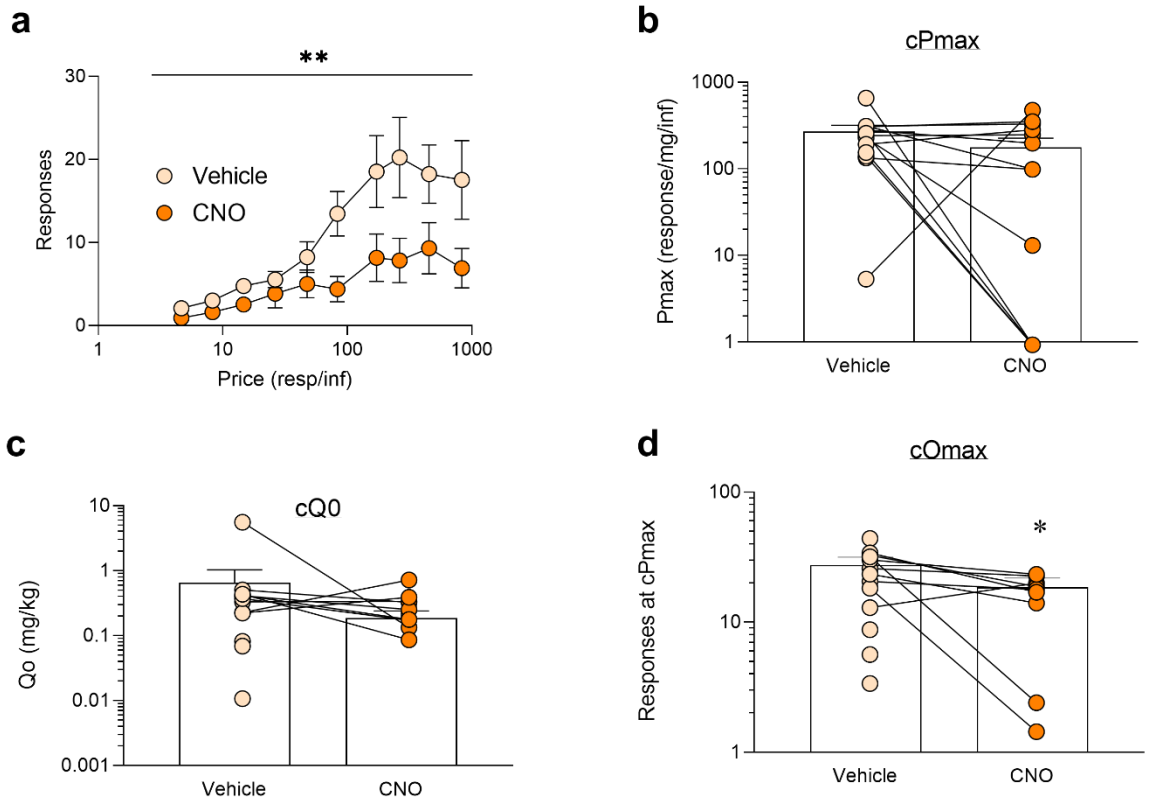



Figure 3: **Inhibition of PT neurons reduces effort exerted to maintain baseline cocaine intake. a.** Demand curve of responding at increasing price per infusion of cocaine during sessions where subjects receive either a pretreatment with CNO (5 mg/kg in 5% DMSO), or a 5% DMSO control solution 30 minutes prior to within-session thresholding (N=13). Responding at dose of 83.3 µg/inf with PT inhibition significantly differed in control sessions. **b.** Responding at calculated Pmax in vehicle vs. CNO test sessions. **c.** Calculated no-cost intake during vehicle vs. CNO test sessions. **d.** Calculated responding at Pmax is significantly lower with PT inhibition than during control treatment. Data is reported as mean ± SEM. *- $p < 0.05$ for Treatment, **- $p<0.01$ for Price, Treatment, and Treatment x Price

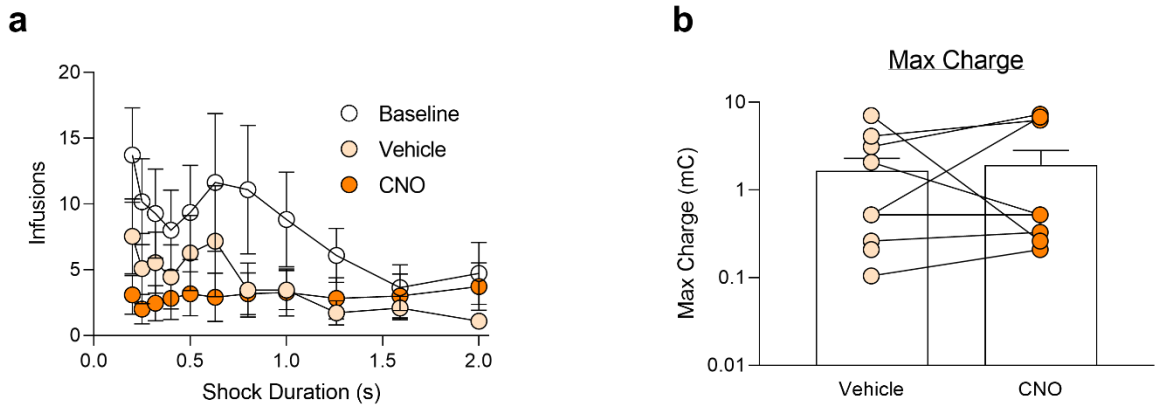



**Figure 4. PT inhibition does not affect punished cocaine consumption. a.** Infusions of cocaine at each 10-minute shock duration bin throughout cocaine punishment-thresholding sessions prior to treatments (Baseline), with a DMSO control (Vehicle), or CNO (N=11). **b.** Max charge (infusions x cocaine dose x shock duration (s) x shock amplitude (0.25)) willing to be paid for cocaine administration did not differ with PT inhibition. Data is reported as mean ± SEM.

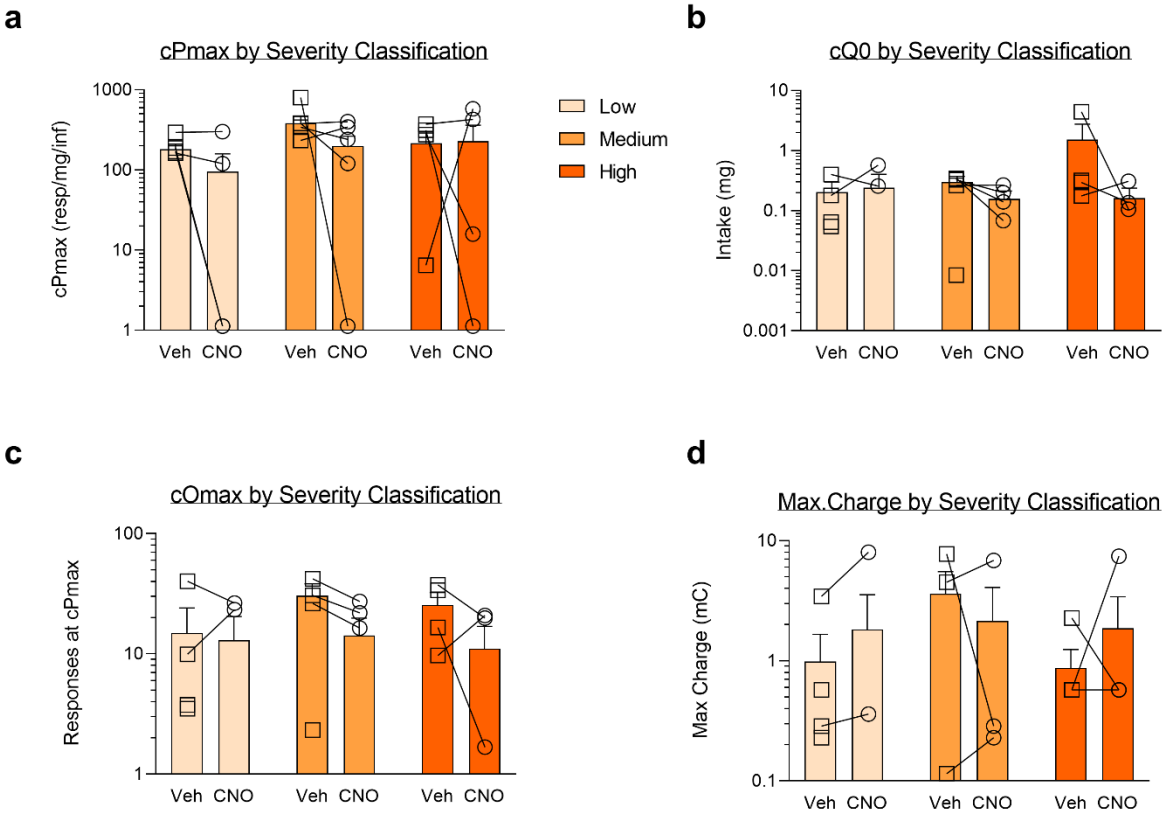


**Figure 5: Motivation and effort to maintain cocaine consumption is not distinguished by self-administration risk severity. a.** Calculated Pmax for within session-threshold fit by an exponential demand curve (N=13). Severity classifications are based on Z scores with SD < -1 considered “Low-Risk”, SD > 1 considered “High-Risk” and between -1 – 1 “Medium Risk” (O’Neal et al., 2020). **b.** No-cost intake ($Q_0$) calculated from an exponential demand curve (N=13) **c.** Calculated responding at calculated Pmax based on exponential demand curve fitting for Low, Medium, and High-Risk SA scores (N=13). **d.** Max charge (cocaine dose x responses x charge magnitude (mA) x shock duration (s) by SA severity classification (N=11). Data is reported as mean ± SEM.

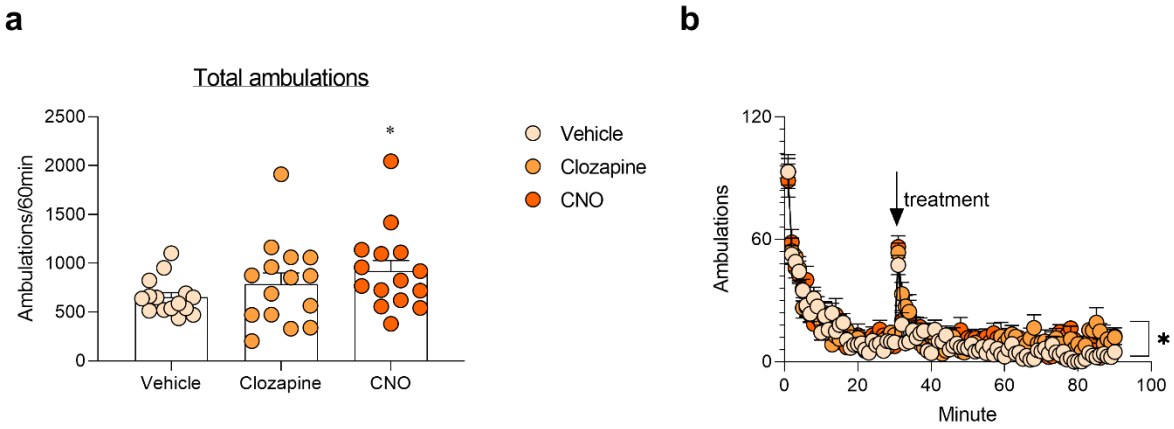



**Figure 6: Reduced responding to cocaine thresholding not from loss of locomotor activity.** All rats (N=15) received ip injections of 6% DMSO vehicle control, 5 mg/kg CNO, and 0.1 mg/kg clozapine in three separate, 90 minute open field sessions. **a.** Total ambulations following ip injections. **b.** Timecourse of activity prior to and following treatment with either vehicle, CNO, or clozapine in 1 minute intervals. Data is reported as mean ± SEM. * - $p<0.05$ vs. vehicle.

## Chapter 5:

## Investigating cell-type contributions to decision-making

- This data is prepared for a publication with the following co-authors: Zackari D. Murphy, Reiley Durre, Marlaena Nooney, Derek MacDougall, Jordyn Richardson, Grayson Baden, and Susan M. Ferguson


### Abstract

Risk-taking behaviors, which are a hallmark of neuropsychiatric diseases including substance use disorders and ADHD, are regulated by cortical circuits. However, studies examining the contributions of these circuits to decision-making strategies have largely focused on regional activity rather than delineation of cell-type specific contributions. The cortex is primarily comprised of two highly heterogenous but physically intermingled populations of glutamatergic projection neurons, intratelencephalic (IT) and pyramidal tract (PT), and the role of these cell-types in complex decision-making has not been investigated. To begin to address this, we used combinatorial, chemogenetic viral approaches to assess how IT and PT neurons regulate performance during probability discounting and reversal tasks. The ventral and lateral orbitofrontal cortex (vOFC and lOFC) were targeted for neuronal manipulation because Fos activity was

elevated in these areas following probabilistic choice. There was no effect of chemogenetic inhibition of IT or PT neurons on probability discounting. However, IT inhibition increased completed within-session reversals, whereas PT inhibition impaired performance following outcome-specific responses. Together these results suggest that, while OFC glutamatergic projection neurons are not necessary for regular choice selection in risk-based models, these populations are involved in distinct aspects of response flexibility. Thus, this study establishes a role of these subtypes in uniquely mediating behaviors involving motivated response strategies.

**Significance Statement:** While it is known that cortical activity is essential for complex decision-making and executive functioning, there are still outstanding questions on the contribution of regional and cell-type cortical activity to decision-making processes. Therefore, parsing the role of distinct populations of cortical projection neurons in a regional manner during these complex behaviors is necessary for clarifying underlying mechanisms as well as elucidating how these processes may be dysregulated in neuropsychiatric disorders. As such, we sought to determine the impact of manipulating cell-type specific activity during probabilistic decision-making and reversal learning tasks to examine the role of these cortical populations in risk assessment and behavioral adaptability.

## Introduction

Decision-making strategies, including those involved in risk valuation, are largely mediated by the executive functions of the prefrontal cortex (PFC) (Floresco et. al, 2008; Knutson et al., 2005; Wrase et al., 2007). Alterations in decision-making processes are common among neuropsychiatric disorders, including substance use disorders and ADHD, but how computations of value and risk are normally regulated by the PFC and become dysregulated in these diseases is still not fully understood. Manipulations to several regions of PFC alter decision-making in various risk and impulsivity models, including medial PFC (Knutson et al., 2005; Stopper et al., 2014) and orbitofrontal cortex (OFC) (Pais-Vieira et al., 2007; Wrase et al., 2007). However, differences in task structure and regional targeting have produced conflicting results (Mai & Hauber, 2012; Mobini et al., 2002; St. Onge & Floresco, 2010), suggesting that the cortical processes underlying decision-making are complex and not fully understood.

Adding to this complexity, the PFC is primarily comprised of two distinct classes of glutamatergic projection neurons; intratelencephalic (IT) neurons which project ipsi- and bi-laterally within the cortex and to the striatum, and pyramidal tract (PT) neurons which project ipsilaterally to the striatum and brainstem structures. IT and PT neurons are morphologically distinct, have

distinguishable firing patterns, and are differentially affected by various neuromodulators (Dembrow et al., 2010; Shepherd, 2013; Stephens et al., 2014). In addition, IT neurons are present across layers II-VI of cortex, with layers II and III characterized by cortico-cortical projections and layers V-VI characterized by both cortico-cortical and cortico-striatal projections. PT neurons are restricted to layer VB, but are notable for being highly multi-projectional, innervating multiple targets in the striatum, midbrain, and brainstem (for review, see Shepherd, 2013). To date, the contributions of these different cortical projection populations in decision-making processes have not been assessed. Accordingly, the purpose of this study was to determine how these subsets of PFC neurons regulate choice patterns and behavioral flexibility. We first used Fos labeling following probabilistic choice for sucrose rewards to determine regions of interest for subsequent cell-type neuronal manipulation. Then, using an inhibitory chemogenetic approach, the role of IT and PT cells were assessed during probabilistic choice tasks and reversal learning.

## Methods

### *Animals*

All experiments were approved by the Seattle Children's Research Institute Institutional Animal Care and Use Committee and were conducted in accordance with National Institutes of Health guidelines. Male Long Evans rats (N=59, Charles River) weighing 251-275g upon arrival were pair-housed in a temperature- and humidity-controlled vivarium on a 12-hour light/dark cycle. Rats were acclimated for at least three days prior to any experimental manipulations. Rats were food restricted and maintained between 85-90% of their free-feeding bodyweight throughout the study with 20g per cage of chow pellets given daily. Water was provided *ad libitum*.

### *Drugs*

Clozapine-N-Oxide (CNO) was obtained from the National Institute of Health through the Rapid Access to Investigate Drug Program and prepared by dissolving in dimethyl sulfoxide (DMSO; Sigma Aldrich, D650) in a hot water bath, then further diluted in sterile water to 6% DMSO. CNO was prepared prior to test sessions at a concentration of 5 mg/kg CNO and administered at 1 mg/kg *ip*. Vehicle injections were 6% DMSO in sterile water administered at a volume of 1 mL/kg *ip*.

### *Surgery*

Rats were anesthetized with isoflurane (2-5% inhalation, Patterson Veterinary) and were administered meloxicam (0.2mg/kg *sc*, Patterson Veterinary) for analgesia prior to stereotaxic surgery. Rats underwent post-procedure monitoring a minimum of three days following surgery.

*Viral strategy*

To achieve selective DREADD expression in IT neurons, rats received unilateral infusions of CAV-CRE (from the lab of John Neumaier, University of Washington) and $AAV_{rg}$-EF1α-FLPO (Addgene #55637) into different hemispheres of the NAc (NAc: A/P: +1.2, M/L: ±2.5, D/V: -7.4 mm relative to bregma; 1000 nl/side, 200 nl/min) along with infusions of the complementary CRE- and Flp-dependent AAV-$hM_4D_i$ into the contralateral OFC (A/P +3.7, M/L ± 2.5, D/V -5.4 mm relative to bregma; AAV1-DIOFRT- $hM_4D_i$-EYFP from the lab of Larry Zweifel, University of Washington, AAV8-DIO- $hM_4D_i$-mCherry from Addgene #44362; 750 nl/side, 150 nl/min) (Figure 2C). To achieve selective DREADD expression in PT neurons, rats received bilateral infusions of CAV-CRE into the caudal pontine nucleus (PNc: A/P: -9.2, M/L: ±0.2 or 0.5, D/V: -9.4 or -9.8 mm relative to bregma; 750 nl/side, 150 nl/min), along with bilateral infusions of AAV8-DIO- $hM_4D_i$-mCherry into the OFC (750 nl/side, 150 nl/min) (Figure 2D). To target DREADD expression non-selectively to all OFC neurons, rats received bilateral infusions of AAV8-hSyn-$hM_4D_i$-mCherry into OFC (Addgene #50475; 750 nl/side, 150 nl, min) (Figure 2B). For targeting DREADD expression to both IT and PT neurons, the same regions were targeted as described for the selective expression, with the exception that FLPO was unilaterally infused into one hemisphere of the PNc ipsilateral to the Flp-dependent DREADD, while CAV-CRE was unilaterally infused into the other hemisphere of the PNc ipsilateral to the corresponding Cre-dependent DREADD (Figure 2C).

*Behavior Apparatus*

Behavioral testing occurred in standard operant self-administration chambers equipped with retractable levers, stimulus lights, a house light, a feeder, and a metal grid floor (Med Associates ENV-007CT). The front wall housed two white stimulus lights, one located above each lever, with an additional green stimulus light above the white stimulus light on one side. The back wall contained a white house light.

*Lever Press training*

A representative timeline of all behavioral testing is outlined in Figure 2A. Rats were trained under a fixed-ratio 1 schedule to a criterion of 100 pellets obtained in a single, 30-minute session on

one lever before undergoing training on the opposite lever (~3 days to train both levers), as in previous studies (Cardinal & Howes, 2005; St. Onge & Floresco, 2009; Stopper et al., 2014).

*Probability training*

Sessions began with levers retracted and operant chambers in darkness. The food receptacle and house lights were presented at the beginning of each trial (every 40s) to signal a response (nosepoke) to occur within 10s. If no respond was recorded within this timeframe, the lever was retracted and the trial was counted as an omission. If a response was made, a single pellet was delivered with 50% probability and continuous illumination of the food receptacle light and house light (3s) up to a 10s collection time limit. In every trial, the left or right lever was presented once in a randomized fashion. Training occurred over a five to six-day period to a criterion of ten omissions or less in a session (Cardinal & Howes, 2005; St. Onge & Floresco, 2009).

*Probability discounting*

Rats were run five to seven days a week. Sessions consisted of 96 trials, separated into four blocks of 26 trials (~70-minute session time) and began in darkness with both levers retracted. Trials began every 40 s with the house light and food receptacle light illuminated. To initiate forced or choice trials, rats nose-poked into the illuminated food receptacle. If a rat successfully initiated the trial, either one lever (forced choice) or both levers (choice trials) were extended. Pressing a lever resulted in cue light presentation (3 s illumination) and food pellets dispensed into the food receptacle. If a press resulted in no reward administration, a green cue light above the white cue light associated with the risky lever was illuminated to indicate reward omission. If a rat did not nosepoke into the food receptacle to initiate the trial within 10 s, or if a rat did not press a lever within 10 s, the trial was scored as an omission. The risky lever was associated with descending probability of administration of four sucrose pellets across the four trial blocks: 100%, 50%, 25%, and 12.5% probability of reward delivery, whereas the safe lever always resulted in administration of one food pellet (St. Onge & Floresco, 2009). After a minimum of 25 sessions on the full discounting task, training continued until stable performance was achieved, as determined statistically with no interaction between session and performance across three sessions. Following training, CNO (5 mg/kg in 6% DMSO in sterile water) or vehicle (6% DMSO) was administered 30 minutes prior to testing in a counterbalanced fashion, followed by two days of re-baselining in between each test session. The discounting rate for the risky reward was calculated using the following equation, where $p_x$ represents the percent of risky choices at a given probability $x$ during choice trials*:*

$$Discounting = \frac{|(p_{100} + p_{50})/2 - p_{12.5}|}{3}$$

The percentage of optimal choices was obtained by taking the average percent choices for the risky reward at $p_{100}$ and $p_{50}$ and the safe choice at $p_{12.5}$. The 25% probability block was not included, as both options were equally valued (adapted from Verharen et al., 2018).

To examine neuronal activity during probabilistic choice, rats (N=20) were trained as described above. On the final behavioral session, the discounting session was altered to maintain a constant probability for the larger reward (100%, 50%, 25%, and 12.5%) across all four blocks. Rats were perfused 75 minutes after the start of the discounting session to examine cFos activity.

*Reversal task*

Following discounting tests, rats underwent a within-session reversal task (adapted from Verharen et al., 2018) consisting of 150 total trials of FR1 pressing, where an active and inactive lever were extended. Initial active levers were chosen based on the “safe” lever association from the probability discounting task. Selection of the active lever resulted in delivery of one sucrose pellet, illumination of the white cue light above the active lever for 3s, and retraction of both levers. Selection of the other, inactive lever resulted in illumination of the green cue light for 3s and an additional 8s time-out period whereby the houselight was turned off and the levers were retracted. A new trial began 8s after the last response, signaled by illumination of the houselight. After five consecutive responses on the active lever, the “active” lever was switched to the previously inactive one, prompting a shift in behavioral strategies to obtain sucrose pellet rewards. A minimum of six sessions was given on this task to assure stable performance before undergoing test sessions. During testing, CNO or vehicle was administered 30 minutes prior to testing in a counterbalanced fashion, followed by one to two days of re-baselining in between each test session.

*Immunohistochemistry*

Following completion of behavioral paradigms, rats were anesthetized with Beuthanasia-D and transcardially perfused with 1x phosphate-buffered saline (PBS; pH = 7.4 on ice), followed by 4% paraformaldehyde (PFA) in PBS. Brains were extracted and fixed overnight in PFA and subsequently stored in 30% sucrose (in PBS) prior to being coronally sectioned on a vibrating microtome (40 µm thickness). To confirm DREADD expression, tissue sections were washed in 1x PBS (3 x 10 minutes) prior to incubating in blocking buffer consisting of normal goat serum (5% NGS), Triton-X (0.25%), and PBS (120 minutes). Sections were then transferred from

blocking solution to primary antibody solution (2.5% NGS, 0.25% Triton-X, 1:1000 rabbit polyclonal anti-GFP, ThermoFisher # A-11122, 1:400 mouse monoclonal anti-mCherry, Clontech # 632543, or 1:800 rabbit polyclonal anti-cFos MilliporeSigma # ABE457, 24 hours), before being washed in PBS (4 x 10 minutes) and subsequently incubated in secondary antibody solution (2.5% NGS, 0.25% Triton-X, 1:500 Alexa Fluor 488 goat anti-rabbit ThermoFisher# A-11034, 1:400 Alexa Fluor 568 goat anti-mouse ThermoFisher# A-11004, 120 minutes). Finally, sections were washed in PBS (3x10 minutes), mounted on slides, and coverslipped with Vectashield mounting medium with DAPI (Vector Laboratories H-1500). Slides were imaged with a Zeiss LSM 710 confocal microscope. For cFos imaging, N=3 images were collected from the following cortical regions, with at least one image from each hemisphere obtained: anterior cingulate (ACC), infralimbic (IL), prelimbic (PL), medial OFC (mOFC), ventral OFC (vOFC), and lateral OFC (lOFC). Image collection of z stacks was set to 20x magnification for 9µm range and cFos counts were obtained using Image J (NIH) analyze particles (thresholded to 2% signal and using parameters for sphericity=0.15-1 and size=250-1500 pixels$^2$) and Adobe Photoshop software.

*Experimental design and statistical analysis*

Data was processed using customized python scripts (available upon request) and analyzed using GraphPad Prism 8.4.3. cFos counts were averaged (N=3 images for each region for each subject) and analyzed using two-way ANOVA of cortical region by probability of the risky reward. Pearsons correlations were analyzed at each reward probability for associations between average cFos counts and the percentage of choice selections for the larger reward in each region. For probability discounting experiments, the primary dependent variable was choice on the risky lever on free choice trials (number of responses on risky lever/total number of choice trials in a block). For reversal experiments, the primary dependent variable was the number of completed reversals within a session, with additional variables examined including the number of trials required to reverse the rewarded lever, and preservative responding (defined as the average number of incorrect choices made after a reversal occurs before selecting the correct lever in a session). Choice across probabilities was analyzed using two-way repeated measures (RM) ANOVA with Geisser-Greenhouse's correction for potential differences in variance. Discounting rate, optimal choice, number of reversals, preservative responding, and trials to first reversal were calculated as previously described (Verharen et al., 2018) and analyzed using a two-tailed paired t-test comparing performance between control and test treatment sessions. Choice patterns (based on if the selection resulted in a reward (win) or no reward (loss), and if this were followed by selection of the same lever (stay) or switching to the other lever (shift) during discounting) were analyzed

with a three-way ANOVA with Geisser-Greenhouse's correction. Trials to reversal based on outcome, defined as either selection of the rewarding lever (win) or incorrectly staying on the previously rewarded lever (loss) at the start of a reversal block, were analyzed with a restricted maximum likelihood (REML) mixed-effect analysis.

**Results**

To determine the cortical areas activated during probabilistic decision-making, rats were trained on the probability discounting task and split into one of four groups: A group receiving the larger reward on 100% of trials, 50% of trials, 25% of trials, or 12.5% of trials (N=5 for each group). cFos was used as a correlate for neuronal activation (Dragunow & Robertson, 1987) during the task, and was quantified in seven potential regions of interest known to be involved in affective and motivated behaviors: ACC, IL, PL, mOFC, vOFC, lOFC, and insula. cFos expression was highest in vOFC and lOFC across all probabilities (Figure 1A, Two-way ANOVA main effect of region: $F_{(6, 112)} = 19.08$; $p<0.0001$), and significantly higher in post hoc comparisons to all regions but the ACC (Table 1). However, activity did not differ based on the probability of the larger reward, but was higher at the 50% reward probability, suggesting overall activity was higher when it was advantageous to choose the larger, riskier option (Figure 1A, Two-way ANOVA main effect of probability: $F_{(3, 112)} = 5.739$, $p=0.0011$). To determine if activity was related to preference for the "riskier" option, regional cFos at each probability was compared to the frequency of choosing the larger reward when given a choice between the risky and safe options (Figure 1 I-O). Interestingly, IL activity at 50% probability revealed significant associations between risk preference and cFos activity, but no other significant associations were found. Together, these results suggest that, while not exhibiting changes in activity based on probability, ventral and lateral OFC are engaged during decision-making with probabilistic options. Based on these results, we targeted subsequent manipulations to these cortical areas. Rats underwent stereotaxic surgery to target DREADD expression to either ventral and lateral OFC (referred to generally as OFC), which exhibit overlapping connectivity (Hoover & Vertes, 2011), or selectively to IT, PT or both neuronal populations in OFC (Dual IT and PT; Figure 2C-E). Once rats reached stable performance on the full probability-discounting task (minimum of 25 sessions with no session effect for final three sessions; Figure 2A) they were administered a pretreatment of 5 mg/kg CNO or vehicle 30 minutes prior to the start of the probability-discounting task, in a counterbalanced fashion.

All groups exhibited sensitivity to the change in probability of the larger reward across a session (Figure 3A-D: Repeated Measures (RM) two-way ANOVA, Main effect of probability of risky choice: Nonspecific: $F_{(1.960, 9.799)} = 14.35$, $p<0.0013$; Dual IT and PT: $F_{(2.462, 14.77)} = 21.91$, $p<0.0001$;

IT: $F_{(1.975, 19.75)}$ = 84.47, p<0.0001; PT: $F_{(2.098, 25.18)}$ = 33.49, p<0.0001). While inactivation of OFC activity altered discounting of the risky option (Figure 3A, Nonspecific, RM two-way ANOVA, Main effect of treatment: $F_{(1.000, 5.000)}$ = 13.89, p=0.0136), inhibiting IT and/or PT neuronal subtypes did not alter probabilistic discounting (Figure 3B-D: RM two-way ANOVA, Main effect of Treatment: Dual IT and PT: $F_{(1.000, 6.000)}$ = 0.07080, p=0.7991; IT: $F_{(1.000, 10.00)}$ = 2.366, p=0.1551; PT: $F_{(1.000, 12.00)}$ = 0.1522, p=0.7032), nor were there effects specific to the probability of obtaining the larger reward for any OFC manipulations (Figure 3A-C, 3E: RM two-way ANOVA, Interaction of Probability x Treatment: Nonspecific: $F_{(2.383, 11.92)}$ = 0.4954, p=0.6525; Dual IT and PT: $F_{(1.672, 10.03)}$ = 1.049, p=0.3726; IT: $F_{(2.080, 20.80)}$ = 1.027, n.s.; PT: $F_{(1.983, 23.79)}$ = 0.1193, n.s.). This result is consistent with the absence of effects found for discounting rate of the risky option (Figures 3E-H, Two-tailed paired t-test, Nonspecific: t=0.1085, df=5, p=0.9179; Dual IT and PT: t=0.6378, df=6, p=0.5472; IT: t=1.381, df=10, p=0.1975; PT: t=0.3954, df=12, p=0.6995) and optimal choice (Figures 3I-L, Two-tailed paired t-test: Nonspecific: t=0.4443, df=5, p=0.6754; Dual IT and PT: t=0.4201, df=6, p=0.6891; IT: t=0.05814, df=10, p=0.9548; PT: t=0.3475, df=12, p=0.7343). Additionally, none of the manipulations increased omissions during the task (Supplementary Figure 1A-D, Two-tailed paired t-test: Nonspecific: t=0.2745, df=5, p=0.7946; Dual IT and PT: t=0.6195, df=6, p=0.5583; IT: t=0.000, df=10, p=0.9999; PT: t=0.9856, df=12, p=0.3438).

Choice patterns, however, appear to involve OFC activity, as general inhibition of OFC alters choice depending on prior outcomes (Figure 3M, Three-way ANOVA, Nonspecific: Treatment x Outcome: $F_{(1.000, 5.000)}$ = 7.619, p=0.0398) and trends towards alterations in subsequent choices to either stay on the previously selected lever or shift to the alternative option (Figure 3M, Three-way ANOVA, Nonspecific: Treatment x Choice: $F_{(1.000, 5.000)}$ = 5.183, p=0.0718). Chemogenetic inhibition of both IT and PT OFC neurons trends towards changes to choice patterns based on outcome and an interaction between outcome and subsequent choice (Figure 3N, Three-way ANOVA, Dual IT and PT: Treatment x Outcome: $F_{(1.000, 6.000)}$ = 4.158, p=0.0876; Treatment x Outcome x Choice: $F_{(0.5954, 3.572)}$ = 6.337, p=0.0759). However, neither subtype is predominantly implicated as manipulations specific to each population had no effect (Figures 3O-P: IT: Treatment x Outcome: $F_{(1.000, 10.00)}$ = 0.2880, p=0.6032; Treatment x Choice: $F_{(1.000, 10.00)}$ = 0.04736, p=0.8321.;Treatment x Outcome x Choice: $F_{(0.5047, 5.047)}$ = 0.6497, p=0.3498; PT: Treatment x Outcome: $F_{(1.000, 12.00)}$ = 0.9953, p=0.3381.; Treatment x Choice: $F_{1.000, 12.00)}$ = 0.09529, p=0.7629.; Treatment x Outcome x Choice: $F_{(0.4839, 5.807)}$ = 0.004099, p=0.800). Taken together, these data suggest that although OFC activity is involved in probabilistic decision-making, activity of each individual glutamatergic subtype is not necessary. Nonetheless, the combination of connectivity

between both subtypes to other neuronal subtypes within OFC and/or to other projection regions may impact outcome-dependent choice.

OFC activity is linked to response adaptation and flexibility, with inactivation of OFC producing preservative responding and impaired reversal learning (Chudasama & Robbins, 2003). To determine if either IT or PT populations are necessary for reversal learning, a within-session reversal paradigm was used to assess the effects of cell-type chemogenetic inhibition on performance, as previously described (Verharen et al., 2018). Nonspecific inhibition of OFC did not change the number of completed reversals within a session (Figure 4A, Two-tailed paired t-test: t=0.1667, df=6, p=0.8731). Furthermore, the simultaneous inhibition of both cell populations did not affect completed reversals (Figure 4B, Two-tailed paired t-test: t=1.595, df=7, p=0.1548). However, IT inactivation significantly increased the number of completed reversals (Figure 4C: Two-tailed paired t-test: t=2.246, df=10,p=0.0485), while PT inactivation had no impact on the number of reversals (Figure 4D, Two-tailed paired t-test: t=0.3413, df=12, p=0.7388). To determine if general inhibition of OFC activity, or if inhibition of particular excitatory subtypes, was sufficient to alter preservative responding, the average number of responses on the previously active lever following a reversal was compared between vehicle and CNO treatments. None of the manipulations were sufficient to significantly alter preservative responding (Figure 4E-H, Two-tailed paired t-test: Nonspecific: t=0.7491, df=6, p=0.4821; Dual IT and PT: t=0.01698, df=7, p-0.9869; IT: t=1.932, df=9, p=0.0855; PT: t=0.4436, df=12, p=0.6653), though preservative responding with IT inhibition tended to be lower on average. The trials required to reach the first reversal were also not significantly different with any manipulation (Figures 4I-L, Nonspecific: t=2.180, df=6, p=0.0721; Dual IT and PT: t=1.366, df=7, p=0.2141; IT: t=1.396, df=10, p=0.1928; PT: t=0.000, df=12, p>0.999), but inhibition of all OFC activity tended to slow the initial acquisition of lever pairing with reward outcomes. When examining outcome-based performance, measured by determining the average trials to reach a reversal following the start of a reversal block with either administration of reward (press on new active lever), or with reward omission (preservative responding with press on previously inactive lever), nonspecific, dual IT and PT, and IT manipulations did not impact performance (Figure 4M-O: Mixed-effect analysis: Nonspecific: main effect of Treatment $F_{(1.000, 6.000)}$ = 0.9368, p=0.3705, main effect of Outcome $F_{(1.000, 6.000)}$ = 0.4195, p=0.5411, Treatment x Outcome $F_{(1.000, 1.000)}$ = 1.956, p=0.3952; Dual IT and PT: main effect of Treatment $F_{(1.000, 7.000)}$ = 3.453, p=0.1055; main effect of Outcome $F_{(1.000, 7.000)}$ = 0.2705, p=0.6190.; Treatment x Outcome $F_{(1.000, 4.000)}$ = 0.2182, p=0.6647; IT: main effect of Treatment $F_{(1.000, 10.00)}$ = 0.02588, p=0.8754; main effect of Outcome $F_{(1.000, 10.00)}$ = 0.05880, p=0.8133; Treatment x Outcome: $F_{(1.000, 8.000)}$ = 0.1215, p=0.7364). Interestingly, however, inhibiting PT neurons in OFC

resulted in slower acquisition of reversals following a winning outcome (Figure 4P: main effect of Treatment $F_{(1.000, 12.00)} = 4.587$, p=0.0534; main effect of Outcome $F_{(1.000, 12.00)} = 1.410$, p=0.2581; Treatment x Outcome $F_{(1.000, 5.000)} = 6.806$ p=0.0477), suggesting a role for PT neurons in outcome-based response flexibility.

**Discussion**

The PFC is heavily involved in value-based decision-making and behavioral flexibility (Li et. al, 2016; McAlonan & Brown, 2003; McDannald et al., 2014; Padoa-Schioppa & Assad, 2006). For example, recordings of OFC neurons during operant tasks have found that these neurons exhibit changes in background firing reflective of outcome-based modifications to signal firing (Kravitz & Peoples; 2008). These changes are particularly evident with established action-outcome contingencies (Shoenbaum et al., 1999), and are also seen in tasks involving risk-based subjective value (Jo & Jung, 2016). Nonetheless, the precise role of cortical subregions remains to be fully characterized, and the contribution of different cortical projection populations has not been investigated. Here, we utilized an inhibitory chemogenetic viral approach to determine the role of the two major subtypes of glutamatergic projection neurons (IT and PT) in probabilistic valuation of a food reward (St. Onge & Floresco, 2009), as well as in response adaptability in a within-session reversal task (Verharen et al., 2018). We focused our neuronal manipulations on the vOFC and lOFC, because these regions were most active across all probabilities for the larger reward (Figure 1). Overall, we found that general inhibition of OFC impacted probabilistic risk discounting, but that IT and PT activity differentially impacted reversal performance.

Prior studies using pharmacological or lesion approaches to examine the role of the OFC in risk-based decision-making have yielded mixed results, with reports of increases, decreases and no changes in task performance (Mobini et al., 2002; Orsini et. al, 2015; Pais-Vieira et. al, 2007; Stopper et. al, 2014; St. Onge & Floresco, 2010). Consistent with some of this work, we found that transient inhibition of OFC reduced risk preference across reward probabilities (Figure 3A) with OFC inhibition altering subsequent choice (Figure 3M). Importantly, the alteration to choice preference that we observed is not indicative of attentional deficits reducing task engagement, as no differences were seen in omissions or response latencies when administering CNO compared to control treatments in any of the manipulations (Supplementary Figure 1). However, these results are contrary to previous work using the same discounting paradigm (St. Onge & Floresco, 2009). The differences in these results could be based on the type of inactivation strategy, as the previous study used GABA agonists to decrease OFC activity whereas we utilized activation of a $G_i$-coupled pathway. Nonetheless, these results suggest that the role of the OFC in decision-

making may involve interactions with inhibitory microcircuitry as well as additional projection-types.

Surprisingly, neither IT or PT neuronal activity seems to be necessary for the regulation of these alterations to probabilistic valuation, suggesting that other neuronal subtypes within the OFC are regulating outcome valuation and subsequent choice. However, the trend effects observed on subsequent choice following inactivation indicates that both IT and PT neurons may have some role in this processing. It is also possible that the cell-type specific targeting strategies that we used preserved some cortico-cortical or cortico-subcortical connections. For example, the value information that is encoded by OFC neurons is subsequently sent to anterior cingulate cortex, a region that interfaces with sensory and limbic processing centers and is found to exhibit both value and action-based representations (Rolls, 2019). Investigating this cortico-cortical connection, particularly with respect to IT neurons, may provide greater clarity into their role in choice evaluation and selection. Additionally, as mOFC manipulations have been shown to increase risky preference in the task that we used (Stopper et al., 2014), examining IT and PT neurons within mOFC may reveal further clarify the roles of these two neuronal populations in probabilistic discounting and risk-based decision-making.

How the OFC regulates decision-making and choice processes varies across tasks and task structure. For example, the OFC has a differential role (i.e. increases or decreases to risk sensitivity) in regulating risk assessment under ambiguous conditions versus established probabilities (Floresco et al., 2008). In addition, recent work recording OFC activity during choice tasks suggests that lOFC is critical for initial learning of economic choice and updating subjective preference, but is not required when these values are established (Gardner et al., 2017, 2019, 2020). Although we did not observe a role for IT and PT neurons in probability-discounting, the task that we used requires extensive training to establish the probabilities presented in each block (St. Onge & Floresco, 2009; Stopper et al, 2014). Thus, it is possible that IT and PT neurons are important in regulating choice during the initial acquisition of discounting baselines, rather than once these risk-based decision-making processes have been established.

OFC manipulations impair value updating for guiding subsequent choices (Groman et al., 2019), and impair reversal learning in multiple paradigms (Chudasama & Robbins, 2003; Izquierdo et al., 2004). Previous work has found that ablation of all OFC projections to the nucleus accumbens (NAc) impairs reversal performance (Meyer & Bucci, 2016). Unexpectedly, we found that IT inhibition, which disrupts a subset of OFC projections to the NAc, resulted in an increase in the number of reversals and reduced preservative responding following the initiation of a new reversal

block, suggesting improved response selection and adaptability to changing contingencies. Although these data are consistent with OFC activity underlying response selection and execution (Shoenbaum & Gallagher, 1999), they are less so with frameworks attributing OFC to encoding the promotion of associative flexibility for projection targets (Marquardt et al., 2019; Schoenbaum et al., 2007) as they indicate an instability in response selection, but no impairments in value updating.

Alterations in OFC activity regulate the selection of goal-directed versus habit-based actions, with inactivation shifting responding towards habit-based behaviors (Gremel & Costa, 2013, Zimmermann et al., 2017). Furthermore, reversal learning performance is differentially regulated by specific striatal subregions (Haluk & Floresco, 2009; Sala-Bayo et al., 2020). Our cell-type specific manipulations targeted OFC to NAc projections, as the NAc is involved in risk-based assessments and valuation (Knutson et al., 2005). Consistent with our cell-specific results, D1 receptor antagonism reduces preservative responding and improves reversal learning whereas D2 receptor antagonism impairs reversal performance (Sala-Bayo et al., 2020). Given that IT neurons are thought to preferentially project to D1 MSNs (Shepherd, 2013), selectively inhibiting OFC IT neurons is likely to reduce D1 NAc activity, resulting in greater behavioral flexibility and goal-directed behavior. In contrast, PT neurons are thought to preferentially project to D2 MSNs, which are hypothesized to encode the value of a selected action based on outcome (Morita & Kawaguchi, 2019), consistent with the outcome-dependent effects we found with PT inhibition. The Opponency and Temporal Difference Model has hypothesized that IT neurons encode an action value, while dMSNs encode action utility and iMSNs encode benefit and cost. PT neurons are thought to integrate action and utility inputs and represent the selected action, likely through sustained activity to iMSNs (Morita & Kawaguchi, 2019). In addition, this model suggests that different subsets of iMSNS represent value based on outcome of a selected action and encode the cost of a proposed action, contributing to reward prediction error. Our results with PT inhibition are in alignment with this model, and suggest that the impairment we observed on outcome-based performance was due to an alteration in outcome value.

In conclusion, our findings build on previous research establishing an important role of OFC activity in complex decision-making. In addition, this work is the first to assess the role of specific populations of glutamatergic projection neurons (IT and PT) in risk-based decision-making and behavioral flexibility. Given the complexity of cortico-cortico and cortico-striatal networks, it will be critical to continue to tease apart how subsets of cortical and striatal neurons interface with one

another to regulate decision-making processes, and how these different networks become altered in neuropsychiatric disorders.

## Figures

**Table 1: Ventral and lateral OFC fos activity is higher across probability discounting.** Bonferroni post-hoc corrections for main effect of regional fos. AI – Anterior Insular Cortex, ACC – Anterior Cingulate Cortex, IL – Infralimbic Cortex, lOFC – lateral Orbitofrontal Cortex, mOFC – medial Orbitofrontal Cortex, vOFC – ventral Orbitofrontal Cortex, PL – Prelimbic Cortex. Data are presented as Mean ± Standard Error of the mean (SEM). *p<0.05,** p<0.01,**** p<0.0001 with respect to differences in counts between regions.

| Bonferroni's multiple comparisons test | Mean Diff. | 95.00% CI of diff. | Summary | Adjusted P Value |
|---|---|---|---|---|
| AI vs. ACC | -12.25 | -19.74 to -4.763 | **** | <0.0001 |
| AI vs. IL | -7.633 | -15.12 to -0.1463 | * | 0.0413 |
| AI vs. lOFC | -17.63 | -25.12 to -10.15 | **** | <0.0001 |
| AI vs. mOFC | -3.917 | -11.40 to 3.570 | ns | >0.9999 |
| AI vs. PL | -7.300 | -14.79 to 0.1871 | ns | 0.0635 |
| AI vs. vOFC | -20.90 | -28.39 to -13.41 | **** | <0.0001 |
| ACC vs. IL | 4.617 | -2.870 to 12.10 | ns | >0.9999 |
| ACC vs. lOFC | -5.383 | -12.87 to 2.104 | ns | 0.5749 |
| ACC vs. mOFC | 8.333 | 0.8463 to 15.82 | * | 0.0161 |
| ACC vs. PL | 4.950 | -2.537 to 12.44 | ns | 0.8854 |
| ACC vs. vOFC | -8.650 | -16.14 to -1.163 | * | 0.0103 |
| IL vs. lOFC | -10.00 | -17.49 to -2.513 | ** | 0.0014 |
| IL vs. mOFC | 3.717 | -3.770 to 11.20 | ns | >0.9999 |
| IL vs. PL | 0.3333 | -7.154 to 7.820 | ns | >0.9999 |
| IL vs. vOFC | -13.27 | -20.75 to -5.780 | **** | <0.0001 |
| lOFC vs. mOFC | 13.72 | 6.230 to 21.20 | **** | <0.0001 |
| lOFC vs. PL | 10.33 | 2.846 to 17.82 | *** | 0.0008 |
| lOFC vs. vOFC | -3.267 | -10.75 to 4.220 | ns | >0.9999 |
| mOFC vs. PL | -3.383 | -10.87 to 4.104 | ns | >0.9999 |
| mOFC vs. vOFC | -16.98 | -24.47 to -9.496 | **** | <0.0001 |
| PL vs. vOFC | -13.60 | -21.09 to -6.113 | **** | <0.0001 |

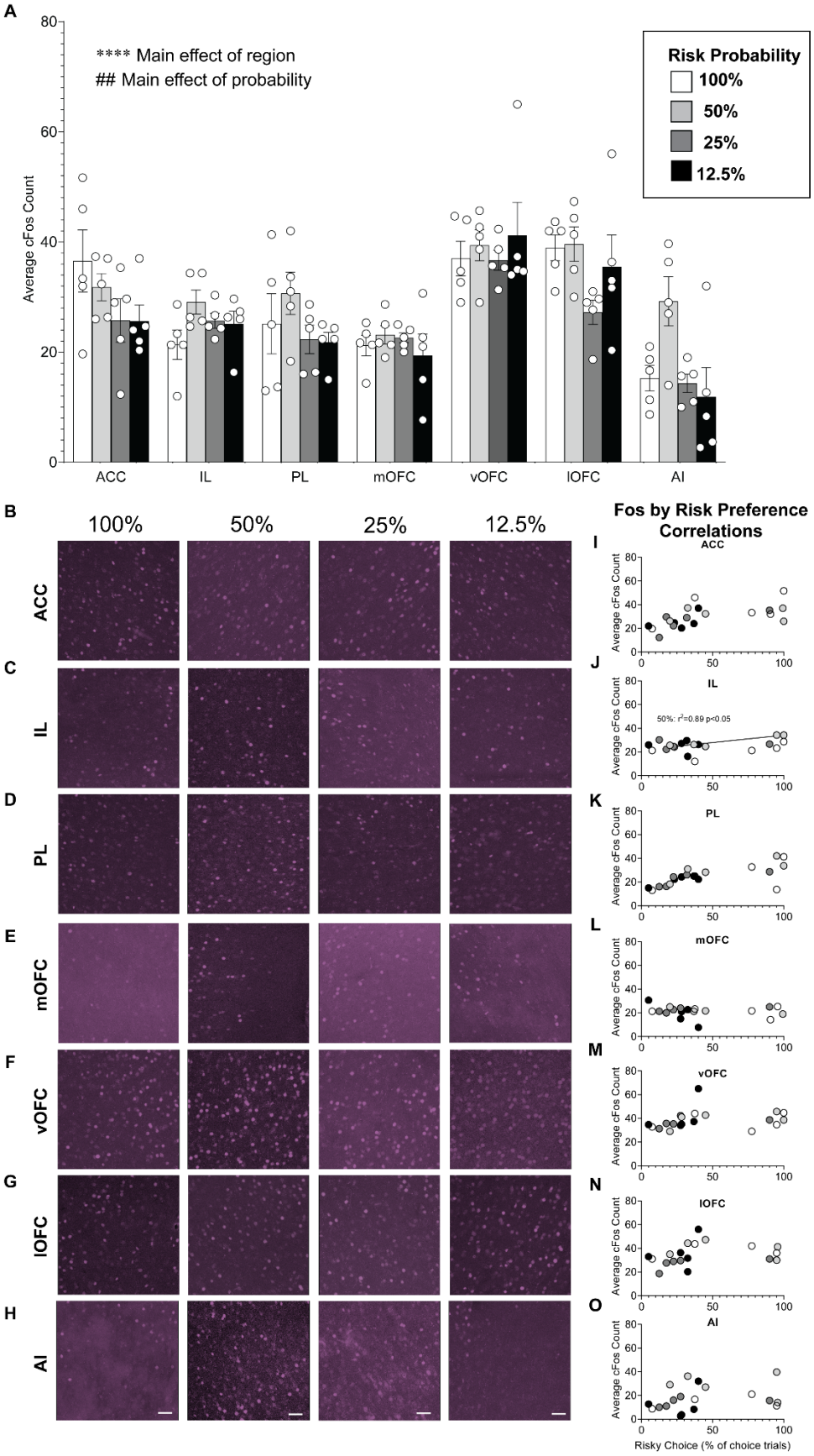

A
**** Main effect of region
## Main effect of probability
Risk Probability
100%
50%
25%
12.5%
Average cFos Count
ACC
IL
PL
mOFC
vOFC
lOFC
AI
B
100%
50%
25%
12.5%
C
D
E
F
G
H
Fos by Risk Preference Correlations
I
J
K
L
M
N
O
50%: r²=0.89 p<0.05
Risky Choice (% of choice trials)

**Figure 1: cFos activation following probabilistic rewards is highest in ventrolateral orbital cortex A**. Average cFos counts from medial and orbital cortical areas by probability of obtaining the larger ("riskier") reward (100, 50, 25, and 12.5%,). **B-H**. Representative regional cFos images at each probability of the larger reward. **I-O**. Correlation between average cFos in each region and the percentage of choice trials where the riskier reward was selected. Trend lines are displayed where a significant $r^2$ was observed, which was found for IL at 50% probability. Data is displayed as mean ± SEM.**** - main effect of region, p<0.001; ##- main effect of probability of riskier reward, p<0.01. N=5 for each probability. Scale bar = 50 μM. Legend: ACC – Anterior Cingulate Cortex, IL – Infralimbic Cortex, PL – Prelimbic cortex, mOFC – medial Oribtofrontal Cortex, vOFC – ventral Orbitofrontal Cortex, lOFC – lateral Orbitofrontal Cortex, AI – Insula.

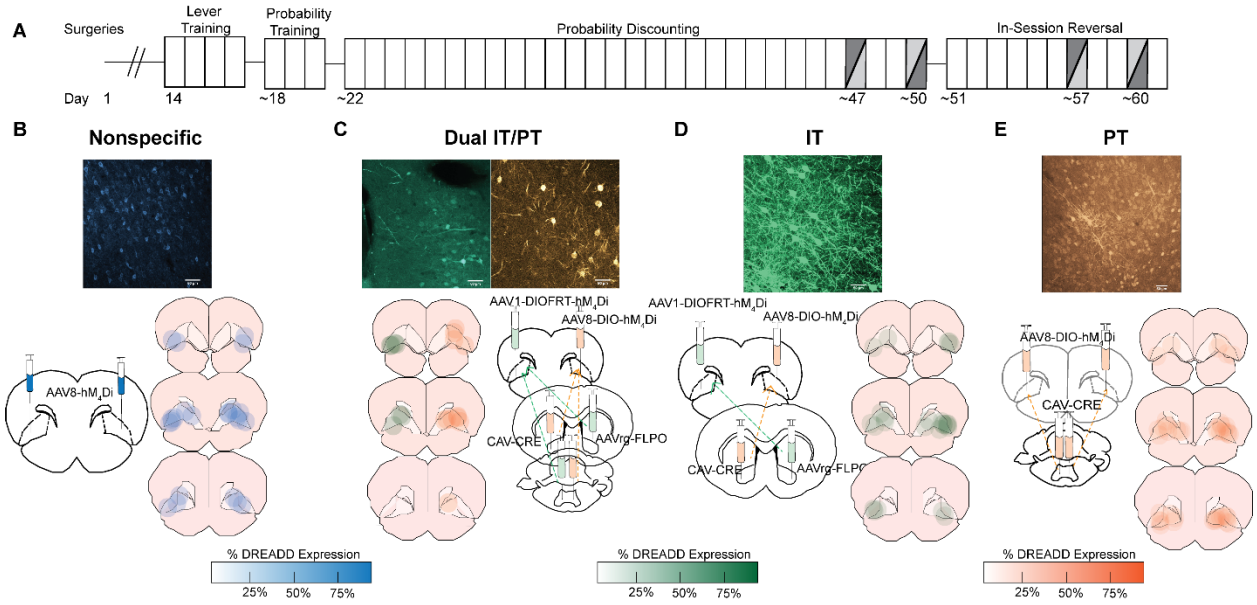



**Figure 2: Viral strategy for chemogenetic inhibition of OFC cortical neurons. A.** Experiment timeline with test days (CNO/VEH) highlighted in gray. **B.** To target all OFC neurons (Nonspecific), a constituitive DREADD (AAV8-hSyn-$Hm_4D_i$) was injected bilaterally into ventral and lateral OFC. **C.** To target both IT and PT OFC neurons (Dual IT/PT), a retrograde FLP ($AAV_{rg}$-EF1a-FLP) was injected into the left hemisphere pontine nucleus and the right hemisphere nucleus accumbens (NAc), while CAV2-CRE was injected into the right hemisphere pontine nucleus and left hemisphere NAc. Inhibitory DREADDS dependent on FLP (Left hemisphere, AAV1-DIOFRT-$Hm_4Di$-EYFP) or CRE (Right Hemisphere, AAV8-hSyn-DIO-$Hm_4Di$-mCherry) were injected into the OFC. **D.** To selectively target IT OFC neurons, CAV2-CRE and a retrograde FLP were unilaterally injected into opposite hemispheres of the NAc whereas the corresponding CRE-or FLP-dependent DREADDs were injected unilaterally into the contralateral OFC. **E.** To selectively

target PT neurons, CAV2-CRE was bilaterally injected into the pontine nucleus, while a CRE-dependent DREADD was injected bilaterally into the OFC. Representative maps of viral spread (% by number of subjects) are included for each targeting strategy.  Scale bars= 50 μM.

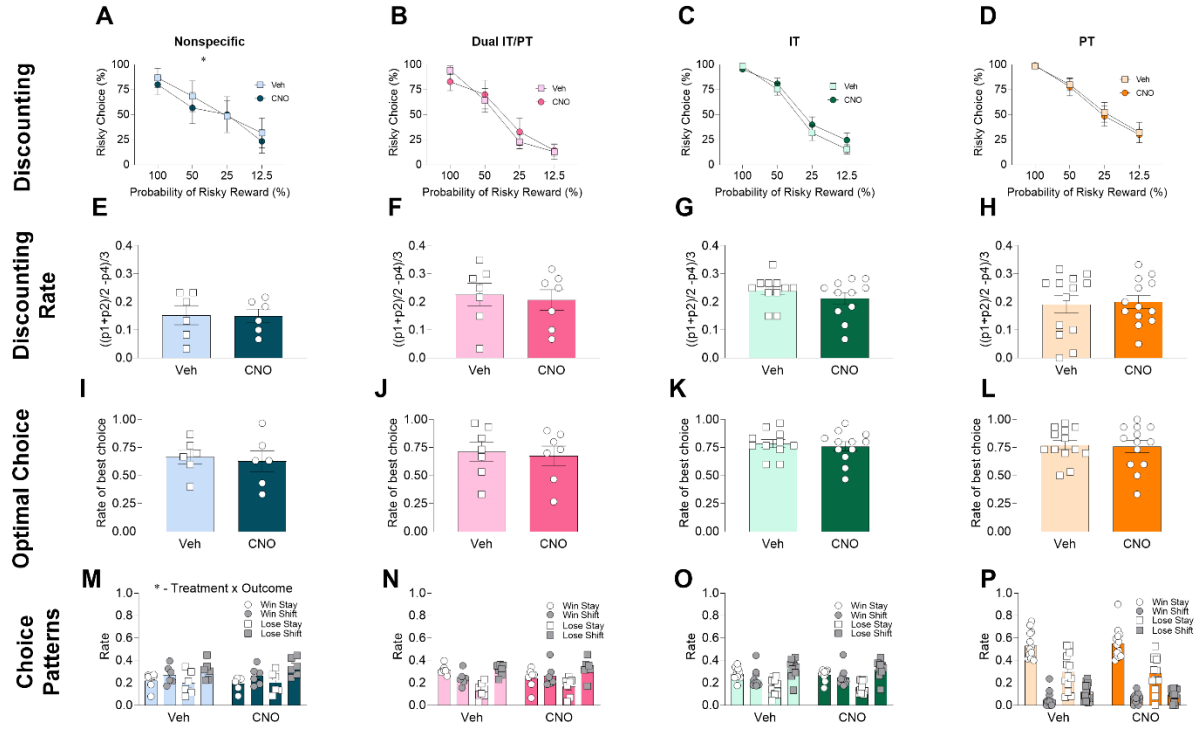



**Figure 3: Nonspecific chemogenetic inhibition of OFC alters risk preference.** Discounting curves for percent choice of the risky reward option across blocks in a probability discounting task corresponding to 100%, 50%, 25%, and 12.5% probability of receiving the larger “risky” reward. General OFC inhibition (**A**, blue) reduced overall choice for the risky reward (two-way ANOVA Treatment x Block main effect of Treatment) while Dual IT/PT inhibition (**B,** pink), IT inhibition (**C**, green), and PT inhibition (**D**, orange) did not significantly alter the discounting curve. Neither the discounting rate (**E-H**, rate per block of risky choice) nor the percentage of optimal choices selected during discounting (**I-L**) were significantly altered with any of the chemogenetic manipulations. When examining patterns of choice based on the outcome (win or lose) and subsequent pattern (stay on option or shift to alternative), nonspecific OFC inhibition (**M**) alters choice based on outcome (three-way ANOVA Treatment x Outcome), while Dual IT/PT inhibition (**N**) alters rates based on both Outcome and whether a choice was on the same lever or shifted to the alternative option from the previous action (three-way ANOVA Treatment x Outcome x Choice). However, neither selective inhibition of IT (**O**) nor PT (**P**) neurons altered choice patterns

during probabilistic decision-making. * - p < 0.05; Data is presented as mean ± SEM. Nonspecific, N=6; Dual IT/PT, N=7; IT, N=11; PT, N=13.

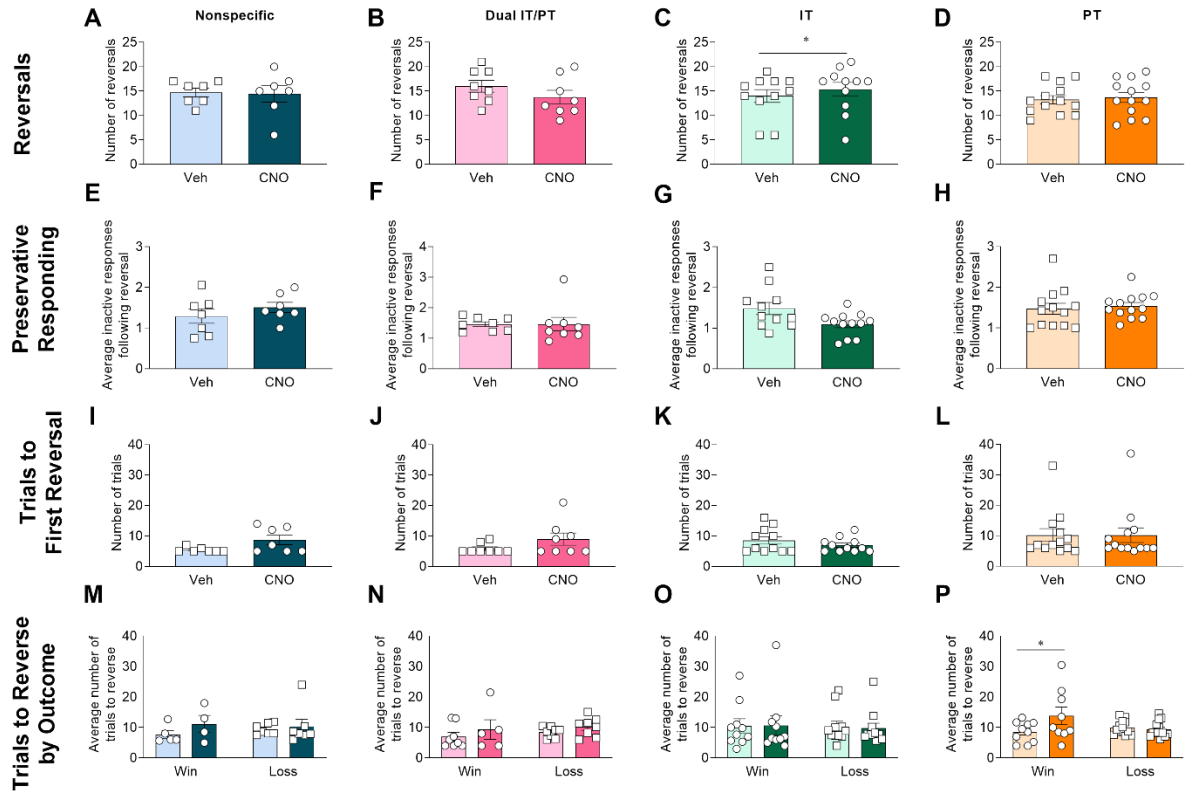
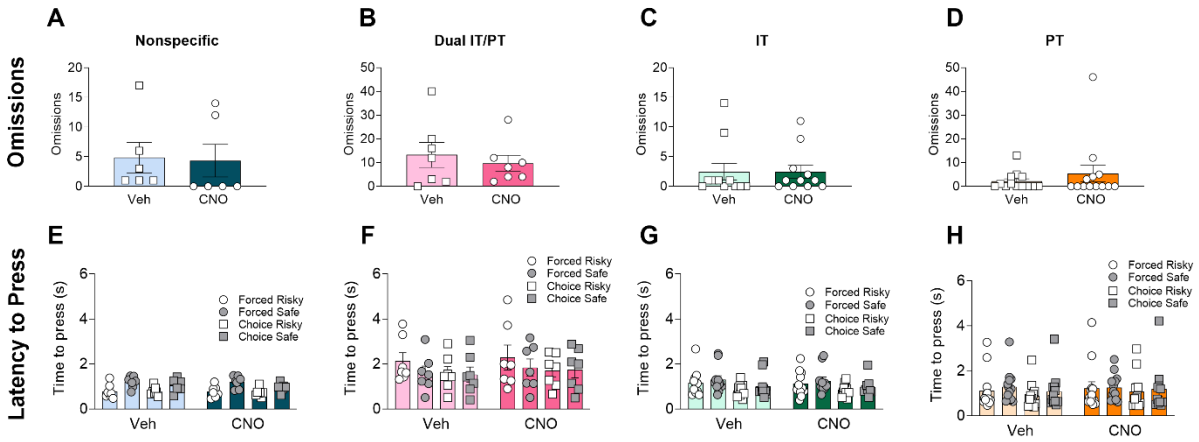


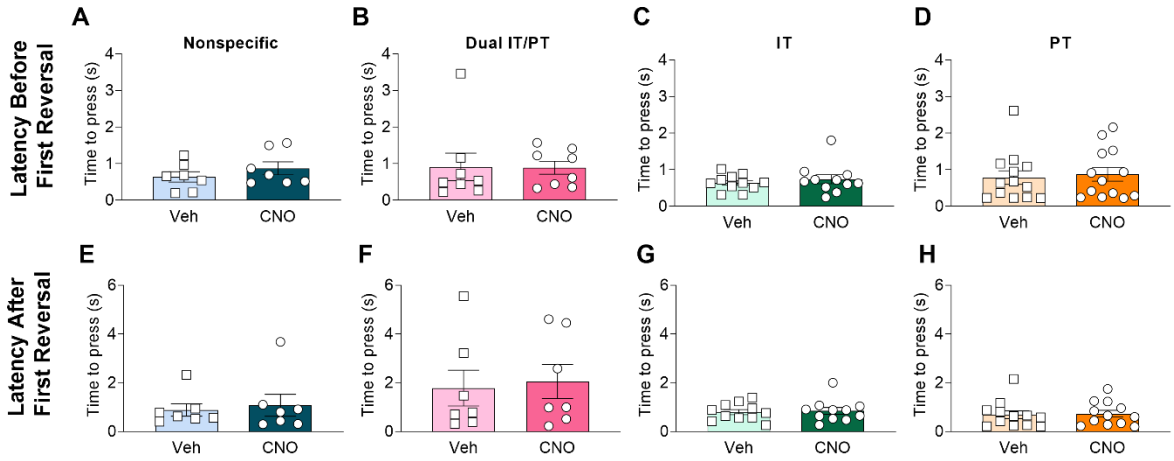

**Figure 4: OFC IT neuronal inhibition increases adaptive responding in a within-session reversal task.** Nonspecific OFC inhibition (**A**), Dual IT/PT inhibition (**B)**, and PT inhibition (**D**) did not significantly change the number of reversals occurring within a session whereas IT inhibition (**C**) significantly increases the total number of reversals (2-sided paired t-test). When examining the number of responses on the previously rewarded lever prior to switching to the new active lever (a measure of preservative responding), none of the manipulations (**E-H**) altered the average number of preservative responses, though there is a trend towards significantly reduced preservative responding with IT inhibition (**G**). The number of trials required to initiate the first reversal was not significantly altered with any of the neuronal manipulations (**I-L**) although it trended towards significance with nonselective OFC inhibition (**I**). Following the first reversal, the subsequent trials to reversal to reach criterion for subsequent reversals were not altered with nonselective (**M**), Dual IT/PT (**N**), or IT (**O**) inhibition. However, if the start of a reversal resulted in a reward (a shift to the new active lever), PT inhibition (**P**) significantly increased the number of trials to reach the subsequent reversal compared to control (three-way ANOVA Treatment x

Outcome). Data is presented as mean ± SEM. * - $p < 0.05$; Nonspecific, N=7; Dual IT/PT, N=8; IT, N=11; PT, N=13.

## Supplementary Figures




**Supplementary Figure 1. OFC neuronal inhibition has no effect on omissions and responding.** Nonspecific (**A**), Dual IT/PT (**B**), IT (**C**), and PT (**D**) inhibition had no effect on responding during discounting sessions. These manipulations (**E-H)** also had no effect on the average time to respond during forced trials or on selection of the risky or safe option. Data is presented as mean ± SEM. Nonspecific, N=6; Dual IT/PT, N=7; IT, N=11; PT, N=13.



**Supplementary Figure 2. OFC neuronal inhibition has no effect on response times.** Nonspecific (**A**), Dual IT/PT (**B**), IT (**C**), and PT (**D**) inhibition had no effect on average time to respond following extension of levers before completing criterion for the first reversal. These manipulations (**E-H)** also had no effect on the average time to respond following completion of the first reversal. Data is presented as mean ± SEM. Nonspecific, N=7; Dual IT/PT, N=8; IT, N=11; PT, N=13.

## Chapter 6: Conclusions and Future Directions

This thesis investigates the significance of drug use patterns, history, and cortical subtype specificity in the manifestation of the rewarding and aversive facets of cocaine use, as well as in alterations in decision-making and behavioral flexibility for nondrug rewards. These studies examined these things from a behavioral standpoint, focusing on the impacts of either types of drug administration, or cell-type specific manipulations on changes to drug-taking and -seeking behavior. However, outstanding questions largely center around the impacts of cocaine on a mechanistic level, and how these changes correspond with the behavioral phenotypes observed with cocaine use.

Chapter 2 investigated behavioral patterns of cocaine and heroin consumption under the lens of polysubstance vs. single substance use history, taking into account combinations of substances self-administered, as well as their impact on effort-based maintenance of no-cost consumption and cue-based reinstatement of drug-seeking. Stark differences were seen in the dose-response demand curve for cocaine versus heroin, as well as in the persistence of responding to heroin-associated cues that was not found with cocaine cues. However, a sequential polydrug history of heroin and cocaine did not produce distinct behavioral responses to drug-seeking and intake, unlike what is frequently reported in patient populations with polydrug histories (Bujarski et al., 2014; Massaro et al., 2017; Wang et al., 2017; Williamson et al., 2006). These findings suggest that: 1. Drugs are differentially impactful on behavioral metrics associated with consumption, seeking, and relapse, and how these unique alterations to different facets of substance misuse should be investigated in other preclinical and clinical models, and 2. Modifications to our initial model could better translate to observations found in clinical populations. Indeed, preclinical and clinical studies have established context-specific preferences in drug-taking which impact the motivation and sensitivity to drug consumption and cues (Caprioli et al., 2009; De Luca et al., 2018; De Pirro et al., 2018). Specifically, cocaine consumption is higher in novel contexts compared to home cages in rats, while, conversely, heroin is preferred in home cages (Caprioli et al., 2009). Additionally, preferred time-of-day for use, while difficult to elucidate from clinical literature, should be incorporated into future models with consideration to environments where drug is preferably sought and consumed. Furthermore, polysubstance criterion may be met by using additionally substances in separate time periods (i.e. different days, or ceasing use of one substance and utilizing another substance during abstinence from the primary substance), by having substances in the same day, but at separate times, or by simultaneous administration of drug combinations. Here, I used

a sequential design where drug use was separated into alternating days. Future work, however, could explore modeling polysubstance use with these additional patterns of drug administration and investigate differences in motivation, seeking, and relapse based on context and timing. Studies have established that patterns of intake within a session as well as the length of self-administration sessions can produce significant differences in escalation of intake, motivation to maintain drug consumption, compulsive seeking despite adverse consequences, and relapse to drug seeking (Ahmed & Koob, 1998; Deroche-Gamonet et al., 2004; Kawa et al., 2016; Zimmer et al., 2012). Investigating how, both between and within these models, polysubstance history uniquely alters different behaviors related to addiction will be necessary for improving the translatability of preclinical models.

While understanding how polysubstance history impacts drug use, abstinence, and relapse, it is also necessary to determine the unique alterations in circuit-level and molecular changes produced with polysubstance use from simultaneous and sequential combinations that are undetected from behavioral assays. Indeed, there is evidence to suggest more deleterious effects with polysubstance combinations than a single substance history alone (Cunha-Oliveira et al. 2010; Gilmore et al., 2018; Kariisa et al., 2019; Pennings et al., 2002). However, unique circuit-level effects, particularly within the cortico-basal ganglia-thalamic circuit (CGBT), are largely unknown. Recent work implicates changes to activation of glutamatergic prelimbic (PL) cortical neurons following alternating, sequential heroin and cocaine use (Rubio et al., 2019) and altered glutamate homeostasis in the nucleus accumbens (NAc) with cocaine and alcohol use (Stennett & Knackstedt, 2020), implicating unique cortico-striatal in polysubstance use that warrant further investigation.

In Chapter 3, we examined subtype-specific cortical activity in a well-established behavioral phenomenon of psychomotor sensitization arising from cocaine treatment. We found that initial expression of cocaine psychomotor activity was augmented with chemogenetic IT inhibition, but that further development of cocaine sensitization was prevent with repeated pairings of IT inhibition with cocaine treatments. As chemogenetic PT inhibition in this paradigm was previously found to dampen the acute effects of cocaine, but enhance the development of cocaine sensitization, this work implicates opponent processing of drug-induced psychomotor activity of cortical, glutamatergic subtypes. While studies have shown differential activity in laterality of forelimb movements following IT or PT innervation (Soma et al., 2017), previous work has not been performed in the context of drug effects. Therefore, the opposing results of IT inhibition on sensitization compared to PT inhibition are significant for establishing a rationale for looking at cortical population microcircuitry and cell-type projections when investigating the

impacts of drug use on the CBGT. While supporting a differential role for IT and PT neurons in mediating psychostimulant effects, imaging IT and PT activity following acute and chronic cocaine administration would clarify activity-dependent changes in cocaine administration. Based on our sensitization results, IT and PT neurons would be expected to demonstrate differential patterns from one another with acute cocaine exposure, which would be further altered following repeated cocaine treatments. Future work will have to determine the mechanisms underlying IT and PT activity through distinct phases of cocaine sensitization, utilizing pharmacological interventions and targeted imaging.

In Chapter 4, I investigated whether modulation of IT and PT neurons is sufficient to induce reward or aversion in the absence of drug treatments. Using a CPP paradigm, I used optogenetic stimulation to activate PT neurons in the ACC during paired saline injections and examined changes to preference for the PT-stimulation chamber versus saline alone. PT stimulation did not alter time spent in the PT-stimulation-paired chamber, suggesting that PT activation alone is not sufficient to produce a place preference or aversion. The role of IT neurons was examined by using DREADDs selectively expressed on IT neurons in the ACC to inhibit IT activity following sucrose administration and determining if the amount of sucrose consumption and time of sucrose drinking was significantly enhanced or reduced in subsequent sessions. As with PT neurons, IT inhibition alone did not produce aversion to sucrose solutions. As previous work found reduced cocaine-induced sucrose aversion with IT inhibition and place aversion with cocaine and PT stimulation treatments (Garcia et al., in press.), this work dispels the explanation of inherent changes to reward and aversion processing by manipulating these cortical subtypes, suggesting that they are involved in the mediation of affective processing involving a cocaine reward.

While their roles in cocaine reward and aversion have been demonstrated, their role in volitional administration and the translation of their activity to affective processing involving motivated behaviors has yet to be established. Here, I began investigation of cortical subtype modulation of motivated cocaine consumption by nonspecifically targeting PT neurons to express an inhibitory DREADD for PT neuronal inactivation during behavioral economic tasks. These paradigms examined motivation to maintain cocaine consumption as effort to achieve desired concentrations is increased, as well as continued consumption as drug consumption is paired with greater aversive consequences (i.e. longer footshocks). While PT inhibition did not impact parameters measuring sensitivity to changes in price of administration on either task, there was surprisingly stark drop-offs in overall responding, suggesting alterations to effort-based drive for cocaine administration. However, consistent with previous work (Garcia et al., in press.), PT

modulation had no effect on aversive-based cocaine administration, further supporting a role of PT activity on processing the rewarding, rather than aversive, aspects of cocaine use. However, this data did not examine the role of PT activity in context-induced seeking or in withdrawal, nor does it establish a role for IT activity in volitional cocaine administration. Future work will have to dissect regional-specific IT and PT activity in cocaine self-administration. While previous studies have investigated IT and PT activity in motor control and in pain models (Soma et al., 2017; Meda et al., 2019), this work is the first to examine cortical subtypes in behavioral-economic cocaine administration models, as well as the first to investigate PT neuronal activity on reward seeking in punished contexts.

Chapter 5 examined the role of IT and PT activity during probabilistic decision-making and in maintaining behavioral flexibility in reversal learning for sucrose rewards. Fos activation during probabilistic administration of larger rewards demonstrated higher activity in orbital cortices, particularly in ventral and lateral areas, suggesting engagement of these regions during evaluation and selection of options where risk is a factor. Lateral orbitofrontal cortex (OFC) has been implicated in behavioral flexibility associated with outcome revaluation, affect regulation, response inhibition, and re-evaluation of stimuli that become aversive (Bryden & Roesch, 2015; Fettes et al, 2017); these behaviors may be mediated through projections to the NAc, a region consistently implicated in risky behaviors (Ghods-Sharifi & Floresco, 2010; Wrase et al., 2007). Similarly, cocaine-mediated alterations in lateral OFC activity produces deficits in signaling cue-associated aversive outcomes, which correlate with reduced flexibility in reversal learning (Stalnaker, et al., 2006), and impairs learned response inhibition when OFC hypoactivation is paired with NAc hyperactivation (Meyer & Bucci, 2016). The contribution of OFC to resolving conflicts and mediating probabilistic decision-making behaviors has been demonstrated in humans (Li et al., 2016; Rogers et al., 1999; Tobler et al., 2007), primates (Hosokawa et al., 2007; O'Neill & Schultz, 2015; Rudebeck et al., 2017), and in rodents (Mobini et al., 2002; St. Onge & Floresco, 2010; Stopper et al., 2014), but results are conflicting and dependent on manipulation strategy and task design. This work expands upon the body of work examining components of CBGT circuitry in these behaviors, particularly in preclinical models. These results suggest that OFC activity is not necessary for probabilistic decision-making. However, medial OFC is implicated in learning through probabilistic feedback and subjective value encoding (Dalton et al., 2016; Fettes et al., 2017) while lateral OFC activity is associated with the probability of improving performance after omission of cued gain and to delivery of cued loss (Wrase et. al, 2007). Discrepancies between studies may reflect cortical subtype differences within medial OFC, rather than the regions targeted in this thesis, or more temporally defined

activity that is necessary for value computations. Therefore, future work could investigate cell type roles in medial OFC in this task, as well as focus more on temporal manipulation (i.e. optogenetic stimulation or inhibition) following omissions to determine if this impacts subsequent choice.

To determine if cell-type specific activity within OFC contributed to decision-making during these types of valuations, IT and PT neurons were chemogenetically inhibited. While no differences were found in manipulating these populations in the OFC during probability discounting, general inhibition of OFC did reduce overall responding for the risky reward, suggesting that populations within OFC are involved in normal valuation of risk and reward, but may be engaging either interneurons within OFC, different projection populations (suggest as CT neurons), or may involve cortico-cortical connectivity of these subtypes, rather than their projections. How OFC neurons process options and guide subsequent choices will require further studies monitoring OFC neurons with targeted cell-type manipulations and possibly greater temporal precision in manipulating cell-type activity. One proposed model of economic decision-making is circuit inhibition of populations encoding unchosen options (Ballesta & Padoa-Schioppa, 2019), which would suggest greater contributions by interneuron inhibition in local microcircuitry.

Our investigation into IT and PT neurons focused on layer V cortico-striatal and cortico-fugal connections. However, it's known that cortico-cortical activity is necessary for integration of sensory and limbic information for mediating actions (Ongür & Price, 2000; Pelekanos et al., 2020) and that cortical neurons project to the corresponding contralateral cortical region (Hoover & Vertes, 2007, 2011). The callosal crossing of IT neurons in layers II/III lend themselves to this cortico-cortical processing and are known to exhibit biased IT to IT and IT to PT projections (Brown & Hestrin, 2009; Kiritani et al., 2012). Investigating these IT projections and how they regulate contralateral IT and PT activity would clarify if cortico-cortical connectivity is significant in these behaviors. Rat connectivity studies demonstrate VOLO projections to contralateral mPFC, including the ACC (Coizet et al., 2017), and connections between the ACC and OFC are implicated in reward valuation and subsequent decision-making (Rolls, 2019). Importantly, ACC was the only region where cfos levels were comparable to those of lOFC and vOFC, supporting the argument for examination of this cortico-cortical circuit in mediating motivated behaviors.

Additionally, associating the differential impact of IT and PT neuromodulation with behavioral outputs has not been directly investigated. Studies have established unique receptor expression and modulation by catecholamines and serotonin (Elliot et al., 2018; Guan et al., 2015; Leyrer-Jackson & Thomas, 2017,2018; Seong & Carter, 2012). For instance, IT and PT neurons have

been shown in both rats and mice to differentially express 5HT1A and 5HT2A receptors (Elliot et al., 2018) with coexpression of 1A and 2A receptors permitting remodeling of integrative cell properties in IT neurons, while sole expression of 5-$HT_{2A}$ receptors elicits depolarization in PT neurons (Elliot et al., 2018). Importantly, dysregulation of serotonergic activity is associated with impaired associative learning (Izquierdo et al., 2012), alters risk-aversion via 5-HT2A receptor activity (Macoveanu et al., 2013), and 5-HT2A receptor activation in OFC neurons in rats is reduced with cocaine self-administration, suggesting deficits in glutamate release from 5-HT2A activity (Wright et al., 2017). Additionally, there is some evidence for subtype-specific differentiation of D1 and D2 receptor expression (Gaspar et al., 1995), with studies demonstrating D1 and D2 receptor-specific modulation in IT and PT neurons, respectively (Gee et al., 2012; Leyrer-Jackson & Thomas, 2017; Seong & Carter, 2012; but see Clarkson et al., 2017; Wang et al., 2004). While effects of pharmacological inhibition of dopamine receptor activity in OFC on impulsivity tasks have been mixed, examination of the roles D2 receptors in a risky decision-making found increased risk preference and response preservation with probabilistic punished outcomes (St. Onge et. al, 2011) with increased OFC D2 receptor expression in risk-taking subjects (Simon et al., 2011). However, dopamine depletion in cortex and NAc does not impact probabilistic choice (Mai & Hauber, 2012). While the significance of dopaminergic transmission is disputable, it's role in progressive escalation and propensity of substance misuse is well-established (for review, see Keiflin& Janak, 2015; Robinson & Berridge, 1993; and Volkow et al., 2017) and could significantly impact self-administration and motivated seeking in a cortical subtype-specific manner. Indeed, human imaging studies have established cortical hypoactivity as a marker of cocaine misuse pathology (Volkow et al., 1992a; Volkow et al., 1993) and studies have demonstrated that dopaminergic innervation of presynaptic cortical terminals blocks calcium influx, reducing cortical activity (Bisagno et al., 2016). Therefore, examining the impact of neuromodulators on cortical cell types would clarify how these cells modulated or are modulated by postsynaptic targets and cortical efferents, as well as how their activity is altered with progressive substance use.

In conclusion, this thesis explores both the significance of drug history and cortical subtype contributions in drug associated and motivated behaviors. Specifically, this work provides a preliminary model for investigating polysubstance use and is novel in its investigation of cortical projection populations in mediating volitional maintenance of cocaine consumption in effort and punished-based conditions. Furthermore, the work in this thesis is the first to explore the role of these populations in complex decision-making and behavioral flexibility. Future work examining polysubstance use with different contexts of administration history and monitoring the activity of

cortical cell-type activity in these behaviors will further our conceptual framework for preclinical modeling of substance use.

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Chapter 2

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**Elizabeth Crummy**
**eacrummy@gmail.com (407) 406-3751 1521 NW 54th St. Seattle, WA 98107**

## EDUCATION

University of Washington, Seattle, WA **9/2016-Present**
Doctoral Candidate, Graduate Program in Neuroscience

The University of South Carolina, Columbia, SC (*Honors College*) **8/2012-5/2016**
B.S., Biomedical Engineering, Neuroscience Minor (*Magna Cum Laude*)

## EXPERIENCE

Dissertation student, Laboratory of Susan Ferguson, Ph.D., Seattle Children's Research Institute **4/2017-Present**

- Projects: Investigating motivation and craving in an animal model of polydrug abuse; contributions of cortical subtypes to risky decision-making
- **Techniques:** operant behavioral tasks (self-administration, conditioned place preference, conditioned taste aversion, sensitization), statistical analysis (GraphPad Prism, R, Python, Excel), rat jugular catheterization, stereotactic viral injection, and fiber implantation surgeries, immunohistochemistry, confocal microscopy

Predoctoral rotation student, Laboratory of Kurt Weaver, University of Washington **1/2017- 3/2017**

- Project: Analyzing coherence in a Go/No-Go Task Paradigm
- **Techniques**: Matlab coding and signal processing

Predoctoral rotation student, Laboratory of Thomas Reh, University of Washington **9/2016-12/2016**

- Project: Generation of stem cell-derived retina
- **Techniques:** stem cell culture, confocal microscopy, retina dissection, cryosectioning, immunohistochemistry, FIJI software

Summer Intern, Vanderbilt University Conte-Sure Program **6/2015-8/2015**

- Project: Proliferation of neural precursors in the presence of apoptotic targets
- **Techniques:** fluorescence microscopy, mouse pup brain dissection, cell culture (neural progenitors, HeLa), PCR, western blot

Undergraduate Research Assistant, University of South Carolina **9/2013-5/2016**

- Research in cell culture models of amyloid-induced neurotoxicity
- **Techniques**: cell culturing (HBMEs, THP1-monocytes, SHSY5Y), immunocytochemistry

## AWARDS

General Conference Student Travel Award **2019**
Seattle Children's Science Communication Scholarship **2018**
National Science Foundation Graduate Research Fellowship **2016-2021**
Peer Leader Spotlight - Office Undergraduate Research, University of South Carolina **2015**
Magellan Scholar - "Assessment of Therapeutic Potential of Polyphenol Compounds in Alleviating Neurotoxicity due to Amyloid Beta in HBMEC Cells." **2014**
Science Undergraduate Research Fellowship (SURF) **2014-2015**
Passport Travel Grant – Comparative Healthcare course, Amsterdam, Netherlands **2014**
McNair Scholar –Out-of-state scholarship at the University of South Carolina. **2012**

**MANUSCRIPTS**

Garcia AF, **Crummy EA**, Webb I, Nooney MN, Ferguson SM (submitted). "Distinct populations of cortical pyramidal neurons mediate drug reward and aversion"

**Crummy EA,** O'Neal TJ, Baskin BM, Ferguson SM (2020). "One is Not Enough: Understanding and Modeling Polysubstance Use" *Frontiers Neuroscience*. DOI: 10.3389/fnins.2020.00569 PMCID: PMC7309369

**Crummy EA**, Donckels EA, Baskin BM, Bentzley BS, Ferguson SM (2019). "The impact of cocaine and heroin drug history on motivation and cue sensitivity in a rat model of polydrug abuse" *Psychopharmacology*. DOI: 10.1007/s00213-019-05349-2 PMCID: 31463541

**PRESENTATIONS**

**Posters**

- **E.A Crummy**, A.F Garcia, I. Webb, Z.D Murphy, and S.M. Ferguson. "Contributions of cortical projection subtypes to locomotor and motivated behaviors in contingent and noncontingent cocaine administration" at Winter Conference on Brain Research, 2020, Big Sky, MT.
- **E.A Crummy**, A.F Garcia, I. Webb, Z.D Murphy, and S.M. Ferguson. "Contributions of cortical projection subtypes to locomotor and motivated behaviors in contingent and noncontingent cocaine administration" at the American College of Neuropsychopharmacology Annual Meeting, 2019, Orlando, FL.
- **E.A Crummy**, E.A Donckels, B.M Baskin, B.S Bentzley, and S.M. Ferguson. "The impact of cocaine and heroin co-use on motivation and drug craving in a rat model of polydrug abuse" at the University of Washington Neuroscience Graduate Program Recruitment Week Poster Session, 2018, Seattle, WA.
- **E.A Crummy**, E. Donckels, A. Surowiecki, S.M. Ferguson. "Craving and addiction severity in a rat model of heroin and cocaine sequential polydrug abuse," at Society for Neuroscience, 2018, Washington, D.C.
- **E.A Crummy**, A. Hoshino, P. Nakamura, C. Zang, R. Wong, F. Rieke, and T.A Reh. "Generation of mouse embryonic stem cell-derived retina" at the University of Washington Neuroscience Graduate Program Recruitment Week Poster Session, 2017, Seattle, WA.
- **E.A Crummy**, F. Hickman, and B. Carter. Neural precursor cell proliferation in response to apoptotic targets for phagocytosis," at the Biomedical Engineering Society Annual Meeting, 2015.
- **E.A Crummy**, K.M Pate, N. van der Munnik, and M.A Moss. "Polyphenols Attenuate Aβ-Induced Apoptosis," at the South Carolina IDeA Networks of Biomedical Research Excellence Conference, 2015 Columbia, SC.

**Talks**

- **E.A Crummy**, The role of drug history and cortical circuitry in substance use for Lobo Lab 2020, July; Baltimore, MD (virtual).
- **E.A Crummy**, The role of drug history and cortical circuitry in substance use for Ahmari Lab Meeting 2020, May; Pittsburgh, PA (virtual).
- **E.A Crummy**, Decision-making in rats: how they beat the game better than a PhD student at Seattle Science on Tap 2020, February; Seattle, WA.
- **E.A Crummy**, S.M. Ferguson. Behavior and circuits in understanding DUD: Investigating polydrug use and cortical subtypes" at the Neuroscience Graduate Program Recruitment Week, 2020, February; Seattle, WA.
- **E.A Crummy**, S.M. Ferguson. "Polydrug use: How heroin and cocaine history impacts motivation and drug craving" at Seattle Science Slam, 2019, November; Seattle, WA.
- **E.A Crummy**, E.A Donckels, B.M Baskin, B.S Bentzley, and S.M. Ferguson. "The impact of cocaine and heroin co-use on motivation and drug craving in a rat model of polydrug abuse" at the Neuroscience Graduate Program Retreat, 2019, September; Seattle, WA.
- **E.A Crummy**, A.F. Garcia, S.M. Ferguson. "Stimulation of cortical subtypes on place preference" at TBSI Seattle Symposium, 2019; Seattle, WA
- **E.A Crummy**, E.A Donckels, B.M Baskin, B.S Bentzley, and S.M. Ferguson. "The impact of cocaine and heroin co-use on motivation and drug craving in a rat model of polydrug abuse" at the Winter Conference on Brain Research, 2019, January; Snowmass, CO.
- **E.A Crummy**, S.M. Ferguson. "Investigating the role of cortical subtypes in orbitofrontal cortex in risky decision-making," at Discovery Breakfast for SCRI Center for Integrative Brain Research, 2018, Seattle, WA.
- **EA. Crummy**, F. Hickman, and B. Carter. "Proliferation of neural precursors in the presence of apoptotic targets," at USC Discovery Day, 2016, Columbia, SC ○ 2nd place for Afternoon STEM A Session

- **E.A Crummy**, F. Hickman, and B. Carter. "Proliferation of neural precursors in the presence of apoptotic targets," at the Vanderbilt Conte-Sure/ASPET Program Symposium, 2015, Nashville, TN.
- **E.A Crummy**, K.M Pate, N. van der Munnik, and M.A Moss. "Attenuation of Amyloid-Beta Induced Apoptosis by Polyphenols," at USC Discovery Day, 2015, Columbia, SC.

## OTHER RELEVANT EXPERIENCE

Allen Brain Institute Summer Workshop on the Dynamic Brain, Friday Harbor, WA **8/2019-9/2019**

- Extraction and analysis of morphological features of neurons from electron microscopy dataset
- **Techniques:** Python (seaborne, numpy, pandas, scikitlearn, matplotlib), dimensionality reduction, model selection, feature extraction

Innovation Development Intern, Co-Motion at the University of Washington, Seattle, WA **5/2019-Present**

- Due diligence for UW inventor technology proposals.
- Literature search for relevant publications and patents of proposed innovations for technologies in life sciences fields.
- Writing innovation assessment and analysis summaries for market evaluation of technologies submitted to technology managers.
- Draft marketing materials and generate contact list of companies with potential partnership or investment interest.

Research Mentor, Seattle Children's STEM Internship Program **7/2018, 7/2019**

- Month-long summer internship for high school students
- Taught high school interns immunohistochemistry, tissue processing, and confocal microscopy

NEURO 302 "Introduction to Systems Neurobiology" **9/2017-12/2017**

- Laboratory practicum teaching assistant

## COMMITTEES

Graduate Program in Neuroscience Admission Committee, University of Washington **9/2019-Present**

- Assist with organizing recruitment week activities and scheduling

Graduate Program in Neuroscience Seminar Committee, University of Washington **5/2017-Present**

- Student and Faculty Host Coordinator

Committee Member, Office for Teaching, Education and Research, Seattle Children's Research Institute **5/2017-Present**
Undergraduate Symposium Abstract Reviewer

- Graduate and Postdoctoral Symposium abstract reviewer and presentation selector

Graduate Program Student Senate Travel Grant Award Reviewer **10/2017-05/2018**

## MANUSCRIPT REVIEWS

Nature Neuroscience **2020**
Frontiers in Behavioral Neuroscience **2017, 2019**
Pharmacological Review **2018**
Neuropsychopharmacology **2019**

## OUTREACH

Winter Conference on Brain Research Outreach Lecturer to Aspen High School, Aspen CO **1/2019**

Pacific Science Center Science Communication Fellow, Seattle, WA **9/2018-Present**

- Developed interactive activity on translating research for general audiences at the Pacific Science Center
- Training on public engagement and public perception and bias recognition

Neuroscience Community Outreach Group – University of Washington, Seattle, WA **10/2016-Present**

- Present and lead neuroscience-themed student activities at public schools
- Fundraiser and organizer for Brain Awareness Week Open House at the University of Washington
- Coordinator and exhibitor at the Pacific Science Center Life Sciences Research Weekend

**STUDENT TRAINING**

*Undergraduate students at the University of Washington since 2018*

- Grayson Baden
- Jordyn Richardson
- Marlaena Nooney
- Reilley Durre

*High School Mentees for Seattle Children's Science Education Department STEM High School Internship Program, 2018*

- Lexi Jones
- Su Cho
- Rumana Ali
- Katie Vo

**REFERENCES**

*Susan Ferguson, Ph.D.*
Assistant Professor, Department of Psychiatry & Behavioral Sciences; Principal Investigator, Center for Brain Research, Seattle Children's Research Institute
smfergus@uw.edu | 206.884.3279

*Melissa Moss, Ph.D.*
Interim Department Chair of Chemical Engineering, Director Biomedical Engineering
Professor; Department of Chemical Engineering, College of Engineering and Computing,
University of South Carolina – Columbia
MOSSME@cec.sc.edu | 803.777.5604