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Cocaine Intake And The Gut Microbiota in Adolescent and Adult Male Rats: A Vicious Cycle?

Gregory Suess

COCAINE INTAKE AND THE GUT MICROBIOTA IN ADOLESCENT AND ADULT MALE RATS: A VICIOUS CYCLE?

by

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Under the Direction of Kyle Frantz PhD & Benoit Chassaing PhD

ABSTRACT

Drug addiction is an intractable psychiatric disorder exerting deleterious impact on public health in the United States and beyond. While the neurobiology of addiction has become clearer over the last few decades, addiction therapies remain largely ineffective. Given recent evidence that a gut-brain axis might influence neuropsychiatric disorders, we explored possible links between gut bacteria and cocaine-related behavior. We hypothesized that gut microbial communities and cocaine intake are linked in such way that microbiota profiles can predict susceptibility to drug use and that drug use alters microbiota composition to enhance drug reward, hence resulting in a vicious cycle of drug use and abuse. Furthermore, although adolescence is a developmental stage associated with high rates of experimentation with drugs of abuse, adolescence is also a period associated with resilience to aversive stimuli. Thus, we predicted that adolescents would be

protected from this vicious cycle. Adolescent and adult male Wistar rats were tested in the intravenous cocaine self-administration model, while their fecal samples were analyzed for bacterial abundance (qPCR) and microbiota profiles (NextGen Sequencing of 16S rRNA). With adult rats, experimentation revealed distinct microbiota profiles among low vs. high responders to cocaine reward and reinforcement, especially after long-access cocaine self-administration. Moreover, the relative abundance of two specific microbial groups at baseline predicted low vs. high addiction vulnerability, perhaps warranting investigation as biomarkers of addiction. After establishing a new white noise training procedure and confirming adolescent resistance to an aversive white noise stimulus, we also manipulated the microbiota to reveal that antibiotic-induced gut microbial depletion increased cue-induced reinstatement of cocaine-seeking after abstinence in a model of drug relapse. Yet this effect was observed only in adult rats, not adolescent-onset groups. Treatment with a probiotic formulation during abstinence rescued normal levels of reinstatement in adult rats, suggesting that probiotics may be effective adjunctive therapies for addiction. Overall, this body of work provides another example of adolescent resilience to the enduring effects of physiological perturbations. This work also supports an important role for the gut-brain axis in drug reward and reinforcement, ultimately suggesting new treatment approaches for addiction.

INDEX WORDS: Addiction, Microbiota, Cocaine, Adolescence, Reinstatement, Gut-brain-axis

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DEDICATION

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LIST OF ABBREVIATIONS

AUC	area under the curve
BLA	Basolateral amygdala
CNS	Central nervous system
DA	Dopamine
DAT	dopamine transporter
db	decibel
EPSP	excitatory post synaptic potential
FR	fixed ratio
GBA	gut brain axis
GI	gastrointestinal tract
I.V.	Intravenous
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis Effect size
LgA	long access
LSA	Loud self-administration sessions
MAO	Monoamine oxidase
NAC	Nucleus accumbens
NTS	nucleus of the solitary tract
OTU	operational taxonomic unit
PCR	polymerase chain reaction.
PFC	Prefrontal cortex

PKA	protein kinase A
PSA	Punished self-administration sessions
QSA	Quiet self-administration sessions
SEM	standard error of the mean
ShA	short access
SHAM	sham surgery
SUD	substance use disorder
TO	timeout
VMAT	Vesicular monoamine transmitter
VTA	Ventral Tegmental area
WNT	white noise training

* All system of measurements are in the International System of Units (SI) Form

1 INTRODUCTION

1.1 Overview

Substance use disorder (SUD), a condition in which chronic substance use leads to mental, physical, and behavioral deficits (American Psychiatric Association: 2013) affects more than 25 million Americans per month (NSDUH, et al. 2013). With regard to cocaine, treatments to prevent continued use and attenuate relapse are ineffective (Fischer et al., 2015; Simpson et al., 1999). The main reason treatments are ineffective is that addiction is associated with long-lasting vulnerability to relapse, even after long periods of abstinence (Cregler et al. 1986).

Recent evidence suggests that microbes in the gut are associated with several neuropsychiatric disorders (Cryan et al. 2019), including substance use disorder (Mekel et al. 2019). We hypothesized that microbiota composition and drug use are linked in such a way that microbiota profiles can predict susceptibility to drug use, and that drug use alters microbiota composition, hence resulting in a vicious cycle of drug use and abuse.

Furthermore, adolescence is the developmental stage when drug use is often first initiated among individuals who identify as addicted later in life (Kandel et al. 1978; Kandel et al. 1984). Adolescence is a period of rapid development, during which hormone levels, CNS circuitry, and behaviors are still maturing (Spear et al. 2000; Spear et al. 2010). The gut microbiota is also shown to be distinct in adolescents compared to adults and susceptible to change (Agans et al. 2011). Based on adolescent resilience to aversive stimuli and some enduring drug effects, we hypothesized that adolescents are protected from the bidirectional impact between the gut microbiome and drug reward and reinforcement.

1.2 Behavioral testing: The Self-Administration and Reinstatement Model

Animal models are used frequently to study the effects of drug abuse, given ethical issues with drug testing in humans. The self-administration and reinstatement model has been the gold standard to study the reinforcing qualities of pharmacological compounds for 50 years. The model has face validity and predictive validity for substance abuse disorder (O'Conner et al. 2011). Based on classic work by Edward Thorndike (Thorndike et al. 1905), B.F. Skinner (Skinner et al. 1953), James Olds, and Peter Milner, self-administration and reinstatement tests are conducted in customized Skinner boxes (operant conditioning chambers). The equipment is used to test whether presentation of a stimulus reinforces (or punishes) a behavior such as lever-pressing. In such chambers, we assess the extent to which a test subject finds intravenous infusions of cocaine to be reinforcing under a variety of conditions. Experimental manipulation of environmental cues in the operant conditioning chambers can reveal additional associations between reinforcing stimuli (e.g. drug infusions) and auditory or visual stimuli (e.g. white noise or cue lights), and we used such cocaine-associated cues in a model of relapse, known as cue-induced reinstatement of drug-seeking after abstinence.

Compulsive drug use despite adverse consequences is a symptom of substance use disorder, and previous studies confirm that rodents compulsively seek drugs similar to humans (Koob et al. 2001). Recently, self-administration models have included negative consequences associated with drug infusions (Barnea-Ygael et al. 2012; Katzir et al. 2007; Holtz et al. 2015) These "punished" models of cocaine self-administration may be considered more translatable to the human condition of addiction than self-administration parameters that do not include such adverse consequences of drug intake. Most humans experience negative consequences in the throes of addiction, such as loss of employment, alienation from friends and family, and

depression or anxiety (Lander et al. 2013). Therefore, one goal of this dissertation was to validate white noise as a method of modeling the aversive nature of substance abuse. This goal emerged after we investigated whether the removal of white noise could be used as a method to facilitate acquisition of lever pressing in operant conditioning chambers. Many different procedures are used for facilitating acquisition behavior, including priming injections of the drug, food restriction and food self-administration, oral tastants on the manipulandum, autoshaping, and fading (Caroll et al. 2011). Each of these procedures has pros and cons, some of which are difficult to overcome when testing different age groups. For example, differential effects of food deprivation across age groups may alter acquisition of lever-pressing through food self-administration. On the other hand, white noise is an alternative that is not consumable and non-invasive. We will show validation of this white noise in the self-administration model later in this dissertation and its use for facilitating acquisition of lever pressing will be used in subsequent behavioral experiments to address behavioral questions asked in this dissertation.

1.3 Experimental Subjects: Wistar rats (*Rattus norvegicus*)

In an attempt to answer questions about human behavior and disease, animal models have been used since the days of ancient Greeks (Ericsson et al. 2013). The research subjects in this dissertation are Wistar rats (*Rattus norvegicus*), a strain of albino rats that have been used in scientific research since the early 1900s (Hatai et al. 1907). Their mainstay in scientific research seems to originate in their propensity for extraordinary memory compared to other rodent models (Watson et al. 1903). Due to similar physiology compared with other mammals, including humans, they often have similar disease phenotypes and sequelae, and they are the most studied animal model (Aitman et al. 2016). While the use of rats as an animal model has been challenged, rats in drug self-administration experiments provide high face and predictive validity

for studies on drug addiction (O'Connor et al. 2011;Panillo et al. 2007; Fuchs et al. 2007; Epstein et al. 2003; Shaham et al. 2003).

1.4 The Reward System

External stimuli and internal states that benefit an animal or species generally elicit approach behaviors and are known as rewards. When their presentation reinforces behavioral sequences, these rewards are known as positive reinforcers. Food, water, social affiliation, and sexual interactions all activate neural pathways in the brain that are known as the reward system. Drugs of abuse also activate reward circuits, often faster and to higher levels than natural rewards, such that drug-seeking can supplant more adaptive behaviors in the repertoire of most animals. Major components of the reward circuitry include mesocorticolimbic (MCL) pathways, with dopaminergic cell bodies in the ventral tegmental area (VTA) projecting to the nucleus accumbens (NAC) and prefrontal cortex (PFC), with glutamatergic and GABAergic feedback loops (Olsen 2011; Yager et al. 2015). Cocaine in particular blocks dopamine (DA) reuptake at the dopamine transporter (DAT) (Daws et al. 2002), which results in excess extracellular dopamine, and elevated binding at both D1-like and D2-like receptors. Cocaine also blocks reuptake of norepinephrine and serotonin, but to a lesser extent (Li et al. 1996; Zhu et al. 2000; Sora et al. 2001; Filip et al. 2005). DA transmission remains altered even after the drug is cleared from the body (Bossert et al. 2007; Lepack et al 2020). Long term cocaine use also alters glutamate transmission (Kalivas et al. 2003) including alterations in adaptation, receptor trafficking, and plasticity in several brain regions in the MCL (Wolf et al. 2016). These long-term neuronal adaptations after prolonged drug use likely underlie the transition from casual drug use to addiction and propensity to relapse. Specifics on how dopamine and cocaine alter the reward system will be explored in detail later on in this dissertation. Of additional interest for

this dissertation is how cocaine, dopamine, and the gut exert their combined effects on altering the reward system, drug addictive behaviors, and behavioral reinforcement.

1.5 The Gut Microbiota

The gut microbiota is the collective term used to describe the ecosystem of microorganisms living inside an animal's gastrointestinal tract. Its constitution is substantial, with trillions of individual organisms equaling over 100 times the number of genes in the human genome (Gill et al. 2006, Borre et al. 2014). While some variability in composition depends on the upbringing and environment of the individual host organism, core groups of microbes are found in all mammalian species (Gill et al. 2006). Gut dysbiosis is a term used to define alterations of the intestinal microbiota with detrimental consequences, such as a loss of overall microbial composition, expansion of populations of harmful microorganisms, and/or loss of beneficial microbes (Petersen et al. 2014). Gut dysbiosis can lead to irritable bowel syndrome, increased gut inflammation, and changes in food metabolism, among other ailments (Yatsunenko et al. 2012).

Outside of the gut it has become clear that gut microbiota has influence on the brain and subsequently behavior (Cryan et al. 2012), leading to the prediction that the gut-brain axis may influence neuropsychiatric disorders. Specifically, animals with experimenter-induced gut dysbiosis show heightened anxiety-like (Diaz Heijtz et al. 2011, Neufeld et al. 2011) and depression-like behaviors (Arseneault-Bréard et al. 2011, Naseribafrouei et al. 2014), with clinical studies supporting gut-brain links in depression (Jiang et al. 2015), bipolar disorder (Evans et al. 2017) and autism spectrum disorder (Benach et al. 2012, Rosenfeld et al. 2015). Recently, evidence has emerged that sensitivity to drugs of abuse is also correlated with changes in the gut microbiota in mice and rats (Kiraly et al. 2016; Ning et al. 2017). Clinical reports also

show distinct microbiota profiles in cocaine users vs. cocaine-naïve counterparts (Volpe et al. 2014). While the above studies have established a relationship between SUD and the microbiota, they are limited in some capacities. Conditioned place preference (CPP) used to show links between drug reward and gut microbiota in mice and rats is a valid classical conditioning model, but it does not capture either the compulsion of an animal to seek out a substance or the reinforcing effects of that stimuli. Publications are lacking on a relationship between the gut microbiota and drug-seeking behavior measured by the self-administration and reinstatement model and gut health (specifically the gut microbiota milieu).

1.6 Specific Aims

We plan to test the hypothesis that drug use and microbiota composition are linked in such a way that microbiota profiles can predict susceptibility to drug use, and that drug use alters microbiota composition, hence resulting in a vicious cycle of drug use and abuse. This overarching hypothesis will be tested through three specific aims.

1.6.1 Specific Aim 1: Test the hypothesis that microbiota composition can predict and reflect susceptibility to cocaine addiction in adult male rats.

We will test this aim by analyzing gut microbiota composition in fecal samples from rats that exhibit high vs. low addiction phenotypes, as quantified and described by collaborators at The Scripps Research Institute and the University of California, San Diego. We predict that the gut microbial profile will differ between addiction-prone vs. addiction-resistant adult rats. Furthermore, we predict that specific bacterial taxa will predict future cocaine susceptibility in adult rats.

1.6.2 Specific Aim 2: Test the hypothesis that depletion of the intestinal microbiota in adolescent and adult male rats increases cocaine- related behavioral reinforcement.

We will test this aim by providing oral antibiotic solutions and subsequently measuring cocaine-related behavioral reinforcement with the i.v. self-administration and cue-induced reinstatement models. We will compare outcomes in adolescent vs. adult male rats, and we predict that antibiotic-induced depletion of the intestinal microbiota will increase cocaine-seeking behavior, with greater effects in adults compared with adolescents.

1.6.3 Specific Aim 3: Test the hypothesis that probiotic treatment in adolescent and adult male rats rescues or reduces levels of cocaine- related behavioral reinforcement.

We will test this aim by providing probiotic supplementation for animals that already show gut dysbiosis induced by antibiotics or by giving probiotics to animals as a prophylactic, then measuring cocaine self-administration and reinstatement. We predict that probiotics will rescue normal levels of cocaine-related behavior in animals that already show antibiotic-induced microbial depletion, or reduce cocaine-related behavior in antibiotic-naïve animals. These effects are predicted to be more robust in adults compared with adolescents.

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2 DOPAMINE AND ITS ROLE IN THE BRAIN'S REWARD SYSTEM, ADDICTION, AND ASSOCIATION WITH THE GASTEROINTESTINAL SYSTEM

2.1 Abstract

The neurotransmitter dopamine is involved in many different mammalian biological processes. Dopamine has been considered the principal neurotransmitter in the reward system and certain psychomotor stimulants, such as cocaine, function primary on dopaminergic signaling in the reward system. Outside of the brain, dopamine plays a role in several peripheral processes, including the gastrointestinal tract, where dopamine was first discovered. The goal of this chapter is to present dopamine's role in the reward system in the context of addiction. Furthermore, we establish how dopamine's role in reward is associated with the gastrointestinal system and the gut microbiota. Starting with the dopamine structure and life cycle we transition to describing dopamine's function at the receptor level then branch out to discussing dopamine circuitry and how it plays a part in drug addiction in the brain. We then briefly discuss how the dopamine system develops in mammalian organisms before touching on dopamine in peripheral systems, focusing on dopamine and the gut. This chapter serves as a strong background source to understanding how manipulating gut homeostasis may alter dopamine systems. Furthering this understanding of how cocaine abuse may be related to gut relate signaling may provide new avenues for treating neurological disorders involving dopamine dysfunction.

2.2 Introduction

Dopamine is a signaling molecule that plays a key role in respiration, gastrointestinal motility, blood pressure, circadian rhythms, voluntary movement, sleep, feeding, reward processing, learning, attention, and many other physiological processes – through interactions with receptor proteins on neurons and other biological tissues (Carlsson et al. 2001; Iversen and

Iversen, 2007; Rubí et al 2010; Tritsch et al. 2012). This classic, small molecule monoamine neurotransmitter binds with two main families of G-protein-coupled receptors, located in both the CNS and periphery. With such wide-ranging impact throughout the body, dopamine dysfunction is at the crux of many neuropathologies. For example, the devastating movement disorder, Parkinson's disease, involves a massive loss of dopamine neurons in the midbrain (Ehringer et al. 1960; Surmeier et al. 2017). The genetic mutation that causes Huntington's disease also dysregulates dopamine in the striatum (Cyr et al. 2006), in part through the loss of receptors for dopamine. In terms of mental disorders, antipsychotics appear to ameliorate the symptoms of psychosis mainly by blocking dopamine receptors in the D2 family (Snyder et al., 1974; Stawarz et al., 1975). Schizophrenia, among the most challenging mental disorders, has been linked to abnormally high DA receptor density, coupled with low prefrontal dopamine activity, leading to elevated DA signaling in mesolimbic regions that contributes to both negative and positive symptoms seen in this disorder of thought, perception, emotion, and outward behavior (Howes et al., 2009). Other neuropsychiatric conditions such as substance use disorder are also related to dopamine dysregulation. Drugs of abuse "hijack" midbrain dopamine systems to amplify signaling that would normally mediate responses to novel stimuli, natural rewards, and even salient stimuli with aversive valence (Koob and Volkow, 2010). Heightened dopamine levels are associated with reward, reinforcement, and even euphoria, while subsequent low dopamine levels are components of withdrawal, all part of an addiction cycle (Cooper et al. 2003). Alterations in reward related brain signaling occur during drug seeking tasks (Koob and Volkow, 2010). Specifically, neuronal signaling in the prefrontal cortex, a brain region known to coordinate higher-order cognitive functions, is compromised in chronic drug users compared with healthy controls (Carboni et al. 2001). Other rewarding stimuli, such as high-fat or high-

sugar foods, also elicit a burst in dopamine signaling from midbrain neurons. It comes as no surprise, then, that dopamine dysregulation is identified in obesity. Striatal D2 receptors are reduced in obese patients compared to controls (Volkow et al. 2008), and dopamine transmission during compulsive overeating is similar to that observed during drug intake (Johnson and Kenny, 2010). Obesity can alter levels of extracellular dopamine in the nucleus accumbens, stimulate dopamine release in response to high fat diet, and may blunt dopamine release in response to other stimuli (such as amphetamine) (Geiger et al.2009). First identified in the peripherydopamine's role in cardiovascular, gastrointestinal, and immune dysfunction is less understood. Pioneering work in areas such as the gut-brain axis serves to link peripheral and central dopamine function under both healthy and disordered conditions.

The main goal of this chapter is to introduce the life cycle, signaling mechanisms, brain circuits, and peripheral impact of dopamine, with some focus on its role in reward and reinforcement. This overview also serves to provide a basic understanding of cocaine, a psychomotor stimulant and major drug of abuse. Cocaine's primary mechanism of action is to block reuptake of dopamine, serotonin, and norepinephrine, thereby amplifying their effects on the acute timescale thereby compromising their signals over the long run. Furthermore, dopamine may interact with gut microbes directly or indirectly, thereby influencing outcomes from microbial depletion or amplification with antibiotics or probiotics, respectively, and creating a route for direct cocaine impact on gut-brain signaling as well. Ultimately, manipulations of gut microbes might alter dopamine systems, providing new avenues for treatment of the many neurological disorders involving dopamine dysfunction.

2.3 The Synthesis, Release, and Inactivation of Dopamine

The lifecycle of neuroactive molecules includes synthesis, storage, release, and inactivation. Dopamine is a catecholamine neurotransmitter, i.e. a monoamine that contains a catechol- aromatic ring with a double hydroxyl group (Moore and Bloom, 1978). Variation in functional groups on the R-group distinguishes dopamine from other catecholamines such as norepinephrine which has the addition of a hydroxy group on the second carbon (Moore and Bloom, 1979). The dopamine lifecycle has been characterized and illustrated very well in the past (Figure 2.1.) (Jones et al. 2014).

Synthesis of dopamine initiates with the conversion of tyrosine from the diet (Daubner et al., 2011). A full diagram of the synthesis of dopamine from tyrosine is shown (Figure. 2.1). Tyrosine comes from many components of the diet, with sources including grains and protein-rich foods such as dairy and meat. Tyrosine is converted into DOPA via tyrosine hydroxylase which adds a hydroxyl group to the aromatic ring. DOPA is then converted into dopamine via aromatic amino acid decarboxylase which removes the aldehyde group. Dopamine can then further be converted into norepinephrine via dopamine-beta-hydroxylase with adds a hydroxyl group to the first carbon. Systemic tyrosine levels do not seem to alter catecholamine levels, contributing to conclusions that the action of tyrosine hydroxylase is the rate limiting step in the formation of dopamine and norepinephrine (Medicine et al., 1994). Dopamine can also be synthesized indirectly from the amino acid phenylalanine (Matthewset al. 2007; Moss et al 1940). Dopamine synthesis is primarily carried out in the gastrointestinal tract but also in brain and spinal cord (Hou et al. 2016), making it available for CNS function despite its inability to cross the blood-brain barrier (Hardebo et al. 1980).

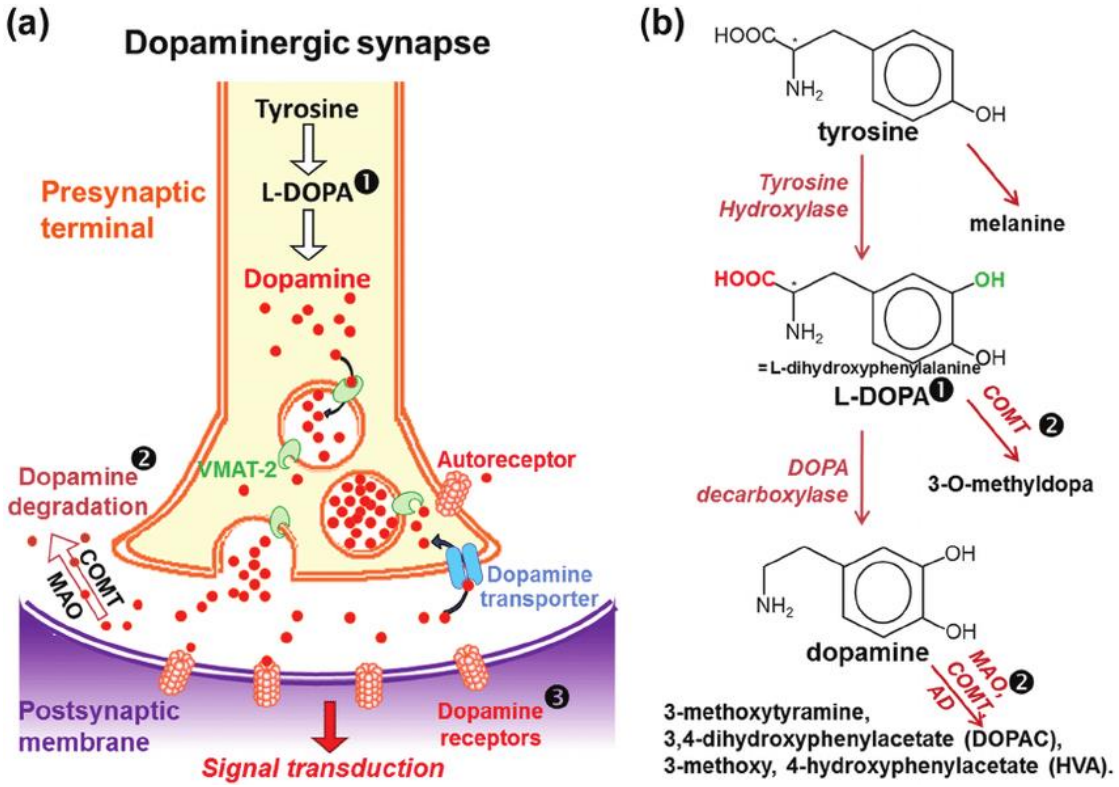

Figure 2.1Dopaminergic synapse and dopamine metabolism
(a) (1) In the presynaptic terminal of dopaminergic neurons, tyrosine is transformed into L -DOPA by the action of tyrosine hydroxylase. L -DOPA is subsequently transformed to the neurotransmitter dopamine (DA) by action of the DOPA decarboxylase. DA is then transferred in vesicles by the vesicular monoamine transporter 2 (VMAT-2). After exocytosis of the DA vesicles, DA binds to DA receptors on the postsynaptic membrane, leading to the transduction of the signal in the postsynaptic neuron. DA is then recycled by reuptake via the DA transporter, or catabolized by the action of monoamine oxidase (MAO), cathecol- O -methyl transferase (COMT) and aldehyde dehydrogenase (AD) enzymes. The dopaminergic synapse is the principal site of action of current PD treatments. By increasing dopamine metabolism (l -DOPA treatment) or by inhibiting dopamine catabolism (COMT or MAO inhibition), or by directly activating postsynaptic dopamine receptors, these treatments boost the activity of the dopaminergic synapses. (b) Illustrates the lifecycle of dopamine starting with tyrosine acquired from the diet. Dopamine can then also be a precursor for other neuroactive molecules (norepinephrine) or be broken down through several mechanisms (MAO,COMPT,AD) (adapted from Jones et al. 2014).

In neurons, tyrosine hydroxylase is synthesized the cell body (Fernstrom et al. 2007), then is transported to the nerve terminal where it converts tyrosine to DOPA (Gervasi et al. 2016; Jarrott

and Geffen, 1972). Dopamine-synthesizing cells exist in many parts of the brain with major groupings in the ventral midbrain and in the hypothalamus (Juárez Olguín et al., 2016).

Once synthesized in the nerve terminal, dopamine is packaged into vesicles for synaptic release. Dopamine is transferred into the synaptic vesicle via vesicular monoamine transporter (VMAT)(Best et al. 2009; Daubner et al. 2011) which uses a form of active transport that relies on a H-ATPase to create an electrochemical gradient by promoting influx of protons into the synaptic vesicles. This gradient drives neurotransmitter into the vesicle (Kandel et al., 2012), creating a high density of some 100,000 times more in the vesicle than in the cytoplasm.

Dopamine release is usually triggered via calcium signaling from arriving action potentials at the nerve terminal (Daubner et al. 2011). Dopamine can also be released at the soma and dendrites, but to a lesser extent (Bergquist et al., 2003; Crocker, 1997). Dopamine neuronal firing and release can occur at a very low frequency (tonic) or at brief, higher frequencies (phasic) (Grace and Bunney, 1984; Rice et al., 2011). Functionally, it has been shown that phasic activity encodes prediction-related information (Schultz, 1998; Schultz et al., 1993) whereas tonic firing underlies the steady-state level of extracellular dopamine in subcortical structures. In essence, tonic release maintains homeostatic dopamine signaling that can restore DA receptors back to normal levels. Being that most tonic release is governed by cortical striatal afferents, this top-down control regulates dopamine homeostasis (Grace, 1991).

Presynaptic autoreceptors contribute to the regulation of release rates using negative feedback mechanisms. Synaptic dopamine release is often accompanied by co-release of other transmitters. For example, glutamate can be co-released along with dopamine (Broussard, 2012), resulting in not only excitation downstream (EPSPs) but also postsynaptic modulation (Broussard, 2012; Chuhma et al., 2004). Small peptides such as cholecystokinin can modulate

release and be co-released with dopamine (Marshall et al., 1991). In the synaptic cleft, dopamine binds to pre- and post-synaptic receptors, with five main families of receptor proteins (see next).

Dopamine activity in the synapse is terminated either by metabolizing synaptic dopamine into non-active metabolites or by presynaptic reuptake. Breakdown in the synapse or cytosol is catalyzed by catechol-o-methyltransferase (COMT). COMT converts dopamine into 3-methoxytryramine which is then subject to conversion by monoamine oxidase (MAO) into homovanillic acid, which is readily excreted (Koreen et al., 1994). COMT modifies all catecholamines by adding a methyl group to the R-group. Another way dopamine is broken down is through deamination by monoamine oxidase (MAO), forming dihydroxyphenylacetic acid (DOPAC). Inactivation by presynaptic reuptake of monoamines is carried out by transporter proteins. The dopamine transporter (DAT) is embedded in presynaptic membranes, mainly on axon terminals outside the synaptic cleft (Cooper et al., 2003) and uses a Na/K-ATPase and an existing Na gradient to fuel transport from the cleft into the nerve terminal cytoplasm (Torres et al., 2003). Sodium gates this action by binding to DAT to change its confirmation, allowing dopamine to pass (Sonders et al., 1997).

2.4 Dopamine Receptors

2.4.1 Postsynaptic receptors

Dopamine receptors have two main subcellular locations, post-synaptic receptors embedded in post-synaptic membranes to mediate downstream signaling, and pre-synaptic autoreceptors positioned on presynaptic membranes to provide autoregulatory feedback on dopamine synthesis and release. There are two families of post-synaptic receptor proteins and five main receptor subtypes (Beaulieu and Gainetdinov, 2011; Tritsch and Sabatini, 2012); all have seven transmembrane-spanning domains and are G-protein coupled on the intracellular

surface. The two families of receptors are D1- and D2-like receptors, found in all dopaminergic pathways but with different expression levels (Hall et al., 1994). The D1-like family consists of D1 and D5 receptors, which share structural similarity and cannot be distinguished pharmacologically. These receptors couple with stimulatory G-Proteins (Gs-alpha and G_{olf}) (Tiberi et al., 1991), which trigger adenylyl cyclase and lead to production of cAMP and activation of PKA (Neve et al., 2004). The result of this cascade is usually the formation of EPSPs. While D1 and D5 receptors are similar, they do differ in terms of expression and affinity. An example is that D1 receptors localize in dendritic spines and D5s are expressed in the nerve terminals of the basolateral amygdala (Muly et al., 2009). Such subcellular differentiation may impart circuit specificity, as this phenomenon of localization on different parts of the neuron is seen in many other brain regions, such as substantia nigra, hypothalamus, and caudate/putamen (Bergson et al., 1995). In the prefrontal cortex, D1 receptors are much more highly expressed compared to D5 (Kandel et al., 2012). With regard to affinity, D5 receptors have a ten times higher affinity for dopamine compared to D1 receptors (Cooper et al., 2003).

The second main family of dopamine receptors is D2-like receptors: D2, D3, and D4. D2 is highly expressed in the brain, whereas D3 and D4 on the whole are more sparse. D2 receptors are mostly located in the striatum. D3 receptors are found mostly in limbic circuits (Cooper et al., 2003). D4 receptors may be concentrated most in pyramidal neurons in the cortex (Neve et al., 2004; Oak et al., 2000; Wang et al., 2002). In contrast to D1-like receptors, D2-like receptors activate inhibitory G-proteins (Gi) which when dissociated inhibits adenylyl cyclase and limits PKA activation (Neve et al. 2004). D2-like receptor affinity for dopamine is about 100x more than for D1s (Titsch et al. 2015) which results in a higher chance of inhibitory dopamine cascades in locations where D1- and D2-like receptors are both present. While no ligand can

fully differentiate between individual receptor types, several medications target one type of receptor vs another (Cooper et al., 2003). An example is clozapine that has its highest affinity for D4 receptors but can still bind to other receptors in the D2- like family (Cooper et al., 2003).

Both D1 and D2 dopamine receptor subfamilies are subject to receptor up- and down-regulation under conditions of diminished or heightened receptor binding with dopamine or its exogenous agonists. Chronic exposure to dopamine receptor antagonists also increases the number of postsynaptic receptors. Conversely, chronic exposure to dopamine agonists decreases binding sites (Cooper et al., 2003). In general, increased postsynaptic receptor stimulation results in decreased nigrostriatal dopamine activity. Binding of dopamine to post synaptic receptors results in several downstream consequences. When dopamine binds to D1 like receptors, dissociation of G_{olf} results in activation of adenylate cyclase , producing the secondary messenger cAMP (Tiberi et al., 1991). When dopamine binds to D2 like receptors the opposite process occurs resulting in less cAMP production (Neve, 2009). cAMP then has the ability to exert its physiological effects on downstream targets through several mechanisms. Perhaps the most studied of these mechanisms is modulation of Protein Kinase A (PKA) (Park et al., 2005), but recently extensive research has been done on Phospholipase C modulating calcium levels via D1 receptor activation (Ha et al., 2012), which has been linked to neuropsychiatric disease (Mishra et al., 2018). Descriptions of second messenger pathways activated by dopamine binding to post synaptic receptors has been covered extensively (Bibb et al. 2005; Lebel et al. 2009; Savica and Benarroch, 2014) (Figure 2.2).

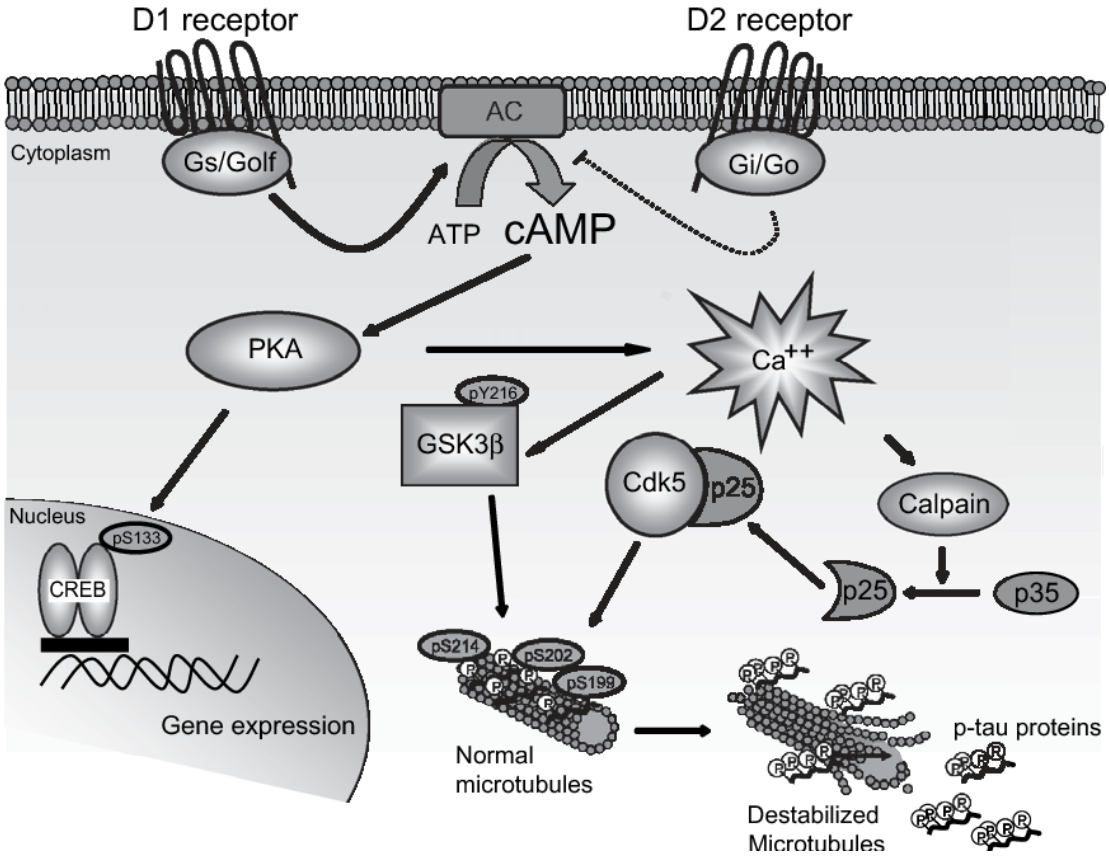

Figure 2.2Downstream second messenger signaling of D1 and D2 like receptors

After D1 receptor stimulation, tau is phosphorylated by activation of PKA, cdk5 and GS to Gs/Golf and stimulate production of the second messenger cAMP. After the increase in CA which binds to definite DNA sequences and modulates the transcription of certain genes. In opposition, the D2 class of receptors (D2,D3, and D4) couple to G_i/G_o and negatively regulate cAMP production, which decreases general PKA activation. D1 but not D2 receptor activation will phosphorylate tau. Through a PKA-dependent intracellular mechanism, D1 receptor activation will increase intracellular calcium levels, leading to both cdk5 activation by calpain proteolysis of p35, and GSK3Beta activation through its phosphorylation at tyrosine 216. Tau hyperphosphorylation may impact neuronal synaptic plasticity as cytoskeletal constituents are involved in the maintenance of dendritic processes, and any changes in their stability could affect major cellular compartments, such as dendrites, spines, and synapses. Plain arrows represent activation or increased levels, whereas the broken arrow represents inhibition. (adapted from Lebel et al. 2009)

2.4.2 Extrasynaptic dopamine function

Dopamine may also alter neuronal functioning in other ways besides synaptic binding to post-synaptic dopamine receptors. The one-to-one synaptic relationship between two cells only

governs a portion of synaptic transmission. Previous reports show that extra-synaptic neurotransmitter modulation occurs frequently with glutamate transmission (Okubo et al., 2010) and now it appears the same is true for dopamine transmission (Daniel et al., 2009; Mani and Ryan, 2009; Taber and Hurley, 2014; Vizi et al., 2010). Volume transmission, which is the concept that neuroactive substances can diffuse over long distance to reach their receptors (Jan and Jan, 1982) has been shown to exist in other types of neural signaling such as in the vasopressin system (Albers, 2015). Through volume transmission, dopamine may exert its effects on neurotransmitter function indirectly. Examples of these outcomes are modulation of vesicular neurotransmitter release via altering axon terminal excitability, calcium signaling, or by interreacting with vesicular release machinery (Tritsch and Sabatini, 2012). This modulation of neurotransmitter release can happen directly via dopamine exiting the synapse and binding to dopamine receptors on cells outside of the synaptic complex (Tritsch and Sabatini, 2012). Through this volume transmission dopamine may modulate the modality and trafficking of receptors on glutamate neurons. In the case of AMPA and NMDA receptors this is primarily through PKA-dependent phosphorylation (Håkansson et al., 2006). Finally, dopamine's widespread effects outside of the synapse may influence cells in other networks resting membrane potential.

2.4.3 Autoreceptors

As mentioned previously, dopamine can also bind to presynaptic autoreceptors. Autoreceptors can be present on dendrites, axons, and axon terminals (Cooper et al., 2003). Presynaptic autoreceptors can regulate dopamine synthesis via phosphorylation of tyrosine hydroxylase (Cooper et al., 2003). Stimulation of autoreceptors on somatodendritic region slows neuronal firing rates, whereas stimulation of autoreceptors on the nerve terminal limits dopamine

synthesis and release. Most autoreceptors can be classified as D2 autoreceptors, although D1 autoreceptors may be transiently expressed during development. While autoreceptors and postsynaptic receptors are structurally similar, presynaptic autoreceptors are 5-10 times more sensitive to dopamine binding. Not all dopaminergic neurons possess autoreceptors, with mesocingulate and mesoprefrontal dopaminergic neurons notably lacking autoreceptors. Dopaminergic neurons with autoreceptors tend to have higher rates of firing, higher dopamine turnover, diminished response to dopamine agonists and antagonists, and lack of receptor tolerance to antipsychotic drugs. In summary, dopamine release can act directly/indirectly to alter neurotransmitter release (presynaptic) or postsynaptic transmission either through dopamine receptors or other neurotransmitter systems.

2.5 Dopamine circuitry in the brain

Dopaminergic neurons are found in almost every part of the brain. The five major dopaminergic pathways in the brain are the nigrostriatal, mesocorticolimbic, tuberoinfundibular, hypothalamospinal, and Incertohypothalamic, with nigrostriatal and mesocorticolimbic being the most studied (Beaulieu and Gainetdinov, 2011). In the nigrostriatal pathway, neuronal cell bodies are located in the substantia nigra with axonal projections into divisions of dorsal striatum (caudate and putamen in primates). Signaling then continues to globus pallidus then to motor cortex via the thalamus. Signals from the motor cortex then descend through spinal afferents to control motor neuron function, therefore the main function of this pathway is the control of movement. Degeneration of neurons in this pathway is a leading cause for several movement disorders such as Parkinson’s disease (Kandel et al., 2012). In the mesocortical limbic pathway, neuronal cell bodies are located in the ventral tegmental area (VTA), with projections into the ventral striatum (nucleus accumbens) as well as areas of frontal cortex modulating behavior

(Arias-Carrión et al., 2010). Specifically, these dopamine projections tend to synapse on medium-spiny GABAergic neurons which then form a mutually inhibitory network (Geldwert et al., 2006). This inhibition which can then directly feedback to the VTA directly or indirectly via the ventral pallidum (VP) or hypothalamus is a way to keep the system in homeostasis (long loop feedback). The mesocorticallimbic pathway is critical for affect, emotion, attention and motivation. Dysfunction in these projections is associated with cognitive disorders, such as schizophrenia and drug addiction (Chen and Kandel, 1995). Despite their distinctions, these two pathways overlap, and the nigrostriatal pathway may contribute to rewarding behavior just as much as the mesolimbic dopamine pathway. For example, electrical stimulation experiments show that stimulating either the substantia nigra or the ventral tegmental area is rewarding in rats (Crow, 1972; Hoebel and Novin, 1982; Routtenberg and Malsbury, 1969). In fact, the anatomical division between the two pathways is blurry (Wang and Morales, 2008),with many researches noting that activation of both areas occurs in response to rewarding stimuli. It is suspected that reward processing involves both circuits to assist with forming habits, maintaining behaviors, and different types of emotional learning needed for reward associations to occur (Wise, 2009). The differences in how these two circuits contribute to reward may not only be attributed to location of the projection but also the receptor subtypes on the dopamine neurons themselves. D1-like receptors are more abundant in the mesocortical limbic pathway, whereas D2 receptors are more abundant in areas of dorsal striatum (caudate and putamen). Considering less evidence of differences in dopamine synthesis and release in these two pathways, receptor expression may be the key to understanding how these circuits operate and contribute differently to reward processing.

Among the lesser studied pathways, the tuberoinfundibular projection originates in the arcuate nucleus and projects to the pituitary gland, with the primary function of controlling hormone release. The hypothalamospinal pathway has cell bodies in the hypothalamus that connect to T1-L2 of spinal cord. Disruption of this pathway is associated with motor dysfunction. The incertohypothalamic pathway has dopaminergic signaling from the zona incerta (a subregion of the hypothalamus) to the thalamus and hypothalamus. Not much is known about this pathway, but some research has shown it relates to sexual function (Giuliano and Allard, 2001).

2.6 Dopamine in reward and reinforcement

Dopamine is the molecule most extensively associated with the behavioral processes of reward, reinforcement, and addiction. Early studies outlined a role for mesolimbic dopamine in the initial approach and exploration of appetitive and novel stimuli, thereby aligning its transmission with salience attribution. Furthermore, mesolimbic dopamine transmission was thought to signal the presence of a reward, given dopamine spikes after initial reward exposure. In the early 1990s, this understanding expanded through a series of studies by Schultz and colleagues. Monkeys trained to associate a conditioned stimulus cue with an unconditioned juice reward showed dopamine surges in the nucleus accumbens before the reward but after the cue (Schultz et al., 1993). Although dopamine had initially risen with presentation of the unconditioned rewarding stimulus (juice), dopamine neuronal activity migrated toward the conditioned stimulus (reward cue) over training trials. The dopamine signal appeared to shift throughout the experiment but could be reset after extinction of the relationship between conditioned stimulus and unconditioned reward. Thus, it appears as though dopamine acts to alert the organism to novelty or error, as in the presence of an unexpected reward or the absence

of an expected reward. Dopamine's role in salience can be extended to both approach and avoidance behavior. While animals approach rewarding stimuli, they avoid aversive stimuli, and stress-induced activation of both dopamine and glutamate circuitry in the prefrontal cortex may contribute to neuropsychiatric disorders, including addiction (Moghaddam, 2002).

2.6.1 Drugs of abuse

Drugs of abuse can alter dopamine function at various points in its life cycle of synthesis, storage, release, binding and/or inactivation. Psychomotor stimulants tend to amplify dopamine transmission either directly or indirectly, as discussed in detail below using cocaine as the prime example. While not addressed in this chapter, several other classes of drugs of abuse such as opioids and cannabinoids appear to enhance dopamine transmission via the endogenous opioid system (Koob, 2001). While the exact mechanisms of how alcohol modulates dopamine transmission are currently unclear (Di Chiara, 1997), the opioid and GABAergic systems act as intermediaries in pathways from alcohol intake to dopamine activation (Cruz et al., 2008; Froehlich, 1997; Tanchuck et al., 2011; Xiao and Ye, 2008).

Cocaine is a psychomotor stimulant that can be administered in solid form through intranasal sufflation of the hydrochloride salt, in liquid form through intravenous injection, or smoked from the modified free base form (crack cocaine). Naturally occurring in the coca plant of tropical environments such as South America and Southeast Asia, cocaine is a psychoactive alkaloid with bitter taste that is likely aversive to insects and other animals. Historical practices of chewing on coca leaves results in very low doses of cocaine crossing into blood circulation. Intravenous and inhaled cocaine appears in higher concentrations in the blood plasma and at a faster rate than cocaine administered through insufflation (Cone, 1995; Jones, 1997). Peak concentrations of cocaine in the blood stream varies depending on administration route but

usually occurs after 30-100 minutes post administration then starts to decline (Jones, 1997). The reported half-life for cocaine is less than one hour. The two major metabolites of cocaine are benzoylecgonine and ecgonine methyl ester (Cone, 1995). Cocaine is metabolized by liver enzymes and excreted through the urine (Stewart et al., 1979). Cocaine metabolites can be detected in the urine approximately three days after initial intake (Chow et al., 1985; Cone et al., 1998).

In terms of pharmacodynamics, cocaine binds to the dopamine transporter (DAT) to block dopamine reuptake, resulting in prolonged dopamine activity in the synapse (Cone et al. 1995; Nestler et al. 2005). Cocaine and several other psychostimulants have a structure very similar to dopamine in that they possess an aromatic ring and similar R groups. Cocaine also binds to the norepinephrine transporter (NET) and serotonin transporter (SERT) but to a lesser extent (Kandel et al., 2012), with binding affinity dependent on specific amino acid residues in the hydrophobic pocket of the DAT, NET, and SERT (Beuming et al., 2008). Under normal conditions, when dopamine binds to the DAT, the gated tunnel of the transporter changes conformation in order to transport the bound dopamine into the intracellular space. When cocaine is present, the molecule gets trapped on the extracellular open state, preventing the conformational change in the gated tunnel (Huang et al., 2009). While cocaine has the ability to bind to all monoamine transporters it exhibits the greatest effect on the DAT because it binds longest (i.e. it has the highest dissociation constant which stems from different amino acids in the binding pocket) (Hasenhuetl et al., 2015). While DAT is found in many parts of the brain, it is abundantly expressed in areas of forebrain, ventral striatum, and slightly less expressed in the dorsal striatum (Ciliax et al., 1995). It is therefore not surprising that the mesocorticolimbic and nigrostriatal pathways are the most influenced by the presence of cocaine or other compounds

that act on dopamine transmission and the dopamine transporter. It is possible that cocaine could have some direct effects in the gut, given the evidence that DAT exists in the epithelial cells of the stomach (Mezey et al., 1999), the colon, and duodenum (Tian et al., 2008).

Cocaine users report feeling euphoric, energetic, alert, awake, and hyper-responsive to sensory stimuli, along with some reports of feeling agitated, irritable, agitated, anxious, panicked, or paranoid. Physiological effects related to DAT blockade in the periphery mimic sympathetic nervous system activation, including vasoconstriction, dilated pupils, increased body temperature, heart rate, and blood pressure.

2.6.2 Withdrawal and Tolerance

After cocaine use has ceased, withdrawal is likely to occur. Cocaine invokes numerous changes in neurophysiology and behavior. Extraceullular DA (and serotonin) levels in the nucleus accumbens drop precipitously initial withdrawal (Parsons et al., 1996; Weiss et al., 2001; Weiss et al., 1992). This alteration in NAC activity may be mediated by dampened activity in the VTA immediately after withdrawal begins (Ackerman and White, 1990; 1992; Henry et al., 1989). DA receptors that are downregulated during chronic administration, start to upregulate during withdrawal (Kuhar et al 1996) Properties of pre- and postsynaptic neurons may be altered as well, with presynaptic neurons showing altered metabolism of glucose at the start of withdrawal and other groups showing changes in postsynaptic D1 receptors in the nucleus accumbens post-withdrawal (Kuhar and Pilotte, 1996; Neisewander et al., 1995). In humans, some evidence has shown that brain physiology is altered during cocaine withdrawal. This is mainly assessed through fMRI, where cerebral blood flow in the PFC is decreased in patients that initiated cocaine withdrawal compared to controls (Volkow et al. 1988). Behaviorally, cocaine withdrawal can alter grooming behavior, locomotion, induce anhedonia, and alter

memory related tasks among other things (Markou and Koob, 1992). These signs of withdrawal are thought to me mediated not only by dopamine post-synaptic receptor functioning but also changes in other neuroactive molecules such as nociception, endocannabinoids, Neuropeptide Y, vasopressin, Substance P ,and norepinephrine (Koob and Volkow, 2010; Markou and Koob, 1991). This is not surprising given the vast crosstalk between dopamine and other neurotransmitter systems. One common trait of withdrawal is the near immediate changes in drug related behavior and neurophysiology with the body's attempt to maintain homeostasis (Kuhar and Pilotte, 1996). In short, the physiological response to withdrawal is the body's attempt to bounce back to a period of homeostasis that was perturbed by chronic drug use.

Tolerance to cocaine is another hallmark of addiction defined by the DSM 5. Tolerance by definition is a diminished response to the same input stimuli. Tolerance usually is seen in laboratory animals after 10 days of chronic cocaine administration (Hammer et al., 1997). It is thought that a reduction in basal dopamine neurotransmitter (Imperato et al., 1992; Maisonneuve et al., 1995), a reduction in stimulated release (Inada et al., 1992; King et al., 1994), and an alteration of dopamine receptors and second messenger systems all contribute to cocaine tolerance (Goeders and Kuhar, 1987; Hammer et al., 1997; Volkow et al., 1993). It is also thought that the opioid system may be involved with the dopamine system to instigate tolerance (Nestler, 2005). Behaviorally, tolerance results in drug intake or approach that results in a shift in the dose response curve to the right (Wood and Emmett-Oglesby, 1986). Tolerance can be achieved in self-administration studies by allowing animals to self-administer for a period of time, then injecting the animal with high doses of cocaine, then observe subsequent behavior. When animals are tested again, both fixed ratio (Emmett-Oglesby and Lane, 1992) and progressive ratio (Li et al., 1994) challenge tests reveal a shift in the dose response curve that

returns to normal after 1-2 weeks. Rodent subjects also had lower reward thresholds when measured by intercranial self-stimulation during a state of tolerance vs no drug exposure or cocaine exposure (Kokkinidis and McCarter, 1990).

Other major psychomotor stimulant drugs include amphetamine, methylphenidate, nicotine, caffeine, and modafinil, among many others. Through direct or indirect action, each of these elevates extracellular levels of dopamine, norepinephrine, and serotonin. The therapeutic advantages of these drugs and their pharmacological activity are well known, as they are used to treat excessive daytime sleepiness (narcolepsy) and attention deficits (attention deficit disorder), used recreationally to enhance wakefulness and focus (caffeine), and may suppress some symptoms of schizophrenia (as nicotine use is highly comorbid with schizophrenia). Yet, their high abuse liability causes billions in healthcare costs related to direct effects (dependence), side effects (organ damage), and is associated with myriad detrimental personal consequences related to directing resources to obtaining and using the drug.

2.7 Dopamine system development

Most dopamine neurons in the brain are located in the midbrain. As such they are called mesodiencephalic dopamine neurons. The number of these neurons is estimated to be about 500,000 (Pakkenberg et al., 1991). Midbrain dopamine neurons are derived from neural tube progenitor cells (Ono et al., 2007). Numerous genes and gene products contribute to the regulation of dopamine neuron development (Bissonette and Roesch, 2016). Dopamine levels fluctuate throughout the life cycle in an inverted u-shape. Dopamine levels rise and peak during adolescence. In addition to this peak, dopamine concentrations shift towards more anterior regions of the brain as mammals develop (Goldman-Rakic and Brown, 1982). This finding mimics the developing brain during adolescence, particularly prefrontal cortex (Spear et al.

2000). A consequence of this rise in dopamine during adolescence could be to promote overall neuronal excitability via dopaminergic modulation of voltage thresholds for firing (Henze et al., 2000). Human dopamine receptor data has shown that D1 and D2 like receptor levels decrease from childhood to adulthood and in fact get to adult levels by five years of age (Montague et al., 1999; Seeman et al., 1987). Rodent studies show that dopamine receptor levels peak between P28 and P42, then level off or decline (Tarazi et al., 1999; Tarazi et al., 1998). There are some discrepancies however to region-specific effects and possible age contributions to certain classes of receptors (Wahlstrom et al., 2010). While ample evidence supports a change in the dopamine system throughout the lifespan, these data are confined to changes in the CNS. Of interest to this dissertation would be to understand whether the timeline of development of dopaminergic neurons in the gut could be similar to what we observe in the CNS.

2.8 Non-neuronal dopamine interactions

2.8.1 Dopamine and Glia

While several neuropsychiatric disorders have been linked to dysregulated dopamine transmission or loss of dopamine neurons, extensive evidence suggests that non-neuronal cells can also play major roles in these pathologies. For example, interactions between dopaminergic cells and glial cells have been implicated in neurodegenerative disorders, particularly Parkinson's disease. Dysfunction among glia increases inflammation which may lead to dopamine cell dysfunction and/or cell death (McGeer and McGeer, 2008). To that end microglia activation may be an early sign of future dopamine dysfunction that may lead to neuropsychiatric disorders (Kanaan et al., 2010). Moreover, dopamine and its receptor agonists or antagonists can alter membrane potentials and evoke small hyperpolarization in astrocytes (Hösli et al., 1987). Alterations on astrocyte function may lead to several pathogenic outcomes, such as loss of

integrity of the blood brain barrier and an increase in neuro and peripheral inflammation. This may be a mechanism on how cocaine induces increases in neuroinflammation (Sil et al., 2019) and inflammation in the gut (Chivero et al., 2019).

2.8.2 Peripheral Dopamine

Beyond the CNS, dopamine and dopaminergic neurons are found in many parts of the periphery. In fact, dopamine was initially identified in the gastrointestinal tract (Carlsson et al. 1958; Clark and Menninger, 1980). While the substance dopamine was first synthesized and identified in the early 1900s, it was not until the 1950s that dopamine was identified as its own neuroactive molecule and not just a precursor to norepinephrine (Carlsson, 1993). The first identification of a binding pocket for dopamine occurred shortly thereafter, along with dopamine's postsynaptic intracellular signaling through G-protein coupled-receptors acting on adenyl cyclase in a molecular cascade (Kebabian et al., 1972). Research in the 1980s and -90s focused on dopamine's role in the cardiovascular system. A breakthrough contribution was that dopamine is vital for proper cardiocyte function (Clark, 1981; Clark and Menninger, 1980). Dopamine was also shown to alter renal resistance and blood flow, which may alter cardiac output, though the mechanism is poorly understood (Gordon et al., 1995).

The role of dopamine in the gut is extensive, but its influence on gut-brain signaling remains to be explored. Dopamine, DAT, and dopamine receptors have all been found in the enteric nervous system, specifically in the jejunum and myenteric plexus (Li et al., 2011;). Dopamine receptors are also observed in the mucosa (Hernandez et al., 1987) and in gastric muscle (Kurosawa et al., 1991). Chromaffin cells in the GI tract contain dopamine receptors and it has been speculated that they may be involved in inter-gut communication (Bigornia et al., 1988). Enteric dopaminergic neurons express tyrosine hydroxylase (TH) and the dopamine

transporter (DAT) but lack dopamine β-hydroxylase (Anlauf et al., 2003). Other catecholamines such as serotonin, norepinephrine, and their synthesis enzymes and transport proteins are present in the gut as well (Gershon, 2004). The purpose of dopamine in the gut is debated, but it appears to be involved in relaxing the intestine and intestinal smooth muscle (Grivegnee et al., 1984; Lucchelli et al., 1990). In addition, D2 receptor agonists alleviate the severity of symptoms in Irritable Bowel Syndrome and Crohn's disease patients, perhaps through a dopamine-mediated inhibition of mucosal permeability and muscle relaxation (Tolstanova et al., 2015). Cocaine administration leads to increases in inflammation, gut membrane permeability (Chivero et al., 2019), and blood clots (Siegel et al., 1999),which may ultimately lead to a decrease in blood flow and nutrient transfer (Bachi et al., 2017). These acute effects can then lead to a maladaptive environment for the gut microbiota, shifting their diversity and abundance (Chivero et al., 2019). The irony of these findings is that cocaine was used extensively in antiquity to restore digestive and gastrointestinal health (Weil, 1981). In fact, up until the mid-20th century, coca leaves were used to alleviate symptoms of diarrhea, constipation, and indigestion (Biondich et al. 2016). Several neuropsychiatric disorders such as Autism Spectrum Disorder and schizophrenia are shown to be comorbid with gut-related diseases and alterations in the gut microbiota (Sgritta et al., 2019). Given that addiction and substance use disorder are also neuropsychiatric disorders, the investigation of how the gut microbiota may play into these pathologies is warranted. Links between gut dysbiosis and neuropsychiatric disorders (Cryan and Dinan, 2012), including substance use disorder (Meckel and Kiraly, 2019), are currently under investigation. Recently a hypothesis for a neural circuit for gut-induced reward has been proposed, such that activity in the gut influences dopamine activity in the striatum via vagus nerve signaling (Han et al., 2018). This pathway passes from the vagal afferents through the right nodose ganglion to the lower

medulla then to the striatum and other parts of the midbrain. This pathway deserves detailed examination with regard to direct influence of gut health on addiction vulnerability.

2.9 Conclusions

Many biological functions have been mapped to dopamine circuits in the brain including arousal, attention, motivation, motor control, and executive function. Neuropsychiatric disorders and pharmacological substances affect the dopamine system by altering synthesis, trafficking, storage, release, reuptake, and/or postsynaptic signaling. With regard to reward and reinforcement, dopamine is a detector for salience and error. One main difference between natural rewards (food, water, sex, etc.) and addictive drugs (cocaine, amphetamine, nicotine, opioids, cannabinoids, alcohol) is that drugs of abuse elevate dopamine levels with great amplitude and duration, across a wider range of conditions, and without as much adjustment after repeated exposure to the same pharmacological stimulus. Thus, drugs are associated more readily with compulsive reward-seeking behavior, withdrawal symptoms, and dependence. Cocaine in particular amplifies dopamine signaling by binding to DAT and decreasing presynaptic reuptake, leaving dopamine in the synapse for longer periods of time and increasing dopamine transmission. While these effects are well-mapped in the brain, to what extent they occur in the periphery, especially in the gut, remains to be understood. There is evidence that dopamine synthesis and transmission occurs in the gastrointestinal tract. The presence of DAT in several parts of the GI tract suggest that cocaine can exert a direct effect on gut function. Altered dopamine signaling in the gut may exert additional indirect impact on reward brain circuitry via pathways that go through the spinal cord, synapse in the nucleus of the solitary tract (NTS), then proceed to the medulla and to parts of striatum. Also, indirectly through its sympathomimetic action, cocaine may alter the gut environment via altering peripheral blood flow, inducing

inflammation, and increasing intestinal permeability. All of these effects are likely to alter the gut milieu and may alter the profile of gut microbes that thrive under cocaine-influenced conditions. This altered profile may result in different levels of microbial metabolites which may then change gut-brain signals. Alterations of brain states may then lead to maladaptive drug-seeking behavior, leading the organism to consume more cocaine and perpetuating a vicious cycle of addiction. These potential mechanisms may contribute to the influence of the gut bacterial environment on long-term vulnerability to drug reward and reinforcement.

2.10 References

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3 EFFECTS OF A WHITE NOISE STIMULUS ON COCAINE SELF-ADMINISTRATION, EXTINCTION, AND CUE-INDUCED REINSTATEMENT IN ADOLESCENT AND ADULT MALE RATS

3.1 Abstract

Adolescent-onset of drug use is associated with deleterious addiction outcomes. In animal models, however, removal of aversive states has not been incorporated effectively into acquisition procedures, and compulsive drug-taking or -seeking remains relatively unexplored among adolescent subjects. We tested a novel method to support acquisition of lever-pressing in operant conditioning chambers, as well as a new approach to investigate compulsive drug-taking. Adolescent and adult male Wistar rats were catheterized and allowed to acquire lever-pressing reinforced by removal of a constant white noise stimulus (4 sessions, 2 hr each). Intravenous cocaine infusions were then added to removal of the white noise as a consequence of lever-pressing (2 sessions). Subsequently, white noise was paired with onset of cocaine infusions (PUNISHED) or removed entirely (QUIET). After forced abstinence, extinction responding and cue-induced reinstatement of lever-pressing were recorded. Removal of white noise supported the acquisition of pressing on the active lever, and cocaine increased rates of pressing in both age groups. Effects of white noise as a positive punisher to decrease lever-pressing were more robust in adults than adolescents, although overall cocaine intake was similar across age and treatment groups. White noise as a punisher did not alter extinction responding, but prior age differences with adults responding more than adolescents was replicated. Cue-induced reinstatement was reduced among punished rats, regardless of age. White noise as a negative reinforcer supports acquisition of lever-pressing, with minimal age differences. White noise as a positive punisher decreases lever-pressing and extends to lower rates of cue-induced reinstatement.

Note: Brett Daniel, Jillian Dawson, Rapheal Williams, Bonnie Williams, and Kyle Frantz are co-authors on the work contained in this chapter.

3.2 Introduction

Adolescent humans engage in more risk-taking behavior than other age groups (Eaton et al. 2006), and experiment the most with drugs of abuse (SAHMSA 2016). Longitudinal and epidemiological studies have found that most substance abusers initiated drug taking during their adolescent years (Chen and Kandel 1995; Palmer et al. 2009; Winters and Lee 2008), with early onset of drug misuse predicting subsequent substance use disorder as well as comorbid mental health disorders (Anthony and Petronis 1995; Stockwell et al. 2005). Cocaine, a psychomotor stimulant, is a highly abused drug among adolescents (Johnston et al. 2016), and the long-term consequences of adolescent cocaine intake may include behavioral, neurochemical, and structural changes in the nervous system (Kuhn et al. 2013; Squeglia et al. 2009; Wang et al. 2013). Using animal models such as intravenous (i.v.) drug self-administration and reinstatement after extinction and/or abstinence, both acute and long-term impact of adolescent exposure to drugs such as cocaine has been investigated. Some results suggest heightened sensitivity to cocaine reward and reinforcement among adolescents compared with adults (Anker and Carroll 2010; Kantak et al. 2007; Lynch and Carroll 2000). Conversely, others suggest some adolescent resistance or resilience (Ator and Griffiths 2003; Frantz et al. 2006; Griffiths et al. 1979; Schramm-Sapyta et al. 2009; Shram et al. 2008). It has also been argued that the stimulus triggering relapse may be responsible for age-dependent effects (Anker and Carroll 2010). Results from our laboratory suggest that adolescent-onset of cocaine, morphine, or heroin self-administration is associated with lower rates of lever-pressing during subsequent tests of extinction responding and/or reinstatement, compared with adult-onset (Doherty et al. 2013;

Doherty and Frantz 2013; Li and Frantz 2009). To replicate and extend this work, we explore herein a novel method to support the acquisition of lever-pressing in operant conditioning chambers and introduce a new approach to investigating compulsive drug intake among adolescent and adult male rats.

An essential element of self-administration studies is acquisition of the interaction between the subject and the operandum that allows access to the reinforcer, e.g. acquisition of lever-pressing that triggers i.v. drug infusions. Various acquisition procedures are common, including spontaneous encounters with the operandum, experimenter shaping of successive approximations of the target behavior, autoshaping procedures that periodically present and retract the operandum, food restriction and initial use of food reinforcers before transition to drug reinforcers, priming injections of the drug itself and/or stress stimuli presented before or during the acquisition sessions. Each acquisition procedure has strengths and weaknesses, with many of the weaknesses including unknown variability across age groups of interest, e.g. potentially greater effects of food restriction in adolescents vs. adults (unpublished data, Frantz Lab), with therefore unpredictable impact on subsequent drug intake. In our own experimentation, we preferred spontaneous acquisition (Doherty et al. 2012; Doherty et al. 2013; Li and Frantz 2009; Shahbazi et al. 2008b), but here we introduce the novel use of a white noise stimulus as a negative reinforcer of lever-pressing behavior in a new acquisition procedure. White noise has long been used as a constant background stimulus during self-administration sessions, except during the time out after reinforced lever presses, when it may have been turned off as part of a cue sequence associated with onset of drug infusion. Preliminary observations in our laboratory revealed, however, that removal of the white noise as a consequence of lever-pressing was actually reinforcing the acquisition of lever-pressing behavior itself, regardless of concomitant

drug presentation. We interpreted this outcome as evidence that the white noise was aversive, and its removal was serving as a negative reinforcer of lever-pressing. The present experiment uses white noise as a reinforcer for both adolescent and adult rats. Given that adolescents appear less sensitive to a variety of drug-related aversive stimuli, such as drug conditioned taste aversion and aversive drug withdrawal (Doherty and Frantz 2013; Hodgson et al. 2009; O'Dell et al. 2007; Schramm-Sapyta et al. 2006; Silveri Marisa and Spear Linda 2006) we hypothesized that adolescents would show lower levels of lever-pressing when reinforced by removal of a white noise stimulus, compared with adults.

Addiction is characterized by physical and emotional dependence on an exogenous substance, often including escalating drug intake over time and continued drug intake despite adverse consequences, with the latter known as compulsive drug-taking and drug-seeking (NIDA 2014). In order to model the compulsive drug-taking component of addiction, some self-administration procedures pair drug presentation with aversive stimuli, such as electric shock to the paws or i.v. histamine (Holtz and Carroll 2015; Katzir et al. 2007) in "punishment" models. A second aim of this study is to introduce the novel use of a white noise stimulus as a punisher of lever pressing behavior, and to use these new parameters to compare compulsive drug-taking across age groups. As above, removal of white noise was used as a negative reinforcer to promote acquisition of lever-pressing, but then it was subsequently eliminated from the background noise and used only as a discrete drug-paired cue, presented for 20 sec as a consequence of pressing on the active lever. Given lower sensitivity to aversive stimuli among adolescents compared with adults, we predicted that any attenuation of cocaine intake associated with the white noise "punisher" would be less robust among adolescents than adults.

Substance use disorder (SUD) is known as a chronically relapsing disorder, so we also explored extinction of lever-pressing in the absence of cocaine after various abstinence periods, as well as reinstatement triggered by re-exposure to environmental and discrete cocaine-paired cues (Bossert et al. 2013; Shaham et al. 2003). To our knowledge, the effects of drug-paired punishers on later extinction and reinstatement have not been reported. If adolescents were less sensitive than adults to the negative effects of a white noise punisher, we would expect that their extinction and reinstatement would be less affected by prior white noise exposure than it would in adults. These results would be coupled, however, with our prior outcomes that adolescents reinstate to lower levels of lever-pressing than adults after "forced abstinence" (Doherty et al. 2013; Li and Frantz 2009). We tested these possibilities by measuring extinction responding and cue-induced reinstatement in a within-session extinction and reinstatement procedure in separate rats at 1-, 14-, or 30-days after the last self-administration session.

3.3 Materials and Methods

3.3.1 Animals

Male Wistar rats (n=65 adolescents, n=65 adults; Charles River Laboratories, Inc, Raleigh, NC, USA) arrived at Georgia State University at either postnatal day 22 (adolescents) or postnatal day 70-74 (adults) and were pair housed in humidity and temperature controlled cages (Optirat Gen II by Animal Care Systems; Centennial, CO), with a reverse light cycle (12:12 hr, lights on at 19:00) and ad libitum access to food and water while in the home cages. Animals were weighed daily to assess general health, except during drug recess when they were weight twice per week (as below). All procedures were conducted in strict adherence to the Principles of Laboratory Animal Care and the National institute of Health Guide for the Care and Use of Laboratory Animals 8th edition (Guide for the Care and Use of Laboratory Animals. 8th edition,

2011) and approved by Georgia State University's Institutional Animal Care and Use Committee.

3.3.2 Surgery

After three days of acclimation to the vivarium, animals were transferred to a surgical suite for i.v. catheter implantation. Catheters were assembled as previously described (Roberts and Koob 1982) with minor modifications including a shorter length of tubing inserted into the jugular vein for adolescents (2 cm) compared with adults (4 cm) (Shahbazi et al. 2008a). All surgeries followed aseptic technique. Rats were anesthetized with 3-5% isoflurane and 1.5-3% isoflurane for induction and maintenance, respectively. Once a surgical plane of anesthesia was achieved, a small mid-scapular incision was made, followed by a ventral neck incision. The right jugular vein was exposed, pierced, and catheter tubing was pulled subcutaneously from the dorsal surface to the ventral incision. Tubing was then inserted directly into the jugular vein and secured with nylon suture thread above and below the insertion point. Incisions were closed with wound clips and animals received 0.2ml Timentin (ticarcillin disodium and clavulanate potassium; 100 mg/ml, i.v.), 0.2 ml heparinized saline (100 USP units/ 1ml, i.v.), topical bacitracin, and carprofen (5mg/kg, s.c.). Two control groups were included: catheterized animals that later received saline infusions instead of cocaine in the operant conditioning chambers (saline controls) and animals that went through all surgical procedures except the actual catheter implantation (sham controls). Animals received twice daily doses of Timentin and heparinized saline over three days post-op and then daily for the duration of self-administration to maintain catheter patency; adolescents received approximately half of the volume of adults.

3.3.3 Equipment

Behavioral tests were conducted in operant conditioning chambers enclosed in sound-attenuating, ventilated environmental cubicles (Med Associates, Inc., St. Albans, VT, USA). Animals were not provided food or water during self-administration sessions. To start each session, the house light was turned on and two levers were extended into the chamber. Presses on an inactive lever were recorded but had no scheduled consequences. Presses on the active lever triggered a syringe pump (variable speed, Med Associates, Inc., St. Albans, VT, USA) to deliver drug solution via a stainless steel swivel (Instech Laboratories, Inc., Plymouth Meeting, PA, USA) and polyethylene tubing attached to a catheter portal on each animal's back. Infusions of cocaine (0.36 mg/kg per infusion) were given to animals under a fixed ratio 1 schedule of reinforcement. Subjects were weighed daily, and infusion volume was adjusted by changing infusion duration, based on a standard 0.1 ml/4s per infusion for a 350-g rat, thereby providing 0.36 mg/kg cocaine per infusion. Saline controls instead received i.v. saline according to the same volume calculations, whereas SHAM controls were not attached to the drug delivery tubing. Each reinforced response lit a cue light above the lever, which stayed on for 2 sec. After a reinforced press the animals entered twenty seconds of time out (TO20) in which presses on levers were recorded with no scheduled consequences. The house light and cue light also were not illuminated during TO20. Two sets of infrared photo beams in the front and rear of the cage were used to record motor activity during the experiment. Drug delivery and data collection were controlled by Med Associates software (Med PC IV). The white noise produced by a generator in the chambers was used for multiple purposes. As above, it was ambient background noise that was turned off in response to active lever-pressing, as part of the reinforcement cue sequence. This occurred for the White Noise Training (WNT) sessions and the Loud Self-Administration

(LSA) sessions. In approximately half the rats, it was subsequently removed entirely from the programming (Quiet Self-Administration; QSA). In the other half, it was switched from ambient on/cue off to ambient off/cue on (Punished Self-Administration; PSA), as below.

Figure 3.1 Timeline of experimentation plots self-administration, abstinence, extinction, and reinstatement
Note that adolescent-onset groups were young adults by the time of extinction, reinstatement, and sacrifice.

3.3.4 Lever-press training with white noise as a negative reinforcer and transition to cocaine self-administration

Per the experimental timeline in Figure 3.1, white noise training (WNT) in the operant conditioning chambers began three days after surgery. All testing was done at the beginning of the dark phase with 2-hr daily sessions over four days. White noise was present in the chamber throughout the session, but pressing on the active lever turned off the white noise (and house light) for the duration of T020. Following the four days of WNT, animals underwent two days of "loud cocaine self-administration" (LOUD; LSA), in which pressing on the active lever not only turned off the white noise (and house light), but also triggered an i.v. infusion of cocaine.

3.3.5 Cocaine self-administration with white noise as a positive punisher

Upon the conclusion of LOUD conditions, animals underwent eight days of cocaine self-administration in either PUNISHED (PSA) or QUIET (QSA) conditions. In PSA conditions, sessions began with the house light on and levers extended, but no white noise. Active lever-presses resulted in a 2 sec burst of white noise, along with presentation of the cue light and a

cocaine infusion. The QSA conditions were the same as PUNISHED except that white noise was never turned on in the session. Saline and sham controls were tested only in QUIET conditions.

WNT and cocaine self-administration took place on weekdays only, with two-day recesses after the WNT and after the first three days of PSA or QSA. Catheter patency was confirmed in all subjects by full loss of muscle tone within 5 s of i.v. infusion of the short-acting anesthetic agent, 1% methohexital sodium, during these recesses and after the last self-administration session. Subjects that failed any catheter test were eliminated from the study.

3.3.6 Abstinence, Extinction, and cue-induced reinstatement

At the completion of self-administration, rats entered abstinence periods of either 1, 14, or 30 days, during which they were handled and weighed twice per week. At the end of abstinence, a within-session extinction and reinstatement test was conducted (Grimm et al. 2001; Grimm et al. 2003). Five 1-hr extinction sessions were separated by five-minute breaks during which the levers retracted, and the house light turned off. During extinction, rats were connected to the metal coil tether but not the infusion tubing; white noise remained off, and the house light remained on. Presses on the active lever were recorded, but no drug was infused, nor were any cues presented. SHAM controls where not attached to the tether. To allow adequate time for blood collection, perfusion, and brain extraction; initiation of extinction session start times were staggered approximately 25 min between animals. For cue-induced reinstatement, animals were tested in the same procedure as QUIET self-administration except no drug solution was infused and no syringe was attached to the pump. No white noise “punisher” stimuli were administered to any subjects, allowing test of the enduring impact of prior punishment, rather than acute effects of the punisher.

3.3.7 Corticosterone analysis

After the last behavioral test, blood samples were collected from the saphenous vein, centrifuged (20,000 RPM, 20 min, Eppendorf 5415R), and plasma stored at -20 °C until ELISA for corticosterone levels, according to the manufacturer's instructions (ab108821: Abcam; K014; Arbor Assays). Absorbance was read at 405 nm (Bio-Rad iMark Plate Reader). Internal quality controls were assessed using internal kit control, and plate control was assessed by conducting analysis in triplicate. Sample control was assessed by devoting two standards to serum taken from adolescent and adult rats that had neither cocaine nor behavioral testing.

3.3.8 Statistics

Self-administration data were analyzed via mixed-models analysis of variance (ANOVA) on active lever-presses, inactive presses, cocaine infusions, and total cocaine intake. The ANOVAs were 2x2x2x3 design with age (adolescents vs adults), drug (cocaine vs saline), and punishment (noise vs no noise) as between-subjects variables, and session (e.g. extinction sessions) as a within-subjects variables, as appropriate. For considerations across experimental phases, lever-pressing was averaged over days in each phase (WNT, LOUD, QUIET/PUN). Change in lever-pressing in LOUD vs. QUIET/PUN phases was also compared, using independent samples t-tests, as was total cocaine intake and plasma corticosterone. All data were analyzed using SPSS v.23 (SPSS, Chicago, IL). Post-hoc tests were conducted to identify individual differences. Alpha was set at 0.05. All Figures were generated using Graphpad/PRISM V.7.

3.4 Results

3.4.1 Active lever-presses

The two control groups (saline and sham) were compared over all self-administration phases by averaging active lever-press data over sessions in each phase. Separate t-tests with Bonferroni's correction revealed no differences in any of the phases (data not shown). Thus, saline and SHAM animals were collapsed into a single control group for all subsequent analyses. Overall differences between controls and cocaine groups were assessed using a four-way ANOVA on active lever-presses, which revealed a significant main effect of drug condition $F(1,123)= 16.12$, $p<.001$ as well as a drug x session interaction $F(1,123)=23.19$, $p<.001$. Data were averaged across phases and compared by individual t-tests with Bonferroni's correction to reveal no drug treatment effects during WNT $t(127)=-0.647$,$p=0.52$, but significantly higher presses among cocaine groups than controls during LOUD ($t(127)=8.60$,$p<0.001$) and during QUIET/PUNISHED ($t(127)=8.57$,$p<0.001$). These data show that all rats acquired lever-pressing to the same degree during WNT, but the introduction of cocaine supported reinforced presses on the active lever, whereas saline infusions (or no infusions) did not, as expected (Figure 3.2). Controls were not included in subsequent analyses.

With focus on subjects that entered the QUIET cocaine conditions (Figure. 3.2, left panels), a two-way age x phase ANOVA shows a main effect of phase ($F(2,84)=20.37$,$p<0.001$), but not age and no interaction. With data collapsed across ages, all phases are different from each other, such that WNT is lower than LOUD $t(96)=-8.92$, $p<0.001$ and lower than QUIET $t(96)=6.67$, $p<0.001$, and LOUD is greater than QUIET $t(96)=-7.83$, $p<0.001$).

On the other hand, for subjects that entered the PUNISHED cocaine conditions (Figure. 3.2, right panels), a two-way age x phase ANOVA shows a significant interaction

($F(2,102)=7.77$, $p=.001$), leading to individual t-tests for age differences in each phase, with adults greater than adolescents in WNT ($t(51)=-2.86$; $p=.006$) and LOUD phases ($t(51)=-2.30$;$p=.026$), but not once the punishment is actually introduced ($t(51)=-0.43$;$p=0.67$). Differences across phases within adolescents showed that WNT is lower than LOUD ($t(24)=-5.36$; $p<0.001$) and lower than PUNISHED ($t(24)= -4.56$; $p<0.001$), and LOUD is greater than PUNISHED ($t(24)=4.45$; $p<0.001$). Similarly, differences across phases with adults showed that WNT is lower than LOUD ($t(27)=-6.53$; $p<0.001$) and lower than PUNISHED ($t(27)=-3.21$; $p<0.001$), and LOUD is greater than PUNISHED ($t(27)=6.22$; $p<0.001$).

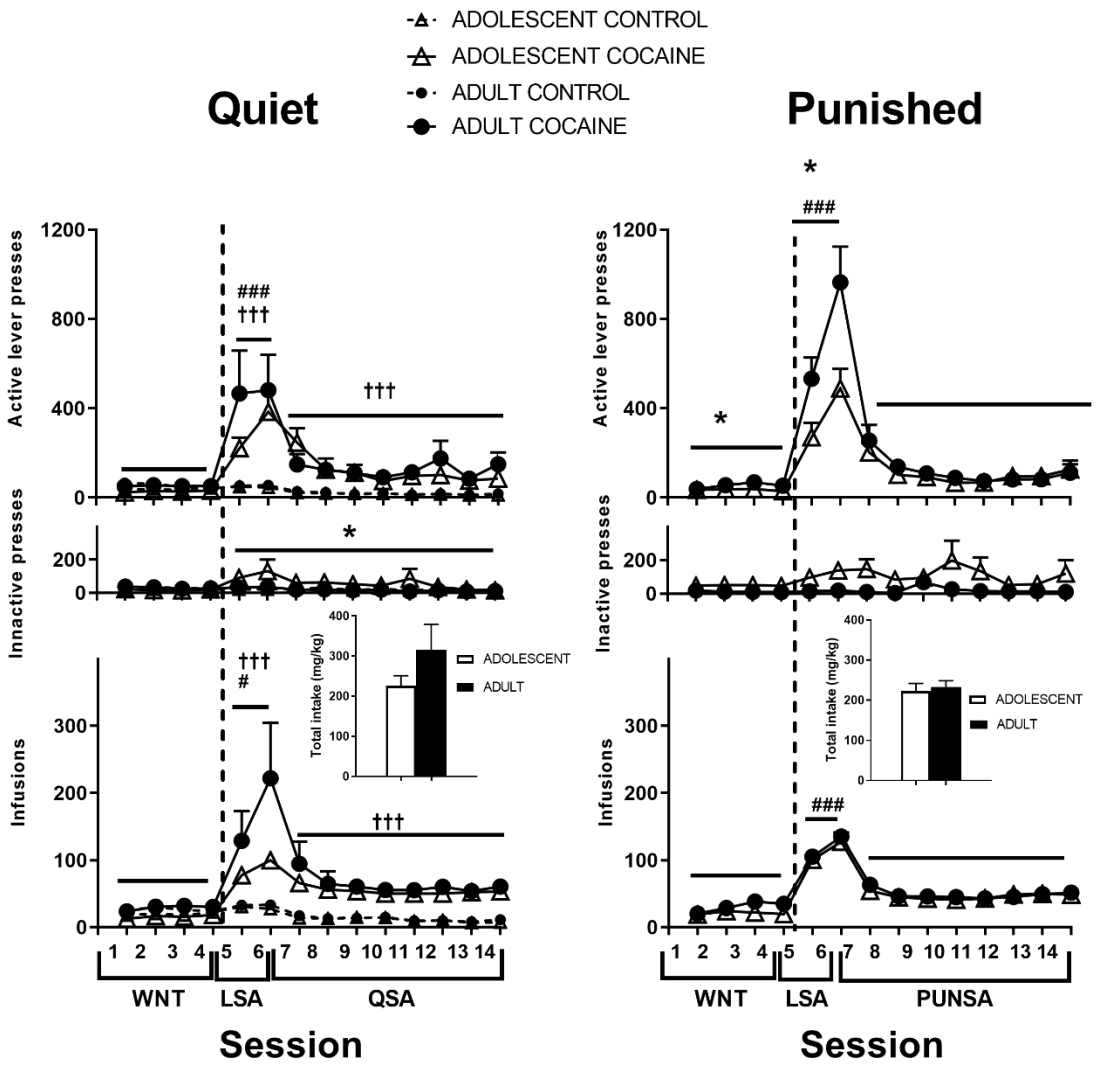

Figure 3.2 Lever-pressing and cocaine intake during two-hour test sessions

Panels a and b: Whereas no differences in lever-pressing during WNT were observed across treatment conditions, cocaine groups pressed more on the active lever than controls when cocaine was available (starting with LSA1), regardless of age or noise conditions (marked on Panel a: †††p<0.001). Lever pressing differed across the three phases of self-administration for both quiet and punished groups, with the highest rates of responding during LSA (###p<0.001), then QSA, then WNT. In the punished subgroup, adults pressed more than adolescents in WNT and LSA (*p<.05). **Panels c and d**: Inactive lever-pressing did not differ across cocaine groups vs. controls, although a main effect of age was observed, with adolescents pressing more than adults when cocaine was available (**marked on Panel c**: *p<0.05). **Panels e and f**: Cocaine groups earned more infusions than controls when cocaine was available (**marked on e**: †††p<0.001). Reinforced presses/earned infusions were higher in LSA than the other two phases (#p<0.05, ###p<.001), regardless of noise condition, but neither the number of infusions nor total cocaine intake differed across age groups. Points and bars represent mean ± SEM.

3.4.2 Inactive lever-presses

An overall four-way age x phase x drug x noise condition ANOVA on inactive lever-presses revealed main effects of age ($F(1,123)=5.73$, $p=0.018$), phase ($F(2,246)$, $p<0.001$), and drug ($F(1,123)=16.12$, $p<0.001$), as well as several two-way interactions: phase x age interaction ($F(2,246)=5.08$, $p=0.007$); phase x noise interaction ($F(2,246)=4.70$, $p=0.01$); and phase x drug interaction ($F(2,246)= 19.57$, $p<0.001$). Pursuing the age differences, adolescents pressed more than adults when cocaine was available, as evidenced by a two-way phase x age interaction among cocaine experienced groups only ($t(95)=2.83$;$p=0.006$, and subsequent t-tests with Bonferroni's correction on age differences during LSA and QUIET/PUNISHED phases ($p<.05$; Figure. 3.2, middle panels). Given that no main effect of drug condition was observed, nor any interactions with drug condition, we conclude that inactive lever-presses do not differentiate cocaine-experienced rats from controls, and they are not considered further.

3.4.3 Cocaine infusions and cocaine intake

Cocaine infusions were analyzed next, using "reinforced responses" as a substitute during WNT when no cocaine was available (Figure 3.2, bottom panels). For this variable, an overall four-way age x noise x drug x phase ANOVA revealed a main effect of drug condition ($F(1,123)=16.12$,$p<.001$), along with main effects of age ($F(1,123)=5.73$,$p=.018$) and phase ($F(1,123)=48.73$,$p<.001$), as well as phase x age, phase x drug, and phase x noise condition interactions. Data were collapsed across age and noise condition to compare cocaine vs. control groups in each self-administration phase separately and showed no significant differences in WNT ($t(127)=0.27$; $p=0.79$). Yet the cocaine group took more infusions than controls in the LSA phase ($t(127)=3.87$,$p<0.001$) and QUIET/PUN ($t(127)=7.15$,$p<.001$). These data suggest that all rats acquired lever-pressing to the same degree during WNT, but the introduction of cocaine

supported reinforced presses on the active lever, whereas saline infusions (or no infusions) did not, as expected. Controls were not included in subsequent analyses.

With focus on subjects that entered the QUIET cocaine conditions (Figure 3.2, bottom left), a two-way age x phase ANOVA revealed a significant main effect of phase ($F(2,84)=11.90$, $p<0.001$). Regardless of age, reinforced presses were lower in WNT vs. LOUD ($t(43)=-3.73$, $p=0.001$), WNT vs. QUIET ($t(43)=-5.93$, $p<0.001$), and higher in LOUD vs. QUIET ($t(43)=2.57$, $p=0.014$). These data indicate that adolescents and adults progressed similarly through the experimental phases of these QUIET conditions.

With focus on subjects that entered the PUNISHED cocaine conditions (Figure. 2, bottom right), a two-way age x phase ANOVA revealed a main effect of phase ($F(2,84)=11.90$, $p<0.001$), such that reinforced presses were lower in WNT compared to LOUD ($t(52)=-13.98$, $p<0.001$) and compared to PUNISHED ($t(52)=-6.54$, $p<0.001$), and higher in LOUD vs. PUNISHED ($t(52)=11.837$, $p<0.001$). As in QUIET conditions, the two age groups progressed similarly through these experimental phases. A two-way age x noise condition ANOVA on total cocaine intake (mg/kg) revealed no significant age differences (Figure 3.2 insets), as expected based on similar numbers of cocaine infusions.

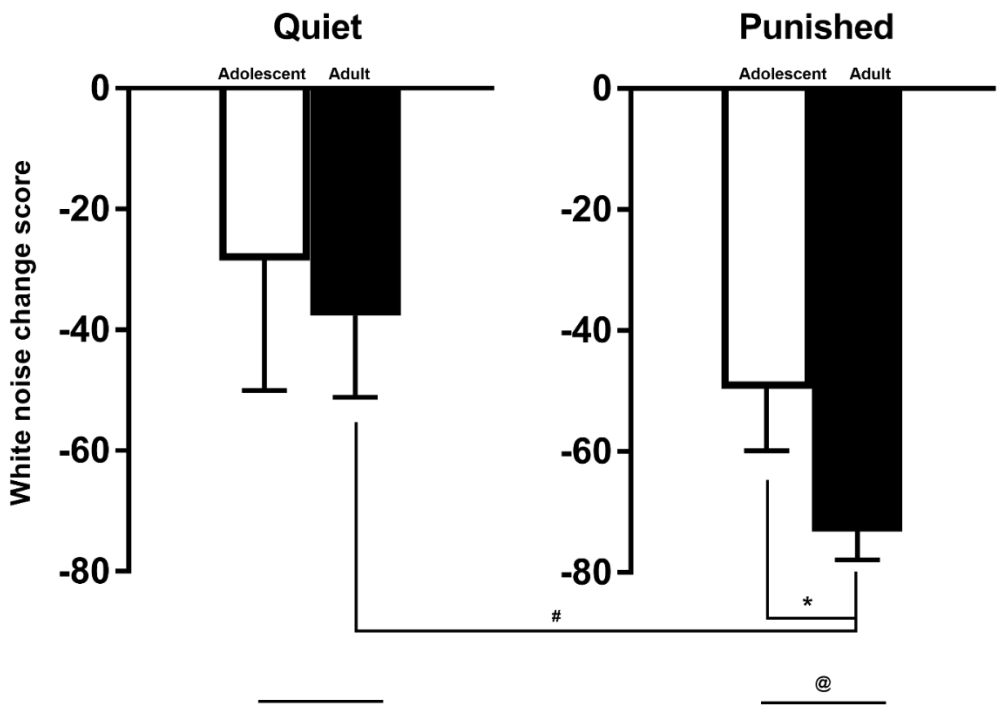

Figure 3.3 White noise change score, i.e. the decrease in lever-pressing in transition from LSA to QSA (Quiet) or PSA (Punished)
Animals that experienced white noise as a punisher had a greater change score compared to quiet animal counterparts (@p=0.038). Adults that received white noise as a punisher showed a significantly greater change score than adults in quiet conditions #p=0.021), and adults in the punished conditions had a greater change score than adolescents in the same punished conditions (*p=0.044).

A major focus of this investigation is the impact of changing the role of white noise from a negative reinforcer to a positive punisher, which occurred in half the subjects during the transition from the LOUD self-administration phase to the PUNISHED phase. In the other half of the subjects, white noise was simply eliminated in transition from LOUD self-administration to QUIET. Therefore, we calculated a Change Score according to the formula below and compared it using individual samples t-tests (Figure 3.3). The greater the change score, the greater the influence of the white noise transition on cocaine infusions. Animals that experienced white noise as a punisher had a greater change score compared to quiet condition counterparts ($F(1,93)=4.41$, $p=0.038$). Adults that received white noise as a punisher showed a significantly

greater change score, compared with adults in quiet conditions (t(23.53)=-2.48), p=0.021). Finally, adults in punished conditions showed a greater change score than adolescents in punished conditions (t(33.60)=2.09, p=0.044.

$$\left[\frac{(\overline{LSAVE} - \overline{PUN\ or\ Quiet\ Ave})}{\overline{LSAVE}}\right] \times -100$$

3.4.4 Extinction and reinstatement after abstinence

Cocaine seeking was assessed in five 1-hr extinction sessions followed by a single reinstatement session after 1, 14 or 30 days of forced abstinence (Figure 3.4). A 5-way age x drug condition x abstinence period x noise condition x session ANOVA on active lever presses during extinction revealed a main effect of drug ($F(1,111)=14.55, p<.001$), so control groups were eliminated from subsequent analyses. A follow-up 4-way age x noise condition x abstinence period x session ANOVA revealed a main effect of session ($F(5,190)=20.98, p<.001$) and an interaction between age x session ($F(5,190)=4.02, p=.002$), but no effect of abstinence period or noise condition. Within the subjects previously tested in QUIET self-administration conditions, no age effects were observed on the individual extinction session, nor the reinstatement session (Figure 3.4, left panels). Within the subjects from PUNISHED conditions, the only age effect within a single session was observed in the first extinction session after the 14-day abstinence period (Figure 3.4, right panels). Given no robust differences over abstinence period, data were collapsed and analyzed with a 2-way age x noise condition ANOVAs on total extinction and the single reinstatement session (Figure 3.5). Extinction testing revealed a significant main effect of age ($F(1,85)=7.18, p=0.009$) but not noise, such that adults made more extinction presses than adolescents overall. For cue-induced reinstatement, a significant main effect of noise ($F(1,85)=10.73, p=0.002$) but not age was observed, such that the prior QUIET conditions supported greater rates of reinstatement than the prior PUNISHED conditions.

3.4.5 Plasma corticosterone

By the end of experimentation, CORT levels (pg/ml) did not differ significantly by age, noise, drug treatment, or abstinence period (data not shown). For example, for all quiet condition rats with viable blood samples for analysis, the mean was 317.3 +/-38.8 (n=46), whereas for all punished condition rats with viable blood samples, the mean was 269.6 +/-28.4 (n=40). Parsing the groups by age instead, adolescents showed 287.04 +/-31.3 (n=51), whereas adults showed 306.88 +/-40.1 (n=35). Data not shown.

3.5 Discussion

The present experiments test the role of white noise as a negative reinforcer during acquisition of lever-pressing and as a positive punisher in a new model of compulsive drug-seeking. The initial phase of the self-administration procedure, white noise training (WNT), demonstrates that removal of white noise is effective at promoting the acquisition of lever-pressing in an operant conditioning chamber. When removal of white noise was combined with cocaine infusions as potential negative and positive reinforcers of lever-pressing, respectively, the number of presses increased significantly, as expected, again with significant age differences only in the group that would subsequently receive white noise punishment.

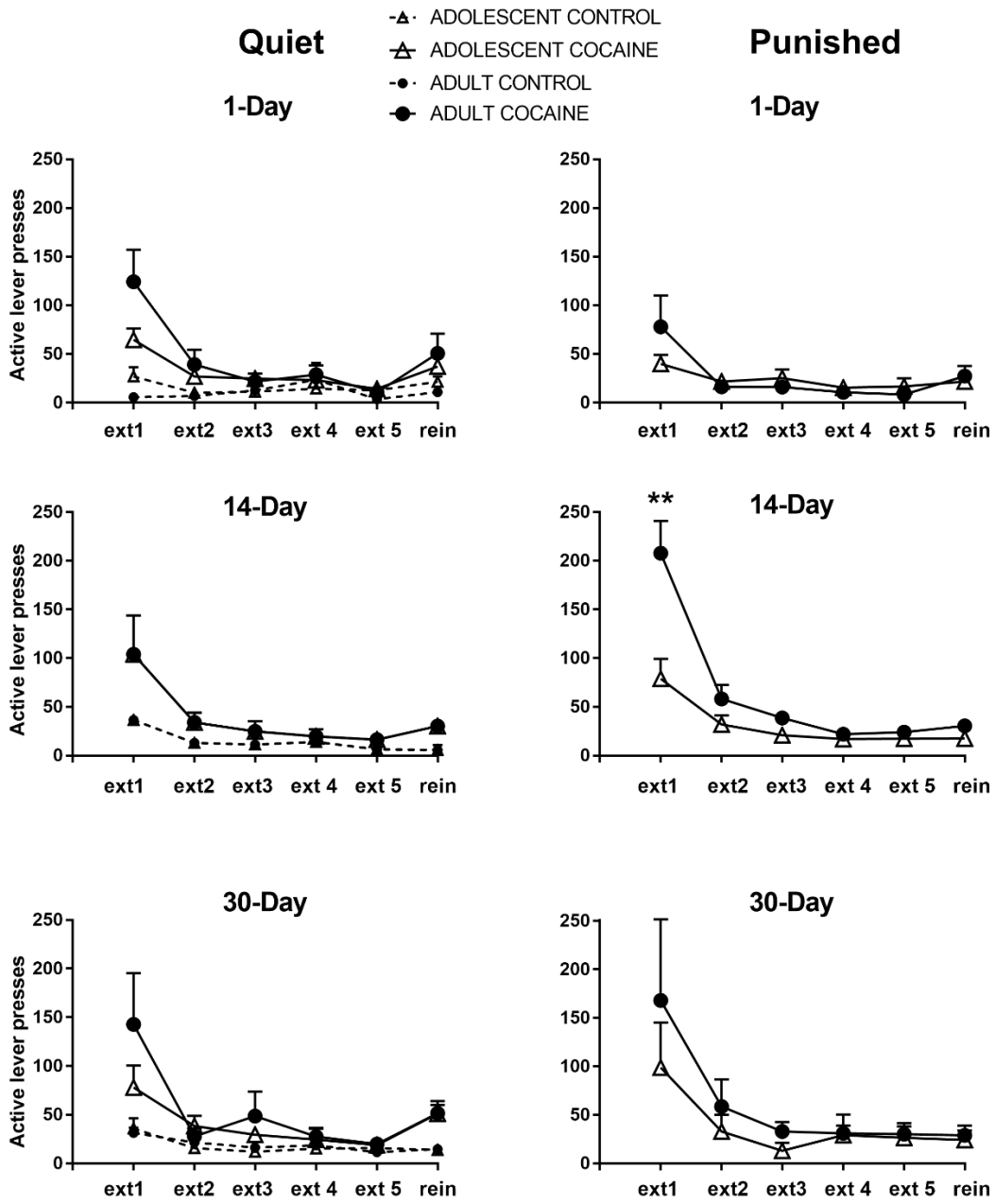

Figure 3.4 Animals that self-administered cocaine had greater extinction responding ($p<0.01$) and reinstatement ($p<0.01$) than control rats

Among those that self-administered, adults pressed more than adolescents in the first hour of extinction, regardless of abstinence period or noise conditions (EXT1; main effect of age not marked, $p<0.001$). Quiet conditions were associated with higher levels of reinstatement than punished conditions (REIN; main effect of noise condition not marked, $p<0.001$). Specific interactions were observed at 14-day abstinence in the punished conditions (**$p<0.01$). Points represent mean ± SEM.

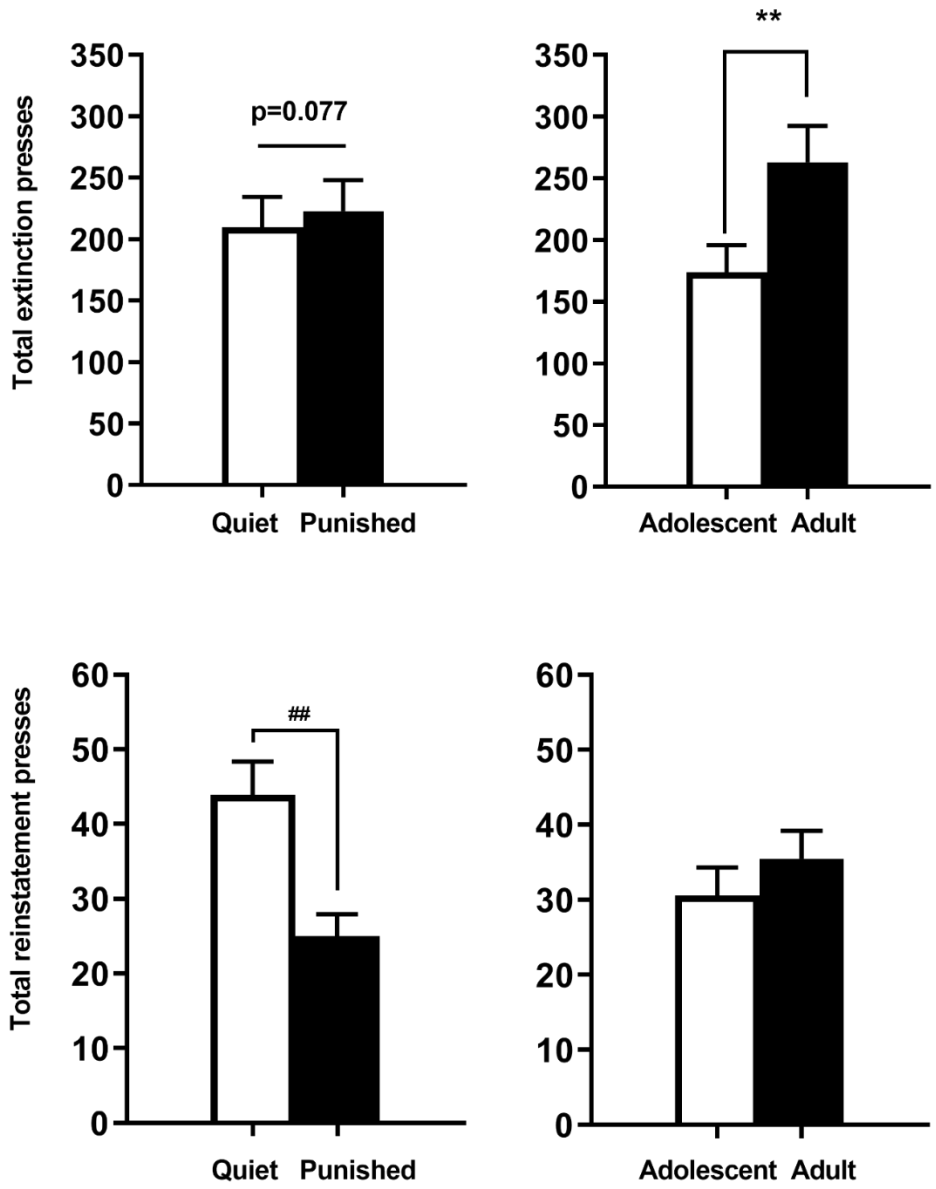

Figure 3.5 Total active lever-pressing in extinction and reinstatement averaged over abstinence periods

In extinction, adults (black bars) pressed more than adolescents (white bars) in punished conditions (**$p<0.01$) and tended to press more in quiet conditions ($p=0.077$). In reinstatement, animals in quiet conditions pressed more than those in punished conditions, regardless of age or abstinence period (##$p<0.01$).

Testing white noise as a positive punisher in the next phase of the experiment produced interesting results; among adults but not adolescents, white noise punishment (PUNISHED) was associated with a greater drop (change score) in lever-pressing than simple removal of the white

noise stimulus (QUIET). The punishment-associated change score was also greater in punished adults than in punished adolescents, supporting our hypothesis that adolescents are less sensitive to the aversive white noise stimulus, while suggesting that acute punishment can suppress drug intake. Moreover, the impact of punishment extended into cue-induced reinstatement of lever-pressing after abstinence, such that rats in the punished group pressed less than those in the quiet conditions, regardless of age group, a result that has important translational implications.

White noise training is a new option for the acquisition phase of operant conditioning. The lever-pressing reinforced by removal of a constant auditory stimulus showed consistency over sessions, discrimination between active and inactive levers, and rapid transition to a new reinforcer (cocaine) after two sessions of simultaneous presentation (LOUD). Levels of cocaine intake during loud conditions were consistent with previous reports from our laboratory when the removal of white noise was used as a discriminative stimulus signaling time out (Li and Frantz 2009). With regard to inactive lever-pressing, higher rates among adolescents compared with adults during drug self-administration was also previously reported (Doherty and Frantz 2012). Overall parallel outcomes across age groups in the rates of cocaine intake are also consistent with our prior reports using similar parameters. Therefore, white noise training can be added to the existing battery of acquisition procedures, with the novel benefit of demonstrating the aversive nature of the auditory stimulus in the same subjects that could be punished with this stimulus in a subsequent experimental phase. Optimizing acquisition procedures has been a challenge since the inception of experimental models of self-administration, with numerous other approaches explored and debated (Carroll and Meisch 2011; Smith et al. 2014).

White noise is also shown here as a viable punisher in models of compulsive drug-seeking. The present results are conceptually similar to prior reports with electric shock to the

paws or i.v. histamine (Holtz and Carroll 2015; Katzir et al. 2007). These "punishment" models may enhance the face validity of research in this area, because they incorporate some element of the negative outcomes often faced by human addicts, such as medical, social, financial, and legal consequences of drug use and abuse (Blanchard et al. 2003; Treatment 2004).

The inference in these procedures that white noise is aversive has support from prior applications of auditory stimuli in various models and approaches. Noise, by definition, is unpleasant, unwanted sound, and has long been recognized as a stressor, with wide-ranging deleterious effects on health and well-being, mediated by activation of sympathetic and/or endocrine systems (Cucurachi et al. 2012). In laboratory settings, white noise at 92 or 102 dB was used in aversive conditioning with human participants, who also filled out an anxiety questionnaire confirming discomfort in white noise conditions (Austin and Duka 2010; Austin 2010). In rodents, auditory stimuli, including white noise, have been investigated as alternatives to electrical shock in aversive conditioning models. For example, in the presence of background, masking noise of 65 dB, a broadband sound at 105 or 125 dB suppressed lever-pressing maintained by food pellet presentation in rats (Reed and Yoshino 2008). Although we are not aware of other reports on the use of white noise to shape behavior in adolescent rats, exposure of adolescent rats to a high amplitude noise resulted in long-term deficits in hippocampal neurogenesis (Jáuregui-Huerta et al. 2011), and adolescents were more sensitive than adults to an intensive light stressor (Slawecki 2005). The present results were conducted under conditions of approximately 74 dB background sound from airflow systems and operant chambers, elevating to approximately 85 db during the white noise stimulus. Age differences under these stimulus conditions occurred only in one subgroup of subjects, suggesting that the settings were aversive but not as intense as the sound or light stressors in prior reports. Indeed at least by the time

plasma samples were collected at the end of the present study, no significant differences in CORT were observed across age groups or noise conditions (data not shown). Although tested here in presumably prepubescent males, WNT would need to be tested in females, especially given suggestions that white noise stress has opposing effects on pain sensitivity in male and female rats (Khasar et al. 2005).

In the transition of white noise from negative reinforcer to positive punisher, the present adolescents were less sensitive than adults, as demonstrated by the lesser change score. These results are consistent with other data suggesting that adolescent subjects are less sensitive than adults to aversive conditions such as drug withdrawal, taste aversion, fear conditioning, and pharmacological stressors (Doherty and Frantz 2013; Holtz and Carroll 2015; O'Dell et al. 2007; Spear 2011; Spear and Varlinskaya 2005). Yet nuances in the present data set call for further exploration. Although significant in only the punished subgroup, lower lever-pressing among adolescents compared with adults in both the WNT and LSA conditions of the present experiment contributed to the lesser change score among adolescents in the transition to punished conditions, calling into question whether the negative reinforcing properties of the white noise or the positive punishing properties of the white noise are the less robust in adolescents. In either case, however, the age difference relies on the aversive nature of the noise. While these results and others cited above suggest that lower sensitivity to aversive stimuli may make adolescents less sensitive than adults to negative reinforcement as a driving force on drug-seeking, adolescents may also be less sensitive to drug-related consequences that essentially serve as punishers. The balance between sensitivity to reward vs. sensitivity to punishment has long been considered in the context of gating disorders, including drug and alcohol misuse, eating disorders, and gambling (Bijttebier et al. 2009; Ernst et al. 2011; Harrison et al. 2010;

Soder et al. 2020), and adolescents have been characterized as having a stronger reward sensitivity compared with harm-avoidant sensitivity (Ernst et al. 2006). In terms of underlying mechanisms, the prefrontal cortex plays a key role in behavioral inhibition (Geier et al. 2009; Halladay et al. 2019; Velanova et al. 2008), shows specific changes that distinguish between punishment-sensitive or -insensitive rats (Blackwood et al. 2020), and is still maturing in adolescence (Casey et al. 2008; Giedd 2004; Giedd et al. 1999; Shapiro et al. 2017; Van Eden and Uylings 1985). On the other hand, differential recruitment of striatal subregions across age groups may be even more consistent than differential prefrontal activation, based on human neuroimaging (Luna et al. 2015).

Despite the ability of white noise to reduce lever-pressing, its overall effects on acute cocaine intake were not significant, as rates of reinforced lever-pressing leveled out similarly over eight days of QUIET vs. PUNISHED self-administration, regardless of noise condition or age. These results contrast some other investigations of acute punishment, such as the ability of co-administration of histamine with cocaine to decrease acute cocaine intake, along with subsequent extinction and reinstatement of drug-seeking after abstinence (Holtz and Carroll, 2015) or the pairing of footshock with drug infusions or alcohol availability that reveals punishment-sensitive subgroups of rats or mice (Barnea-Ygael et al. 2012a; Barnea-Ygael et al. 2012b; Katzir et al. 2007; Le et al. 1999). Again, we conclude that the white noise is aversive, but not as robust compared to other punishers of cocaine behavior. Rats in the PUNISHED conditions pressed less than their QUIET counterparts when cocaine-associated discrete cues were re-presented after abstinence of up to 30 days. These results suggest that even though the acute effects of the white noise punishment on cocaine intake were negligible, a latent effect emerged after abstinence and extinction. The extinction phase itself confirmed age differences

reported by our group and others, such that adolescent-onset groups pressed less than adults (Doherty et al. 2013; Holtz and Carroll 2015; Li and Frantz 2009). Mechanisms for a latent effect of punishment remain to be explored. In studies on pain sensitivity, though, intermittent sound stress enhances pharmacologically-induced hyperalgesia (Khasar et al. 2008), adding another possible dimension to the enduring impact of noise exposure. White noise in the self-administration sessions may have elevated sensitivity to other aversive stimuli later in the experimental timeline, such as the anhedonic state of cocaine withdrawal, making rats more likely to reinstate drug-seeking as driven by negative reinforcement processes that would alleviate withdrawal through reinstatement of drug intake. Moreover, the lack of time-dependent increases in reinstatement of drug-seeking (incubation) suggests an additional benefit of punishment. Incubation was not observed after quiet conditions either, though, so this could be a long-term impact of the white noise training, rather than a punishment effect, used previously.

Enduring effects of the white noise punisher were observed during the reinstatement phase of testing. Rats in the PUNISHED conditions pressed less than their QUIET counterparts when cocaine-associated discrete cues were re-presented after abstinence of up to 30 days. These results suggest that even though the acute effects of the white noise punishment on cocaine intake were negligible, a latent effect emerged after abstinence and extinction. The extinction phase itself confirmed age differences reported by our group and others, such that adolescent-onset groups pressed less than adults (Doherty et al. 2013; Holtz and Carroll 2015; Li and Frantz 2009). Mechanisms for a latent effect of punishment remain to be explored. In studies on pain sensitivity, though, intermittent sound stress enhances pharmacologically-induced hyperalgesia (Khasar et al. 2008), adding another possible dimension to the enduring impact of noise exposure. White noise in the self-administration sessions may have elevated sensitivity to other

aversive stimuli later in the experimental timeline, such as the anhedonic state of cocaine withdrawal, making rats more likely to reinstate drug-seeking as driven by negative reinforcement processes that would alleviate withdrawal through reinstatement of drug intake. Moreover, the lack of time-dependent increases in reinstatement of drug-seeking (incubation) suggests an additional benefit of punishment. Incubation was not observed after quiet conditions either, though, so this could be a long-term impact of the white noise training, rather than a punishment effect.

In summary, our data suggest that white noise can be used as both a negative reinforcer and positive punisher in a rodent model of drug self-administration. White noise at a moderate amplitude can be used to promote acquisition of lever-pressing, whereas switching the role of white noise from negative reinforcer to positive punisher can attenuate cocaine-seeking behavior. Both of these acute effects of aversive white noise are slightly stronger in adults than adolescents. At the point of reinstatement after abstinence, though, both adolescent-onset and adult groups showed lower levels of drug-seeking after punished conditions compared with quiet conditions, suggesting that punishment has latent and enduring effects on cocaine-seeking. Further research with these parameters may reveal new approaches to addiction treatment that involve mildly aversive sensory stimuli.

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4 THE GUT MICROBIOTA MILLIEU IS ASSOCIATED WITH COCAINE BEHAVIOR VARIABILITY AND PREDICTS SEVERITY OF FUTURE COCAINE USE IN ADULT MALE RATS

4.1 Abstract

Bacterial communities in the gut participate in a gut-brain axis that influences the nervous system. Disruption in the microbial composition is associated with neuropsychiatric disorders, including drug abuse. It remains unknown, however, whether gut microbial profiles can predict an addiction phenotype before it emerges, or reflect drug experience after it occurs. This study used behavioral data and biological samples from the Cocaine Biobank to test the hypothesis that the gut microbiota can predict and reflect susceptibility to cocaine reinforcement. Adult male rats were catheterized and allowed to acquire lever-pressing maintained by intravenous cocaine infusions in 2-hr daily sessions (10 days), followed by progressive ratio (PR) testing. Rats were transitioned to long-access daily sessions (6-hr each, 14 days), also followed by a PR test and alternating blocks of footshock testing, long-access, and PR. Fecal samples were collected at three time points, and sequenced using NGS approaches to test for microbiota differences. Specific taxa identified from baseline samples were used as inputs to predict the future cocaine susceptibility in naïve animals. As expected, rats varied in levels of cocaine-related behavior, such that a quartile split identified high and low responders on each measure and an overall addiction index. Although beta diversity at baseline and after short access did not predict membership in high or low addiction quartiles, linear discriminant analysis (LDA) identified specific taxa that were robustly represented in either low or high responders. Beta diversity after long access revealed a difference in microbiota profiles between high and low responders using multiple addiction indices. Again, LDA identified robust bacterial differences

between groups. Plotting baseline samples identified using LDA on a Receiver Operating Characteristic (ROC) curve predicted whether a drug naïve animal would become a future low or high responder. This study is the first to report that microbiota variability reflects variability in cocaine intake and that the microbiota might be used as a diagnostic tool to predict the likelihood of future drug use.

Note: Sierra Simpson, Giordano de Guglielmo, Jennysue Kasiah, Benjamin Anthony, Oliver George, Benoit Chassaing, and Kyle Frantz all contributed to the following experiments.

4.2 Introduction

Substance use disorder (SUD) affects more than 25 million Americans (Bercik et al., 2010), leads to mental, physical, and behavioral deficits (NSDUH, et al. 2013) and yet continues without effective treatments (Fischer et al., 2015; Simpson et al., 1999). Despite the prevalence of SUD, only about 15-30% of individuals who initiate drug use develop chronic use habits and addiction (Wang et al., 2012b; Anthony et al., 1994; Saunders and Robinson, 2013; Franken et al., 2000). The factors that underlie addiction vulnerability are still poorly understood, stimulating continued investigation of novel approaches to treat and prevent drug abuse and drug relapse.

The gut-brain axis is a bi-directional communication pathway between the gastrointestinal tract and the central nervous system (Martin et al., 2018), that is involved in several neuropsychiatric disorders (Kim and Shin, 2018), including substance use disorder (Meckel and Kiraly, 2019; Dinan and Cryan, 2017). The gut microbiota carries out metabolic functions, contributes to immune responsivity, produces molecules that signal locally, activates neuronal projections to the brain, and enters the bloodstream for distribution throughout the body (Cryan et al. 2012). Variability in microbial communities among subpopulations of humans and

other animals may contribute to individual differences in disease, symptomatology, and/or general behavioral characteristics, but the ability of microbial profiles to predict vulnerability to addiction has not been explored.

The gut microbiota is different among individuals abusing alcohol (Engen et al., 2015a), opioids (Wang et al., 2018), or cocaine (Cho and Blaser, 2012; Volpe et al., 2014), compared with healthy controls, but whether the drugs induced the change or the distinct microbiome pre-existed the drug use is not clear. Diet, stress, and other environmental or genetic factors could alter the gut microbiota (Karl et al., 2018; Lyu and Hsu, 2018; Wen and Duffy, 2017), increasing drug sensitivity. Alternatively, gut inflammation, reduced blood flow, and/or ulcers associated with drug use could be mechanisms through which drugs of abuse such as cocaine cause dysbiosis in the gut microbiome (Chievero et al 2019). With regard to opioid intake, gut microbial depletion via non-absorbable oral antibiotics results in widespread changes in neuronal ensembles that are activated by oxycodone intoxication and withdrawal. This suggests that the gut microbiome may play a role in opioid use and dependence (Simpson et al. 2020). In mice, antibiotic-induced gut dysbiosis is associated with elevations in cocaine reward, perhaps related to reduced production of short chain fatty acids (Kiraly et al., 2016), a concept supported by methamphetamine reward among rats as well (Ning et al., 2017). These reports and others drive continued exploration of the gut-brain axis in cocaine-related behaviors.

The present study tested the hypothesis that drug use and microbiota composition are linked in such a way that microbiota profiles can predict susceptibility to drug use and that drug use can alter microbiota composition. Specifically, we predicted that the gut microbial communities characterized through fecal sample analysis would be different between addiction-resistant and addiction-prone adult male rats, with addiction vulnerability defined by a battery of

cocaine-related behavioral tests including self-administration, escalation, compulsive drug-seeking, and irritability.

4.3 Materials and Methods

4.3.1 Animals

Male heterogenous stock (HS) rats were provided by Dr. Leah Solberg (Wake Forest University School of Medicine). Rats were housed two per cage on a reverse 12 hour/12 hour light/dark cycle (lights off at 8:00 AM) in a temperature (20–22°C)- and humidity (45–55%)-controlled animal facility with ad libitum access to water and food. All experiments were designed to minimize animal suffering. All of the procedures were conducted in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute and the University of California San Diego

4.3.2 Drugs

Cocaine HCl (National Institute on Drug Abuse, Bethesda, MD) was dissolved in 0.9% saline (Hospira, Lake Forest, IL) at a dose of 0.5 mg/kg/0.1 ml infusion and self-administered intravenously.

4.3.3 Intravenous catheterization

The animals were anesthetized by isoflurane inhalation, and intravenous catheters were aseptically inserted in the right jugular vein using a modified version of a procedure that was described previously (47, 48). The vein was punctured with a 22-gauge needle, and the tubing was inserted and secured inside the vein by tying the vein with suture thread. The catheter assembly consisted of an 18 cm length of Micro-Renathane tubing (0.023-inch inner diameter, 0.037-inch outer diameter; Braintree Scientific, Braintree, MA, USA) that was attached to a

guide cannula (Plastics One, Roanoke, VA, USA). The guide cannula was bent at a near right angle, embedded in dental acrylic, and anchored with mesh (2 cm square). The catheter exited through a small incision on the back, and the base was sealed with a small plastic cap and metal cover cap. This design helped to keep the catheter base sterile and protected. The catheters were flushed daily with heparinized saline (10 U/ml of heparin sodium; American Pharmaceutical Partners, Schaumburg, IL, USA) in 0.9% bacteriostatic sodium chloride (Hospira, Lake Forest, IL, USA) that contained 20 mg/0.2 ml of the antibiotic Cefazolin (Hospira, Lake Forest, IL, USA). After recovery from surgery, rats were tested in several behavioral assays, per the experimental timeline (Figure 4.1). All behavioral testing was conducted during the dark phase.

4.3.4 Operant training

Self-administration was performed in operant chambers (Med Associates, St. Albans, VT, USA). Each chamber was equipped with two retractable levers. Cocaine was delivered through an infusion pump that was activated by responses on the right lever (active), resulting in the delivery of cocaine (0.5 mg/kg/0.1 ml). Responses on the left lever (inactive) were recorded but had no scheduled consequences. The rats were first trained to self-administer cocaine under a fixed-ratio 1 (FR1) schedule of reinforcement in daily 2-h sessions, for 10 days. A cue light was paired with each cocaine reward for 20-s. During this timeout (TO) period, lever presses were not followed by any cocaine infusion. After the completion of the short access period, the rats were subjected to daily 6-h long access (LgA) self-administration sessions to allow them to escalate their cocaine intake (Ahmed and Koob, 1998) over 14 days.

4.3.5 Progressive Ratio

At the end of the ShA and LgA phases, rats performed a progressive ratio test. The progressive ratio (PR) schedule of reinforcement was used to assess the break point, a valid

measure of the reinforcing value of a reward (Stafford et al., 1998; Hodos, 1961). Following acquisition of cocaine (10 sessions) the rats were also tested in a progressive ratio (PR) schedule of reinforcement. Under these conditions, the response requirements necessary to receive a single drug dose increased according to the equation: $[5e^{(\text{injection numbers} \times 0.2)}] - 5$. This resulted in the following progression of response requirements: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, etc. The breakpoint was defined as the last ratio attained by the rat prior to a 60 min period during which a ratio was not completed. The performance under progressive ratio was repeated two times after the LgA phase.

4.3.6 Compulsivity- like behavior

The day after the last extended access to cocaine self-administration and PR, the animals were placed in the self-administration chamber for a 1 h session, and they were tested for compulsive-like behavior. In this experiment, the rats were allowed to self-administer cocaine on an FR1 schedule of reinforcement, in which 30% of the reinforced responses were paired with a contingent footshock (0.1 mA, 0.5 s). After completion of this phase, the same procedure was used every 3 days, with the only difference that the intensity of the footshock was increased to 0.2 mA, 0.5 s and 0.3mA, 0.5s.

4.3.7 Feces collection

At times marked in Figure. 1a, animals were held by the base of the tail so that the front paws were on a solid surface and the back paws are able to be lifted off the surface. The back paws were gently moved up and down off of the surface until a fresh fecal pellet was released. Immediately the feces were collected into a sterile tube and put on dry ice. Once all feces were collected, the tubes were moved to -80°C for storage, then shipped overnight to Georgia State University's Neuroscience Institute for storage at -80°C until use.

4.3.8 Fecal microbiota analysis by 16S rRNA gene sequencing

DNA was extracted from frozen feces using QIAamp 96 Powerfecal QIAcube HT Kit (Quiagen) with mechanical disruption (bead-beating). Fecal microbiota was analyzed as previously described (Chassaing et al., 2015). Briefly, the 16S rRNA genes, region V4, from each sample were PCR amplified using a composite reverse primer and a forward primer containing a unique 12-base barcode used to tag PCR products from respective samples. PCR products were purified with magnetic beads (Beckman Coulter), visualized by gel electrophoresis and quantified using Epoch Microplate Spectrophotometer (Biotek). A master DNA pool was generated and sequenced using a Illumina MiSeq (2*250 bp, paired end) sequencer at the Cornell University.

4.3.9 Genetic sequence analysis

16S rRNA gene sequence analysis was performed as previously described (Chassaing et al., 2015). Sequences were demultiplexed, quality filtered using the Quantitative Insights Into Microbial Ecology (QIIME) software package (Caporaso et al., 2012), and forward and reverse Illumina reads were joined using the fastq-join method (http://code.google.com/p/ea-utils) (Aronesty, 2013). QIIME default parameters were used for quality filtering and sequences were assigned to OTUs using the UCLUST algorithm (Edgar, 2010) with a 97% threshold of pairwise identity and classified taxonomically using the Greengenes reference database (McDonald et al., 2012). Principal coordinate analysis of the unweighted UniFrac distances was used to assess the variation between samples (beta diversity) (Lozupone et al., 2011). Beta diversity was further visualized via jackknife-supported PCA plots. Alpha diversity and rarefaction curves were calculated and displayed using observed OTUs as the variable of interest. OTU sorting and

sequencing analysis has been previously validated and adapted from previous work in rats (Fields et al. 2018).

4.3.10 Identification of bacteria predicting drug use

Predictive analysis was conducted by generating Receiver Operating Characteristic (ROC Curves) and area under the curve (AUC), as adapted from (Sokol et al., 2019) using the new multiple logistical regression tool in updated Graphpad 8 PRISM (v 2019). In brief, regression plots of species relative abundance was plotted against bacteria present in sample (binary: yes/no). Artifacts due to phylogeny differences were discarded and present curves represent lowest similar taxonomic identification. ROC curve quality was assessed based on previous standards (Mandreka et al. 2010). Curves with AUC less than 0.5 were excluded from analysis.

4.3.11 Statistics

Statistics on behavioral outcomes, including median splits and z-score calculations were conducted with IBM SPSS v. 23, and corresponding behavior graphs were generated using PRISM 7/Graphpad software (San Diego, California, USA). ROC curves were generated using the latest plugin application in Prism/Graphpad (v.8.3.0). Generation of robust bacterial differences between groups, using Linear discriminant analysis Effect Size (LEfSe) was accomplished using the online Galaxy tool (http://huttenhower.sph.harvard.edu/galaxy/). Galaxy was also used to generate Linear discriminant analysis (LDA) bar charts and corresponding cladograms. Determination of predictive biomarkers was accomplished via a combination of LEfSe baseline output data, highly abundant taxonomic groups observed in the present samples, and from the literature on bacterial groups previously associated with substance use disorder or related conditions. Diversity plots and relative abundance stacked bar charts were generated via

QIIME. Differences in distinct clustering in PCA plots was assessed via PERMANOVA method using vegan R-package through QIIME.

4.4 Results

4.4.1 Identification of low- and high-vulnerability subpopulations based on variability in behavioral outcomes

Rats self-administered cocaine under four different experimental conditions, per the timeline in Figure 4.1a. In each condition, variability in response rates allowed division of subjects into low vs. high responder subgroups, using a quartile split separately for each measure (Figure. 4.1 b) During short access self-administration (2 h/day, the number of cocaine infusions (rewards) earned per session was between 0 and 90, with an average of 6.13 +/- 6.17. The bottom 25% of animals, labeled as low or resistant responders, had an average of 0.97 +/- 0.1 rewards earned per session. The top 25% of animals, labeled as high or vulnerable responders, had an average of 16.3 +/- 1.21 per session. The behavior split between all animals divided into quartiles is shown by session (Figure 4.1 a) During long access/ escalation self-administration (6 h/day), cocaine infusions ranged from 0 to 277 with an average of 77.14 +/- 1.59. Low responders had an average of 27.13+/- 2.15 infusions per session whereas high responders had 121.8 +/- 2.05 infusions per session. The behavior split between all animals divided into quartiles is shown by session (Figure e 4.1 b). During PR, we observed that all animals except for low responders had higher presses when PR was conducted after LgA compared to ShA (Figure 4.1 b). This trend was not observed in total active lever presses as only animals binned in the top 50% of responders had higher PR presses after LgA compared to Sha. In animals that were binned into the bottom 50% of responders PR did not different between session day. Low responder animals had an average 1.6+/- 0.33 reward infusions and 3.47 +/- 0.68 active lever presses during PR.

High responders had an average of 9.77 +/- 0.89 reward infusions and 49.7 +/- 7.39 active lever presses during PR. Under intermittent shock conditions, the number of cocaine infusions decreased as the shock intensity increased, but high vs. low responders were differentiated at each shock value (Figure 4.1 c). To summarize, distance from the mean for response groups were transformed into Z-scores on each measure (LgA, PR, and shock), and also were averaged to create an overall Addiction Index that averages individual rat scores on all three self-administration conditions (Figure 4.2). The use of the top 25% (high, vulnerable) of subjects and the bottom 25% (low, resistant) of subjects based on the overall Addiction Index was used for microbiota comparisons. When examining the contribution of cohort to results we saw that there was high variability between groups of animals, a potential limitation to this study (Supplemental Figure. 4.1). Given the evidence that the microbiota and cocaine related behaviors have high variability, we wanted to eliminate cohort-specific effects through the use of multiple groups.

4.4.2 Short-access cocaine self-administration induced modest alteration in the intestinal microbiota composition.

To assess the impact of short cocaine self-administration sessions on the intestinal microbiota, we used the addiction index z-scores to divide subjects into low (Q1) and high (Q4) vulnerability groups and compared the microbiota composition from fecal samples take after short access test conditions. Overall microbial communities do not cluster into separate populations, i.e. beta diversity is not significantly different based on principal coordinates analysis (Figure. 4.3a). However, individual bacterial groups do vary in relative abundance, as demonstrated with LEfSe results plotted in a cladogram (Figure. 4.3 b) and bar chart (Supp. Figure 4.2 b). Specifically, the family Enterobacteriaceae, the order *Enterobacteriales*, the class

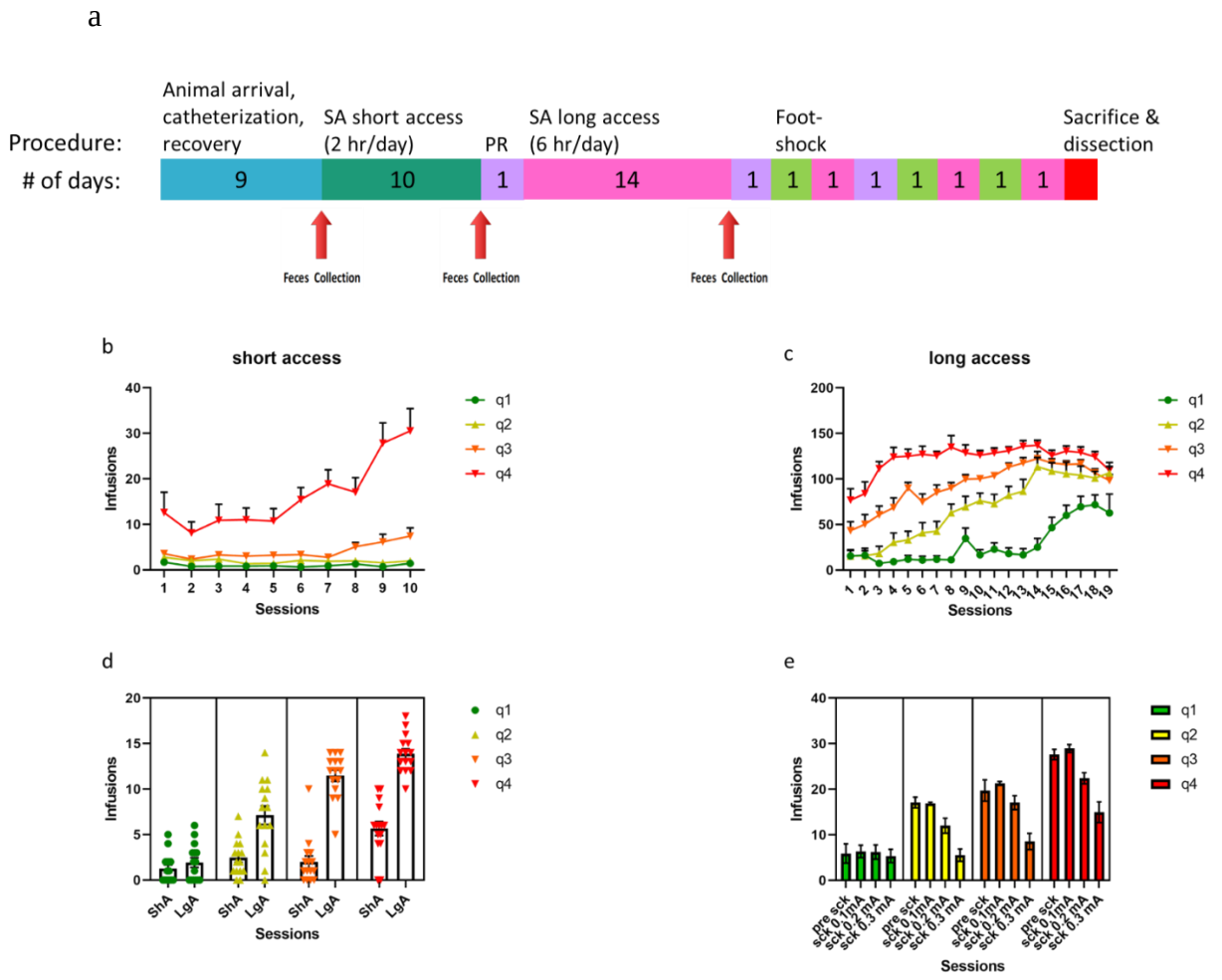


Figure 4.1 Behavioral testing timeline and behavioral outcomes

a. The experimental timeline indicates the order of procedures carried out with all subjects. Fecal matter was collected at timepoints indicated by the arrows. Based on the number of cocaine infusions or behavior on the PR schedule, rats were divided into four quartiles each covering 25% of the subjects (from low to high; green, yellow, orange, red). Panels show rewards earned under **b.** short-access conditions (2 h/day), **c.** long access (6 hr/day), **d.** PR schedule of reinforcement, **e.** or compulsive drug seeking in the face of intermittent footshock paired with cocaine infusions.

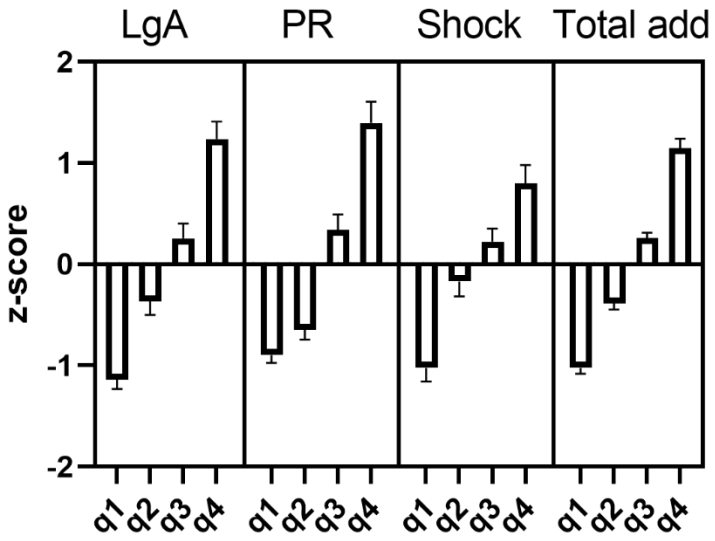


Figure 4.2 Highly variable cocaine behavior sorted into z-scores for microbial analysis The left three panels show individual z-score distributions for low and high responders on the long-access, PR, and compulsive (shock) conditions. The right panel shows an average of scoring on all three measures, creating an overall addiction index and plotting z-score distributions for low and high responders. All subjects self-administered cocaine under all conditions.

Gammaproteobacteria, and the genus *Anaerostipes* are all more robustly expressed in the low responders vs the high responders at this time point, although none of these groups are found in particularly high levels in either addiction group (data not shown) No differences in alpha diversity after short access cocaine self-administration were seen in high vs low responders. (Supplemental Figure 4.2 b).

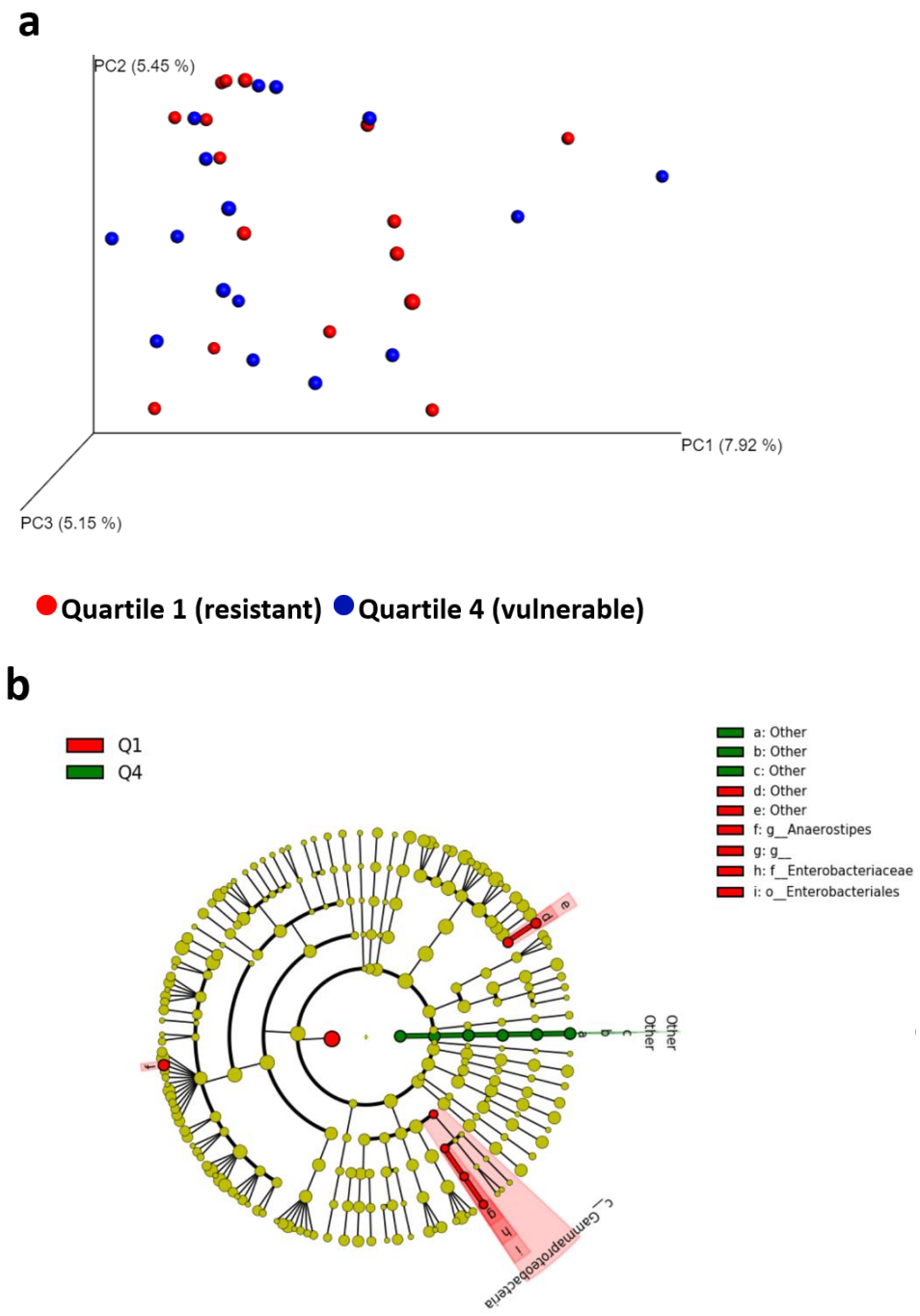


Figure 4.3 Impact of short-access cocaine self-administration on microbiota composition
a. Principal coordinates analysis (PCoA) of the unweighted UniFrac distance matrix of the bottom quartile of cocaine resistant rats on the overall addiction index (Q1, red) and top quartile of cocaine vulnerable animals (Q4, blue), shows no significant clustering based on sequencing 16s rRNA bacterial genes from fecal samples collected after short-access conditions. **b.** Taxonomic cladogram obtained from linear discriminant analysis effect size (LEfSe) highlights specific bacterial taxa that were relatively more abundant in Q1 (red) or more abundant in Q4 (green). Minimum LEfSe score was 2.0.

4.4.3 Long-access cocaine self-administration induced gut microbiota dysbiosis

To assess the impact of longer (6 hr/day) cocaine self-administration sessions on the intestinal microbiota, we used the addiction index z-scores to divide subjects into low (Q1) and high (Q4) vulnerability quartiles and compared the microbiota composition from fecal samples taken after long access test conditions. Overall microbial communities clustered into distinct populations, as determined by PCoA of the unweighted UniFrac distance matrix (Figure. 4.4 a). Moreover, individual bacterial groups varied in relative abundance, as demonstrated with LEfSe results plotted in a cladogram (Figure 4.4 b) and bar chart (Supplemental Figure 4.3). Specifically, the class *Erysipeltotrichi*, the order *Erysipetotrichales* the families *Erysipelotrichaceae* and *Bacteroidaceae* and the genus *Allobacullum* were all more robustly expressed in the high responders vs low responders at this time point, although none of these groups is found in particularly high levels in either addiction group (data not shown). On the other hand, drug resistant animals showed relatively higher abundance of the family *Eubacteriacease* and the genus *Anaerofustis*. Among these, only the phylum *Bacteroidetes* was identified at high proportions in the samples (Supplemental Figure 4.3 b). No differences in alpha diversity after short access cocaine self-administration were seen in high vs low responders. (Supplemental Figure 4.3 a).

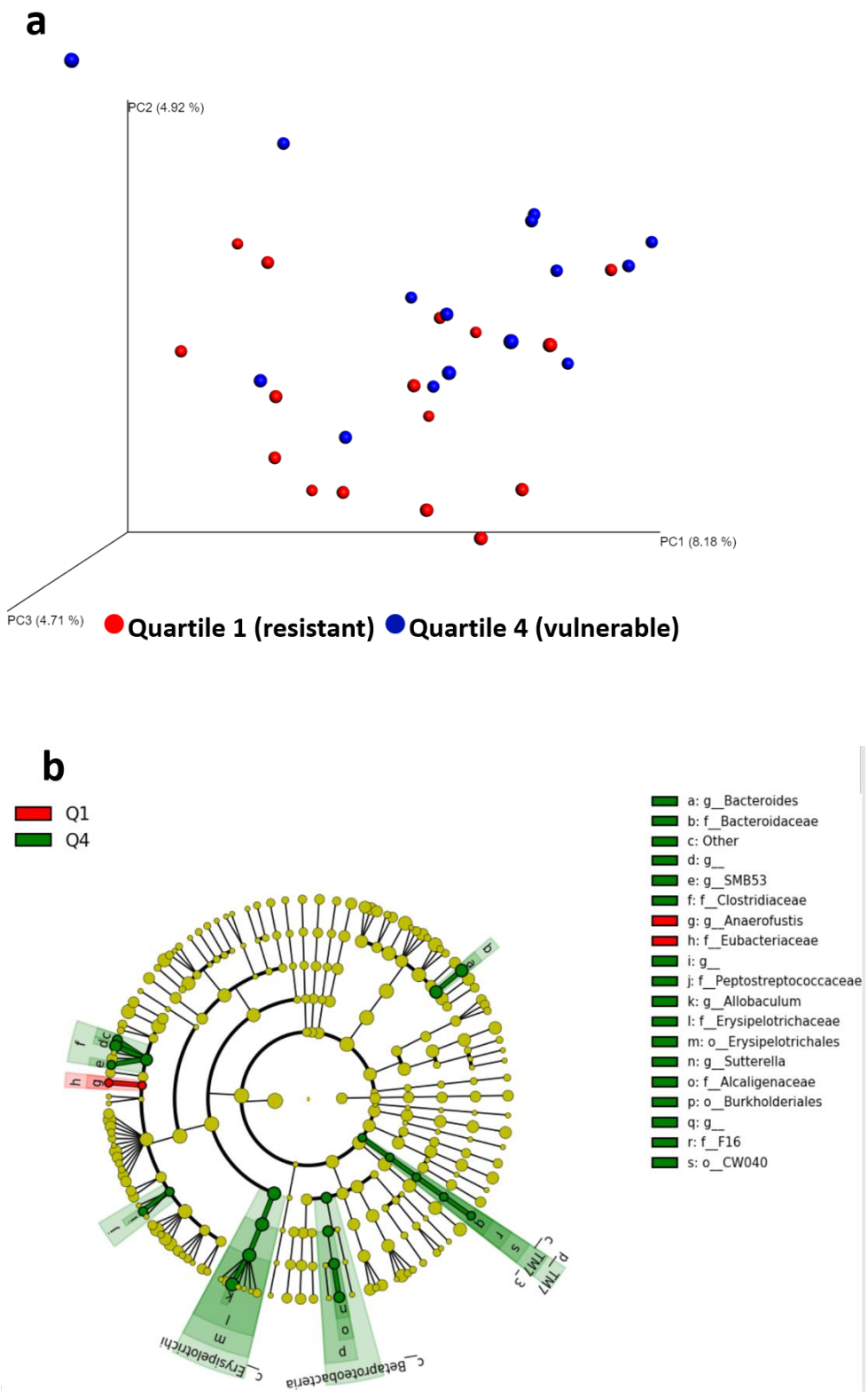
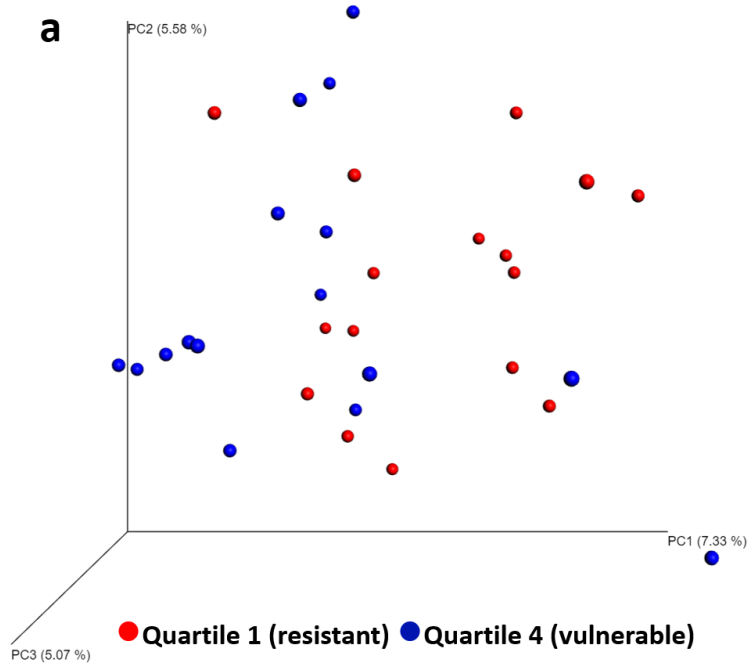


Figure 4.4 Impact of long-access cocaine self-administration on microbiota composition

a. Principal coordinates analysis (PCoA) of the unweighted UniFrac distance matrix of the bottom quartile of cocaine resistant rats on the overall addiction index (Q1, red) and top quartile of cocaine vulnerable animals (Q4, blue), shows differential clustering based on sequencing 16s rRNA bacterial genes from fecal samples collected after these long-access conditions (p=0.017). **b.** Taxonomic cladogram obtained from LEfSe highlights specific taxa that were relatively more abundant in Q1 (red) or more abundant in Q4 (green). Minimum LEfSe score was 2.0.

In addition to using the addiction index z-scores, we also used the long-access (escalation) z-scores to subdivide animals for consideration of the impact of long-access cocaine self-administration on microbial profiles. With this differentiation, we again observed a significant difference in beta diversity between high and low responders (Figure 4.5 a). Overlap in bacterial subpopulations showing greater relative abundance in high responders was observed: family *Clostridiaceae, Erysipilotrichaceae*, family *Peptostreptococcaceae*, genus *Allobaculum*, genus *SMG53*, and order *Erysipelotrichales*.

In addition, several groups showed higher relative abundance in high responders with this z-score but not the prior: family *Elulsimicrobiaceae*, family *Turicibacteraceae*, family *Bifidobacteriaceae*, genus *Bifidobacterium*, genus *Ruicibacter*, order *Bifidobacteriales*, order *Elusimicriobiales*, and order *Turicibacterales*. Conversely, several groups showed higher relative abundance in the low responders: family *Verrucomicrobiaceae*, genus *Ruminococcus*, genus *Akkermansia*, genus *Oscillospire*, order *Verrucomicrobiales*, and order *YS 2*. Using the escalation index as the determining factor for cocaine vulnerability resulted in no differences in alpha diversity (supplemental figure 4.4 a). Individual bacterial contributions to microbial profiles are detailed in supplementary materials through LEfSe (Supplemental Figure 4.4 a) and the overall abundance charts show that some phylum levels are shifted between high and low responders. (Supplemental Figure 4.4 c).



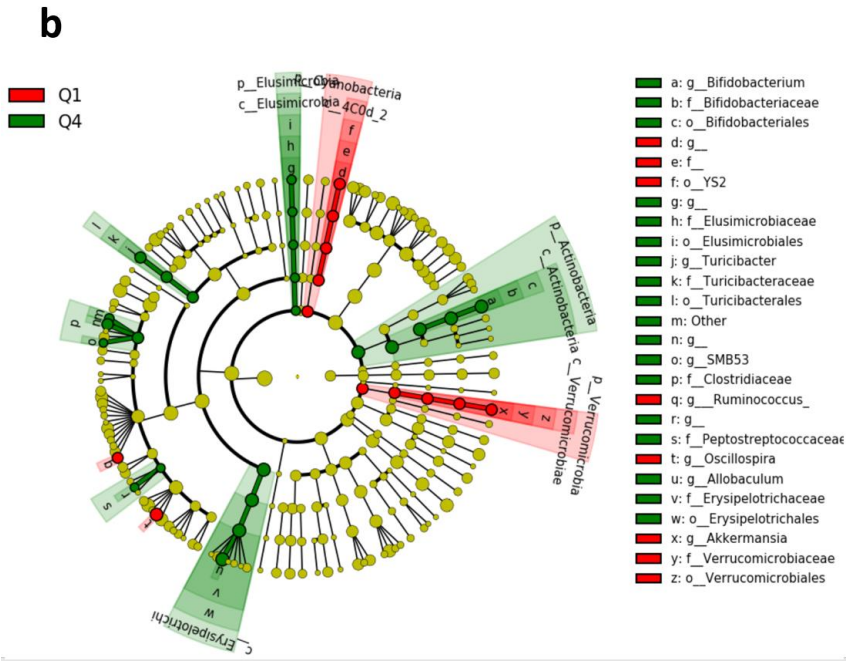


Figure 4.5 Impact of long-access cocaine self-administration on microbiota composition, with subpopulations defined by long-access (escalation) z-score

a. Principal coordinates analysis (PCoA) of the unweighted UniFrac distance matrix of the bottom quartile of cocaine resistant rats on the specific escalation z-score (Q1, red) and top quartile of cocaine vulnerable animals on the escalation z-score (Q4, blue), shows differential clustering based on sequencing 16s rRNA bacterial genes from fecal samples collected after

these long-access conditions (p=.001). **b**. Taxonomic cladogram obtained from LEfSe highlights specific taxa that were relatively more abundant in Q1 (red) or more abundant in Q4 (green). Minimum LEfSe score was 2.0.

4.4.4 Microbial composition may predict future cocaine use

As noted in the timeline (Figure 4.1 a), fecal samples were also collected before any exposure to cocaine. Microbiota composition analysis in these samples demonstrated that before cocaine exposure, microbiota profile does not cluster differentially based on the likelihood of cocaine use later in life, as presented Figure 4.6 when using the Total addiction z-score. However, when looking at individual taxa contribution to future phenotypes via LeFSe analysis (Supplemental Figure 4.5 b) and taxa summarization (Supplemental Figure 4.5 c), we observed that certain bacterial groups were significantly altered in future high versus low cocaine responders. Specifically, *Akkermansia muciniphila* was more highly represented in future low responders versus future high responders. Conversely, the orders *Anaeroplasmatales* and *Turcibacterales*, families *Ruminococcaceae*, *Anaeroplasmataceae*, and *Turicbacteracae*, genera *Ruminococcus*, *Aneroplasma*, *Allobaculum* and *Turicbacter* were all more robustly expressed in future high responders vs future low responders (Figure 4.6 and Supplemental Figure. 4.5a). Some of these clades belong to phyla that are among the most highly abundant in both low and high responders (*Actinobacteria*, *Firmicutes*, *Tenericutes*, and *Verrucomicrobia*; Supplemental Figure 4.5c). Moreover, future high responders have higher alpha diversity compared to future low responders (Figure 4.6 and Supplemental Figure 4.5a).

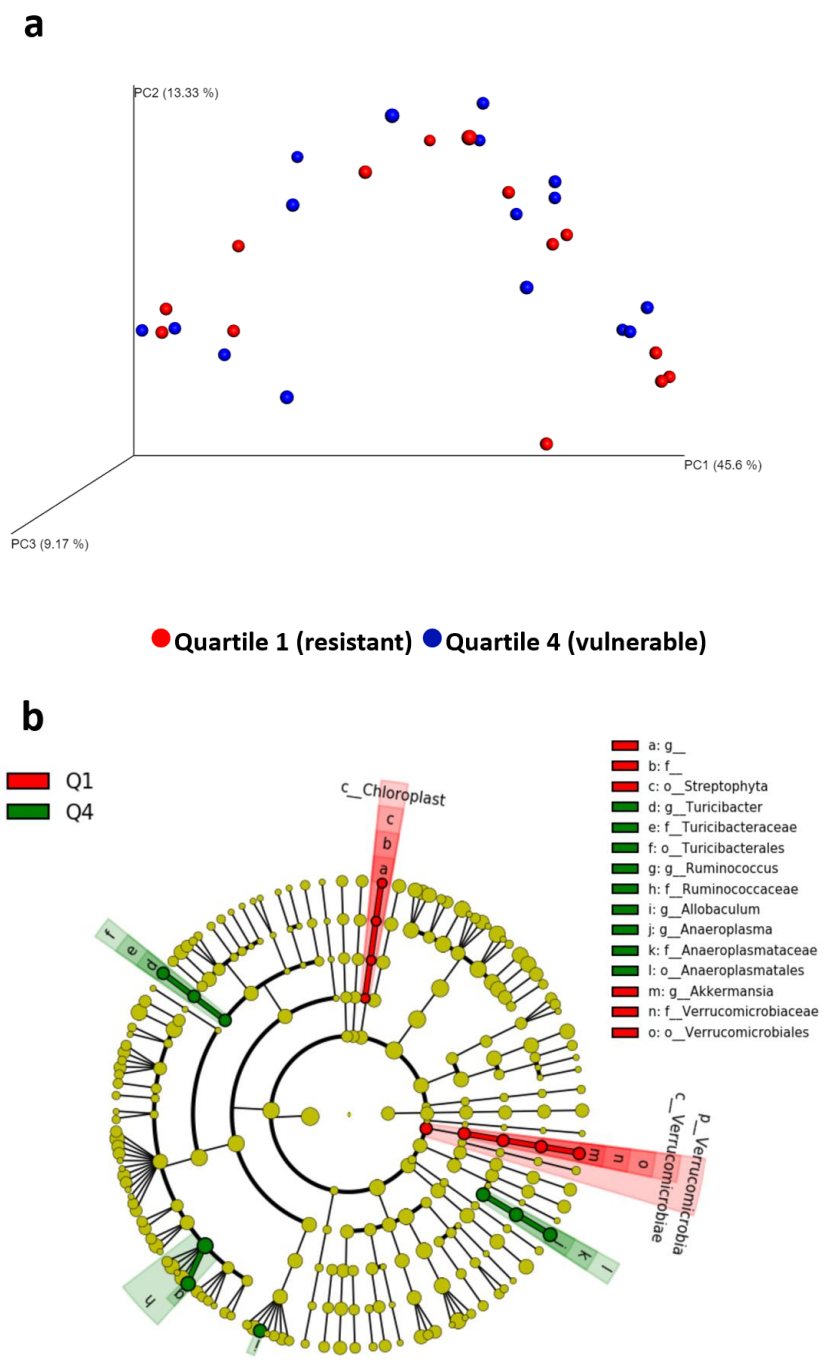


Figure 4.6 Using the microbiome to predict future cocaine sensitivity
a. Principal coordinates analysis (PCoA) of the unweighted UniFrac distance matrix of the bottom quartile of cocaine resistant rats on defined by the overall addiction index (Q1, red) and top quartile of cocaine vulnerable animals on the addiction index (Q4, blue) does not show differential clustering based on sequencing 16s rRNA bacterial genes from fecal samples collected before any cocaine exposure (baseline samples). **b.** Taxonomic cladogram obtained from linear discriminant analysis effect size analysis of fecal 16S ribosomal RNA genes sequencing. Animals that go on to show high sensitivity to cocaine reward and reinforcement have certain bacteria more represented compared to animals that go on to be low responders.

Taxonomic cladogram obtained from LEfSe highlights specific taxa that were relatively more abundant in Q1 resistant (red) or more abundant in Q4 vulnerable (green) subpopulations, even before any cocaine exposure. Minimum LEfSe score was 2.0.

Using output from the LEfSe, we chose two clades to test as potential biomarkers that might discriminate future membership in the high vs. low responder quartiles, based on the overall addiction index. The ROC curve plotting rates of true positive (sensitivity) against false positive (specificity) revealed that higher abundance of the species *Akkermansia muciniphila* showed excellent predictive value for membership in the low responder group (AUC=.8103), whereas the family *Ruminococcaceae* discriminated high responsivity (AUC=0.7388; Figure 4.7).

Based on relevant literature linking the phyla *Firmicutes* and *Bacteroidetes* (or the ratio of their relative abundance) with obesity (John and Mullin, 2016), age (Mariat et al., 2009), and alcohol use disorder (Engen et al., 2015a), we also plotted ROC curves for these clades. *Firmicutes* and *Bacteroidetes* separately showed only fair discrimination of future cocaine sensitivity with AUCs of 0.6429 (Supplemental Figure 4.6a) and 0.6027 (Figure 4.6b), respectively. Similarly, the ratio of the two yielded modest predictive results (AUC=0.625; Supplemental Figure 4.6c).

Evidence suggests that the genus *Lactobacillus*, part of the order of *Lactobacillaceae* are an important bacteria in psychological and social health and well-being. Specifically in rodents, administration of probiotics containing Lactobacillus reverses damage that psychological stress has on the brain (Ait-Belgnaoui et al., 2013) as well as moderate psychological stress responsivity (Messaoudi et al., 2010; Messaoudi et al., 2011). Since there is several lines of evidence that addiction phenotypes are highly comorbid with other neuropsychiatric conditions such as exaggerated stress responsivity (Regier et al., 1990) and that several neuropsychiatric

conditions are related to the gut microbiota (MacQueen et al., 2017), we sought to examine whether *Lactobacillus* related bacteria at baseline could predict future drug use. ROC curves revealed that the order *Lactobacillaceae* showed fair discrimination (AUC=0.6897; Supplemental Figure 4.7a), while the genus *Lactobacillus* was a poor predictor (AUC=0.6272; Supplemental Figure 4.7b).

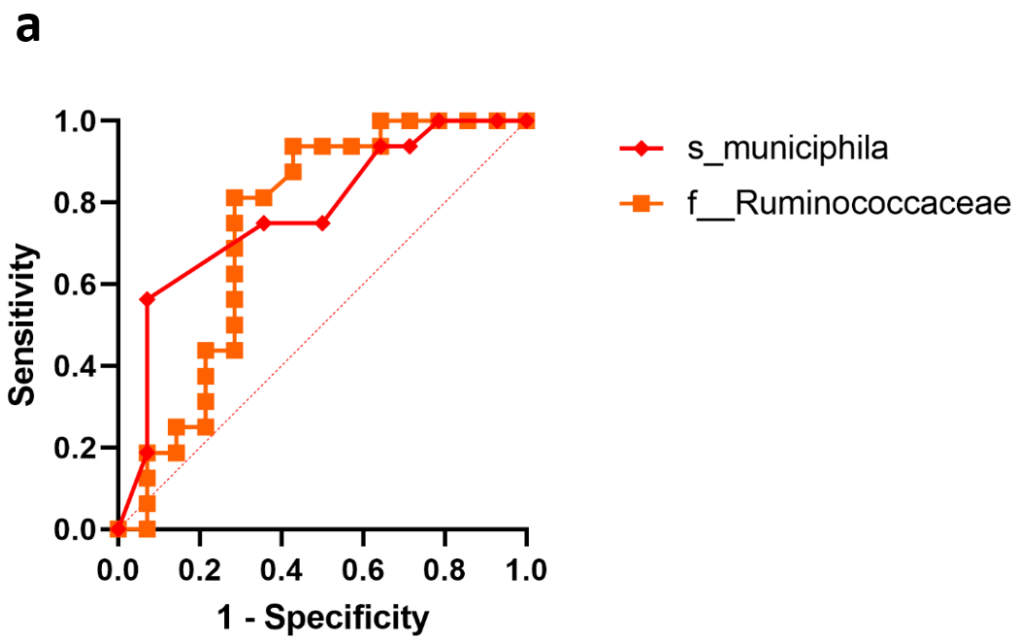


Figure 4.7 Specific taxa predict future cocaine sensitivity
a. Specific microbial signatures were analyzed from fecal samples obtained at baseline before any cocaine exposure, using a random forest algorithm to predict future phenotype contribution. The species *Akkermansia muciniphila* (AUC=0.8103) and family *Ruminococcaceae* (AUC=0.7388) both had strong probability of predicting future addiction index phenotype

4.5 Discussion

Cocaine use is a debilitating condition that is estimated to affect millions of people in the united states per year (Pomara et al., 2012). Despite the chronic strain cocaine use and use of other illicit substances has on the population, uncovering effective therapeutics has been difficult

in part due to the high variability of intake by individuals both in rodents (Panlilio et al., 2003) and humans (Davidson et al., 1993). With several lines of evidence that cocaine seeking behavior and the microbiota are intertwined (Kiraly et al., 2016; Meckel and Kiraly, 2019; Chivero et al., 2019; Cryan et al., 2019) we sought to determine the relationship between microbiota profile and cocaine responsivity as well as ascertain whether the microbiota could be a useful diagnostic tool to predict severity of future cocaine use.

The gut microbial communities in low vs. high addiction-prone rats in the present study were different in ways that not only reflect acute and long-term impact of cocaine intake on bacterial populations but also predict membership in the low or high sensitivity subgroup. For example, while short-access (2 hr/day) to cocaine self-administration was associated with higher relative abundance of a few bacterial subgroups in low vs. high responders, it was the long-access escalation conditions (6 hr/day) that reduced the overall bacterial diversity in the high responder group and also clearly differentiated the bacterial communities, with more than 15 specific taxa showing significant elevations or declines in one population or the other. Baseline sampling prior to cocaine exposure also revealed some potential biomarkers for cocaine resilience or sensitivity.

The behavioral and microbial assays used in this study have been validated previously and are highly relevant to evaluate addiction-like behaviors and microbial composition (Chassaing et al 2012). Rats were tested in a battery of behavioral assays using standard operating protocols that measure different aspects of the reinforcing effects of cocaine. The level of responding for cocaine under a fixed ratio schedule of reinforcement after short vs. long access has been shown to be highly relevant to model the difference between recreational drug use and drug addiction (Ahmed et al. 1998; George et al. 2014) The progressive ratio (PR)

schedule of reinforcement was used to assess the break point, a valid measure of the motivation to seek a reward (Stafford et al 1998; Hodos et al. 1961) The resistance to contingent foot shock has been established as a relevant measure to evaluate compulsive-like responding for drugs (Vanderschuren et al. 2004; Deroche-Gamonet et al. 2004).

Quartile splits in behavioral outcomes were used successfully to show low- and high-responding subgroups for short-access cocaine self-administration (2 hr/day), long-access self-administration (6 hr/day), motivation to seek cocaine on a PR schedule of reinforcement, and compulsive cocaine-seeking despite concurrent footshock. Both raw values and variability in cocaine-related behaviors were consistent with prior reports (Briand et al. 2008; George et al. 2008; Verheij et al. 2018), supporting the reliability and validity of the behavioral outcomes. In addition, microbiome profiling revealed levels of beta diversity and alpha diversity, as well as dominant bacterial taxa, that are similar to those reported in other studies of rodents (Li et a. 2017) and share similarity with clinical reports on human participants (Human microbiome consortium, 2012). Thus, the description of microbial communities within and between the subsets of rats categorized as low and high addiction-prone is based on reliable microbiome profiling.

The impact of relatively low-level cocaine intake was assessed using fecal samples collected after the short-access phase of cocaine self-administration. Although the overall microbiota diversity and richness was not altered between animals that fell into the lowest vs. highest quartiles of the overall addiction index, some interesting individual differences in relative abundance suggest the possibility that bacterial communities in low responders contained higher levels of some beneficial bacterial taxa. For example, the family *Enterobacteriaceae* is abundant in the gut of newborn and juvenile mammals (Bokulich et al., 2016), perhaps suggesting a role in

growth and development, while the overall class of *Gammaproteobacteria*, to which *Enterobacteriaceae* belong, is elevated after gastric surgery in both humans (Aron-Wisnewsky et al. 2014) and rodents (Antonelli et al. 2009), suggesting a role in recovery. Both of these taxa were more abundant in cocaine-resistant animals in the present study. *Anaerostipes* is among the many bacterial taxa known to produce short chain fatty acids (Schwertz et al. 2002; Duncan et al. 2004). Its higher abundance in low responders could support prior reports that exogenous SCFA administration reduces cocaine's reward value in mice (Kiraly et al. 2016).

In contrast to results observed after short-access cocaine self-administration, we observed more robust differences in gut bacterial communities between low and high responding animals after the long-access phase of self-administration, whether the low and high quartiles were defined using the overall addiction index or the specific escalation index that takes into account only the degree of escalation under long-access conditions. Alpha diversity within the sample populations was not different using either z-score subdivision, but beta diversity was significantly different in each case, suggesting that high cocaine intake during the escalation model creates a distinct microbial environment in the gut. With regard to specific taxonomic groups, the two taxa that were overrepresented in the guts of low vs. high responders after short-access cocaine were no longer elevated after long-access. Yet *Anaerofustis* was, and it is also a SCFA producer of the family *Eubacteriaceae* in the class *Clostridia* (Duncan et al. 2007; Poeker et al. 2018). When categorizing rats based on the escalation index alone, two additional clades from the class *Clostridia* were higher among low responders: *Oscillospira*, which is associated with gastric dysfunction (Lam et al. 2012) and *Ruminococcus gnavus*, which is associated with low oxygen conditions and gut inflammation (Henke et al. 2019). Notably, several *Clostridia* species were also higher among non-opioid users, compared with opioid abuse patients (Acharya

et al., 2017). Finally, *Akkermansia muciniphila* was higher among low responders at this time point, and this is a species well known for its inverse relationship with cardiometabolic disease and gut inflammation (Cani et al. 2017 ;Naito et al. 2018). As the name implies, *A. muciniphila* is associated with integrity of the intestinal mucosal barrier, including immune system function as well as the production of SCFA (van Passel et al. 2011). *A. Muciniphila* has high relative abundance among non-obese, healthy controls (Xu et al. 2020, Dao et al. 2016) and those on ketogenic diets (Olson et al. 2018), for example. Therapeutically, chronic alcohol consumption lowers relative abundance of *A. muciniphila* and administration of *A. muciniphila* to patients with alcoholic liver disease ameliorates their symptoms (Grander et al. 2018). It is also shown that *A. Muciniphila*, and subsequently higher levels of SCFAs, are prevalent in rats that consume coffee (Gao et al. 2018).

Regardless of whether the overall addiction index or the escalation index was used to subdivide rats, the highest quartile of cocaine responders showed higher relative abundance of two different clades within the class *Clostridia:, SMB53* and *Peptostreptococcaceae*, along with the genus *Allobaculum* from the class *Erysipelotrichia*. Interestingly, both methamphetamine and cocaine users also show higher relative abundance of *Allobaculum*, compared with healthy controls (Franzosa et al., 2019; Scorza et al., 2019). Moving on to those clades associated with high responders defined by the escalation index, three taxa might be explored for their specific relationship with higher doses of cocaine intake: *Elusicrobiaceae, Turcibacter*, and *Bifidobacterium*. A different set of clades differentiated high responders when defined using the overall addiction index including F16, *Alcaligenaceae, Sutterella*, and *Bacteroides*. Notably, the relative abundance of taxa in the phylum *Bacteriodetes* (which includes *Bacteroides*) predicted cocaine use in humans (Volpe et al., 2014) and is higher among alcohol users compared to non-

user controls (Volpe et al., 2014) (Fan et al., 2018), as well as rodents given chronic alcohol (Yan et al. 2012). Overall, the impact of these changes in the gut depends on the specific functionality provided by the clades independently, as well as their influence on one another in the bacterial communities of the gut. Furthermore, whether or not these changes in bacterial populations are responses for cocaine intake and irritability tests or causes of the differences in responsivity on these tests remains to be determined. One approach in beginning to understand these distinctions is to investigate which clades were already highly abundant in low vs. high responders at baseline before cocaine intake.

Among the most promising translational components of this study is the potential to identify biomarkers of cocaine vulnerability. Thus, the bacterial taxa that were higher at baseline among future low responders could be protective, whereas the taxa that were higher among future high responders could be associated with vulnerability. The most interesting of these might be *Akkermansia muciniphila*, which was higher at baseline among future low responders. Not only that, it remained higher among low responders throughout long-access testing. Further statistical exploration using the ROC curve suggested this species as an excellent predictor of belonging in the low addiction prone subpopulation. Conversely, perhaps playing a role in vulnerability are the *Allobaculum* and *Turcibacter* populations that were higher among high responders at baseline and after long-access testing. The genus *Anaeroplasma* was also higher at baseline among future high responders, warranting further investigation. Interestingly, the genus *Ruminococcus* was higher in high responders at baseline, but the *Ruminococcus gnavus* species was higher in low responders later in the experiment, suggesting that its decline relative to other taxa could be an important factor in the behavioral switch to high response levels. Nevertheless, the *Ruminococcaceae* family was a strong predictor of membership in the high addiction prone

subpopulation, according to the ROC analysis. This family has been associated with drug reward, impulsivity, attention, and locomotor activation in animal models (Ning et al. 2017, Peterson et al. 2020), as well as obesity and Alzheimer's Disease in humans (Crescenzo et al. 2017, Zhuang et al. 2018, Ticinesei et al. 2018), although it is a SCFA producer (Jiang et al. 2015; Acharya et al. 2017), appeared lower in opioid users compared to healthy controls (Acharya et al., 2017), high abundance during abstinence from alcohol (Starkel et al. 2016), and was inversely related to cirrhosis (Ticinesi et al. 2018). The present results did not support the use of *Lactobacillus* as a predictor, nor *Firmicutes*, *Bacteroidetes*, nor the *Firmicutes/Bacteroidetes* ratio, per low discrimination ability in the ROC curve analysis.

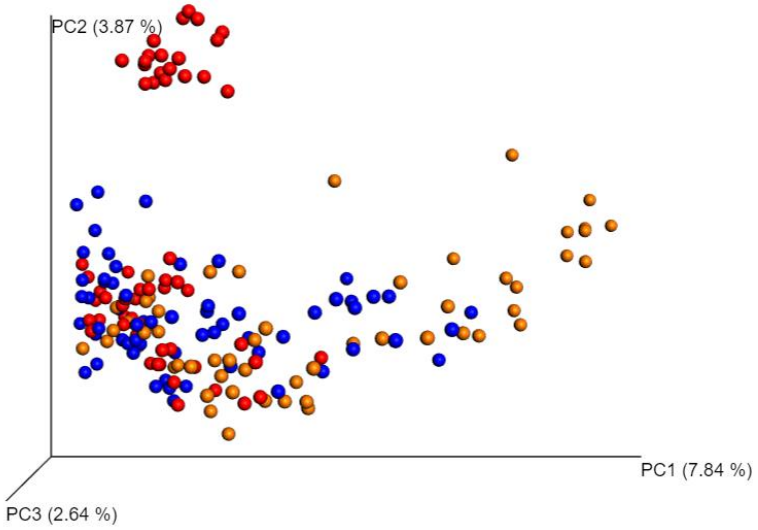
The mechanisms through which cocaine alters the gut microbiome remain to be determined. General perturbations to gut function have long been associated with cocaine use and abuse (Brown et al., 1994; Gibbons et al., 2009). Cocaine in mice issues proinflammatory responses and compromises the mucosal lining of the gut, along with changes in microbiota composition (Chivero et al., 2019). As a sympathomimetic drug, cocaine also activates alpha-adrenergic receptors in the mesentery, leading to gastric ulcers and perforations in the gut membrane (Gourgoutis et al. 1994). Cocaine also exerts major impact to reduce blood flow to the gut, disrupt healthy diet, and disrupt exercise patterns (Cregler et al. 1986; Gibbons et al., 2009), each of which also affects the gut microbiota (Luna and Foster, 2015; Maslowski et al. 2011; Sandhu et al., 2017; Kang et al., 2014; Allen et al., 2015; Tang et al., 2017). These changes in the gut milieu are likely to favor certain bacterial populations while inhibiting the growth of others.

Although this is among the first reports to characterize the impact of cocaine on the gut microbiota while also suggesting diagnostic tools to predict the likelihood drug vulnerability, several limitations are noted in this work. First, microbial profiling from fecal samples rather

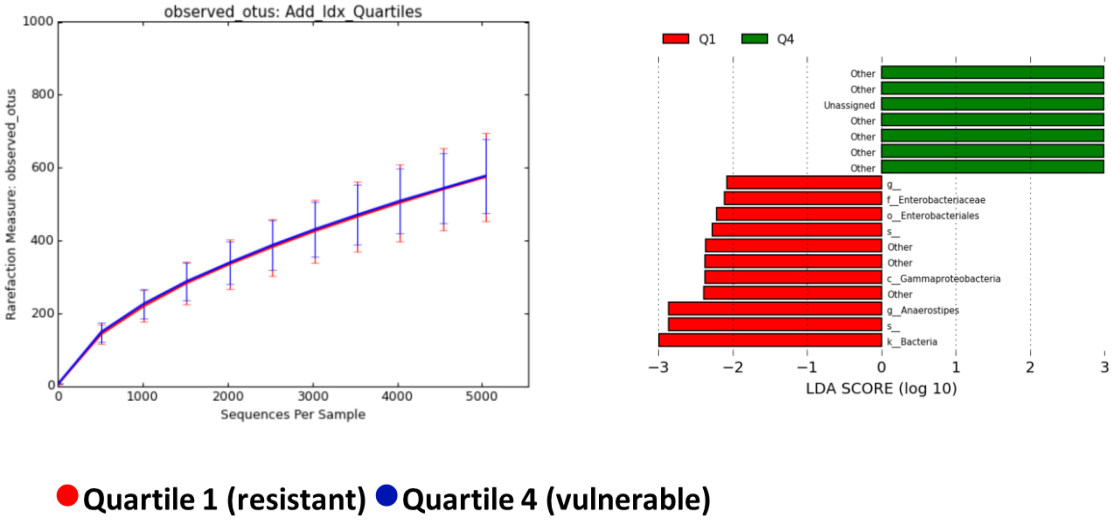
than colon tissue restricts the view of the actual microbial environment in the gut (Stanley et al., 2015). Second, the present animal subjects were tested in three cohorts, which showed baseline differences in the microbiome, but were combined for all analyses. Cohort effects and differences across populations of clinical and animal study participants call into question the generalizability of the identification of specific bacterial groups in correlation with disease and/or behavioral outcomes. Finally, as noted above, the mechanistic relationships between gut microbes and reward-related behaviors remain to be explored.

In summary, several lines of evidence suggested that cocaine-seeking may be influenced by the gut-brain axis (Kiraly et al., 2016; Meckel and Kiraly, 2019; Chivero et al., 2019; Cryan et al., 2019) and the present work extends this concept by identifying microbial populations that are associated with specific phases of cocaine experience, including candidate biomarkers of cocaine resilience or vulnerability. Given that only a subset of cocaine-experienced individuals proceed to develop substance use disorder (McLellan, 2017), predictive gut profiles could suggest new treatment approaches to identifying addiction-prone populations. Pre- and probiotic formulas that promote the growth of bacterial species associated with low cocaine vulnerability may ultimately serve as effective adjunct therapies to treat addiction.

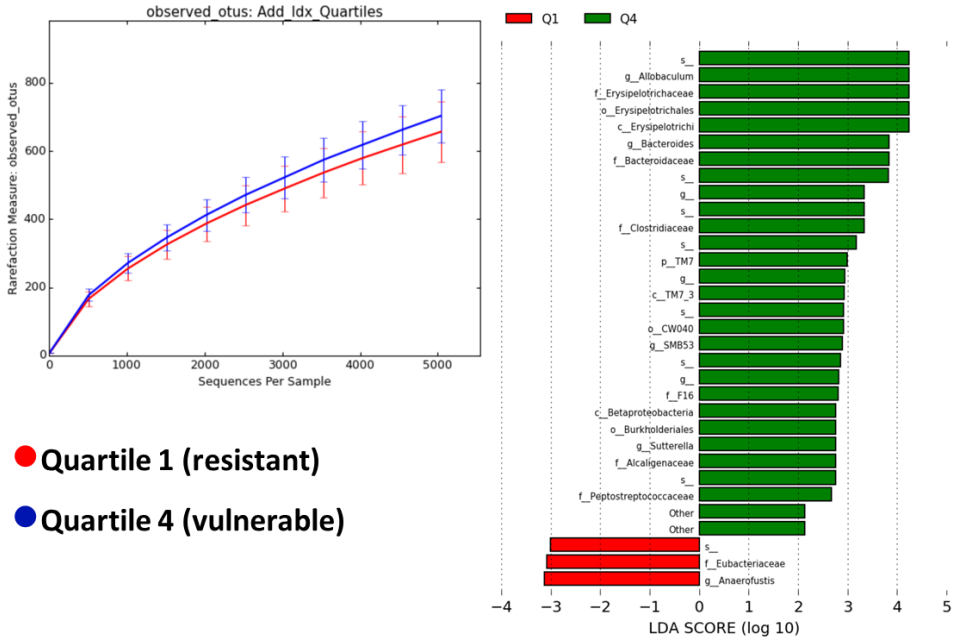
4.6 Supplemental Figures

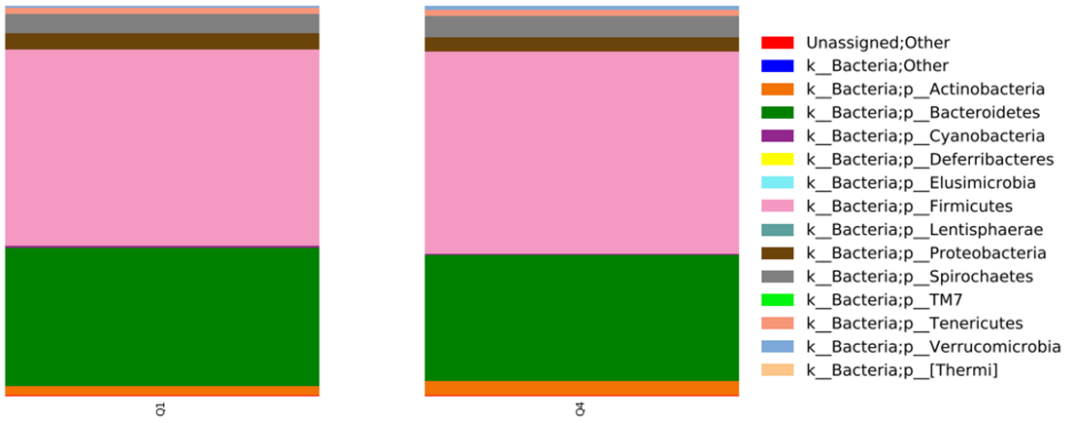


Supplemental figure 4.1.1: Comparison of microbiota diversity between cohorts
Principal coordinates analysis (PCoA) of the unweighted UniFrac distance matrix of all fecal samples taken for this experiment by cohort (red= cohort 1, blue= cohort 2, gold= cohort 3). Given that these animals were outbred it comes as no surprise that microbiome composition varies between groups.

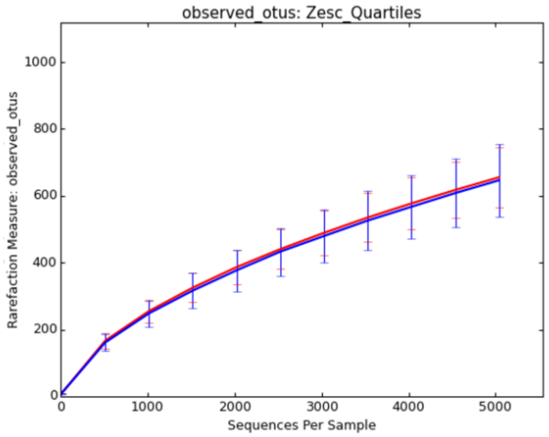


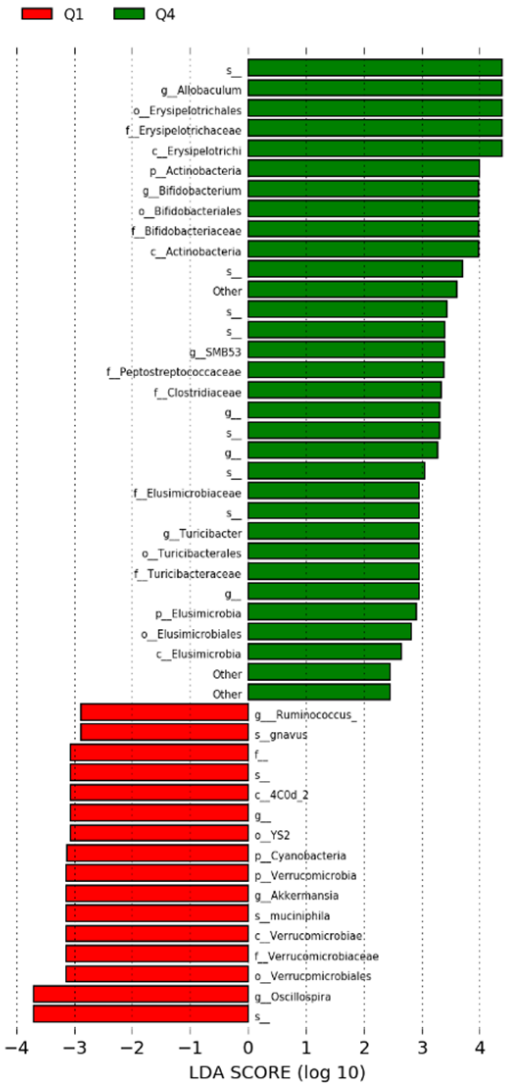
Supplemental Figure 4.2: Characterization of microbiota after short access cocaine self-administration correlates with drug seeking phenotype
a. α-diversity in fecal samples after short access cocaine self-administration looking at observed OTUs. **b.** Linear discriminant analysis effect size was used to investigate whether individual bacterial taxa were more robustly represented in low vs high cocaine responders **c.** Taxa summarization performed at the phylum level in animals after short access self-administration.





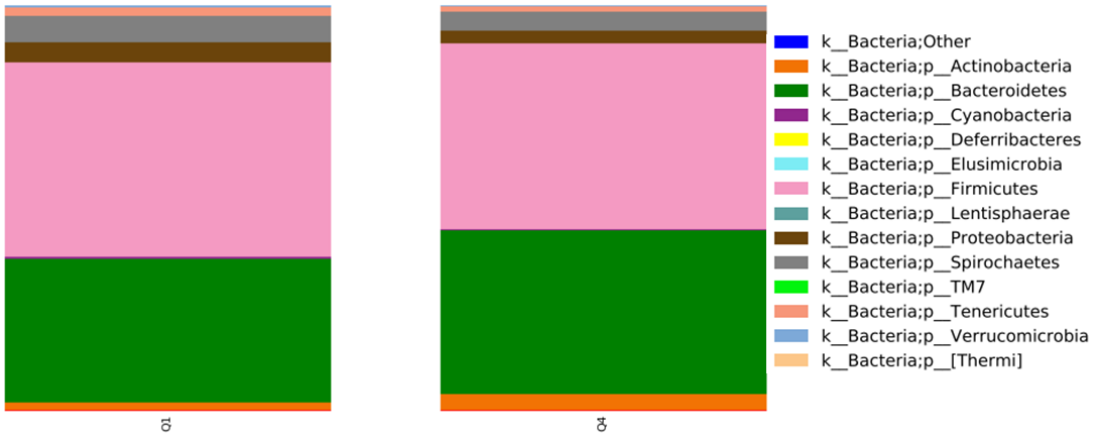
Supplemental Figure 4.3: Characterization of microbiota after long access cocaine self-administration correlates with drug seeking phenotype
a. α-diversity in fecal samples after long access cocaine self-administration looking at observed OTUs**. b.** Linear discriminant analysis effect size was used to investigate whether individual bacterial taxa were more robustly represented in low vs high cocaine responders after prolonged cocaine exposure. **c.** Taxa summarization performed at the phylum level in animals after long access self-administration.





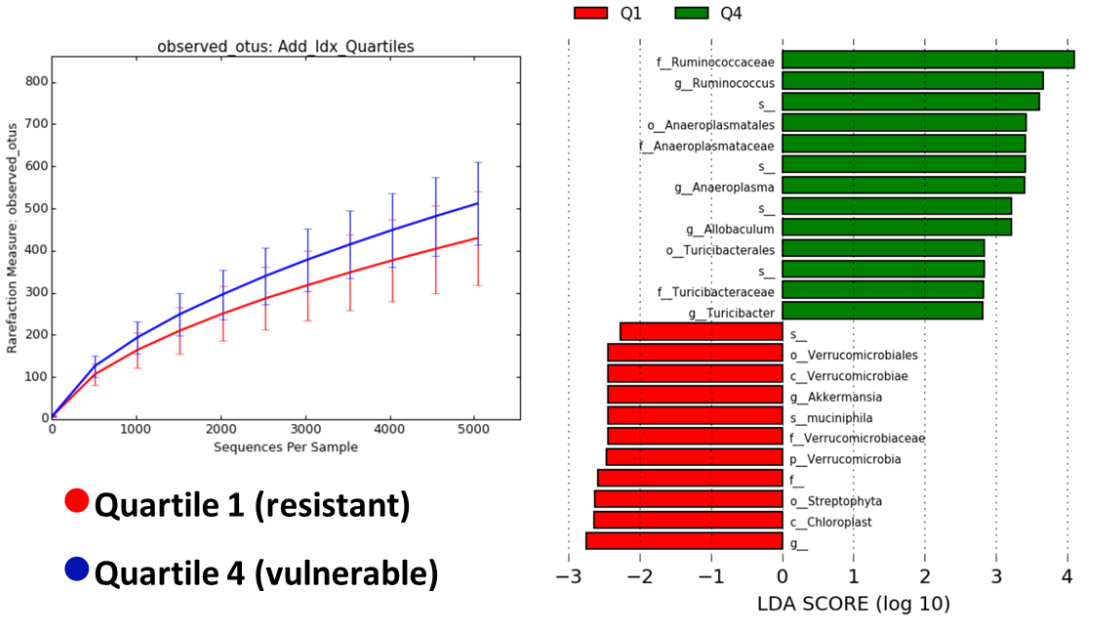
Quartile 1 (resistant)

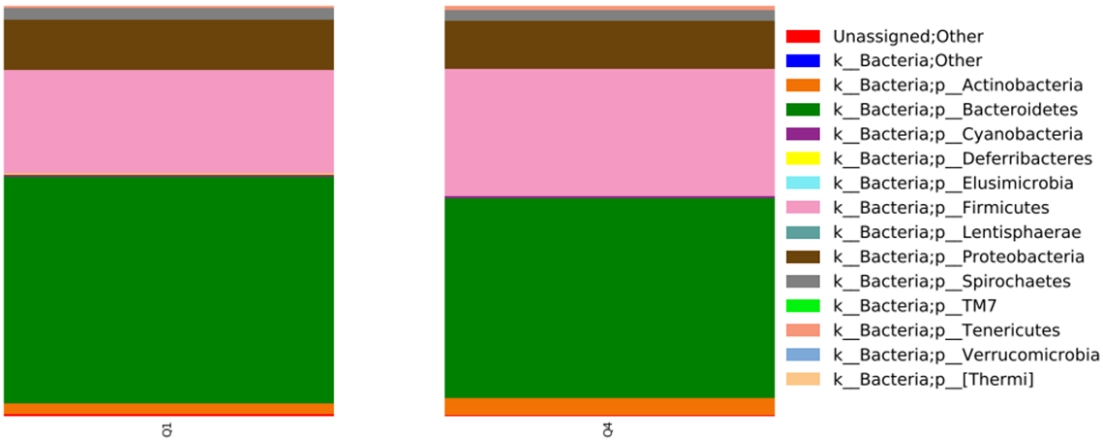
Quartile 4 (vulnerable)



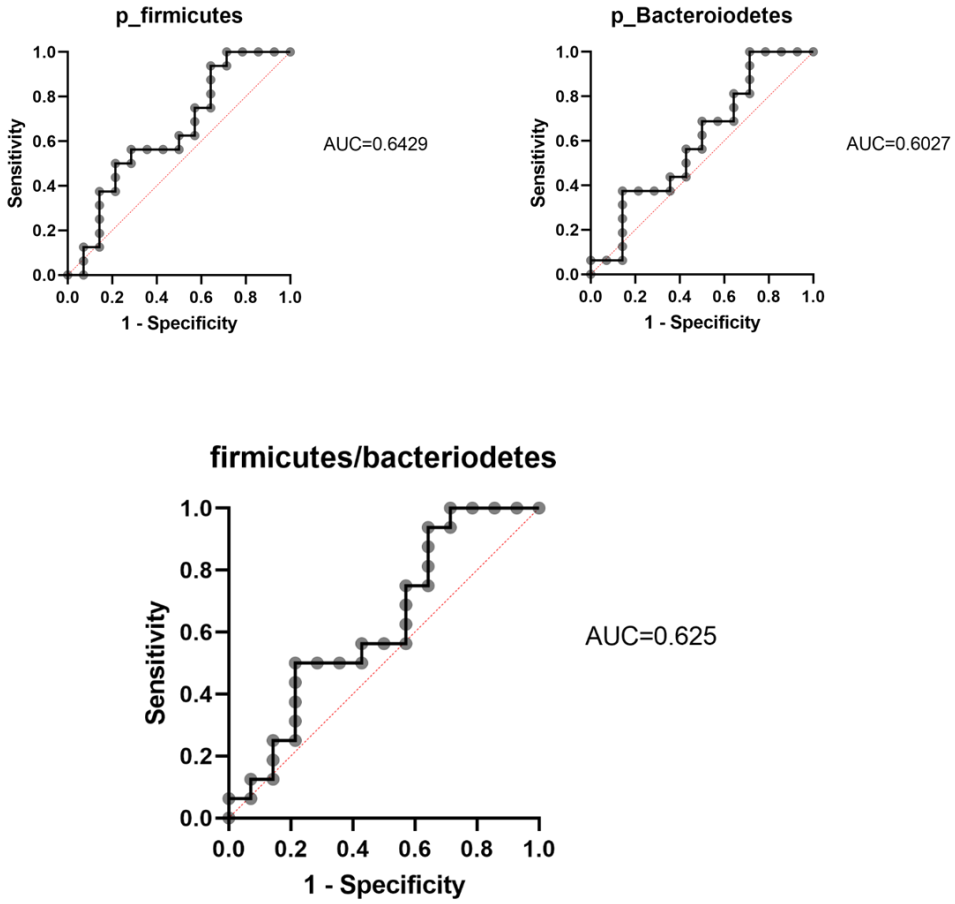
Supplemental Figure 4.4: Characterization of microbiota after long access cocaine self-administration correlates with drug seeking phenotype when using different measures of behavioral dependent measures.

a. α-diversity in fecal samples after long access cocaine self-administration when using the escalation index as a measure of cocaine resistance or vulnerability. **b.** Linear discriminant analysis effect size was used to investigate whether individual bacterial taxa were more robustly represented in low vs high cocaine responders after prolonged cocaine exposure using the escalation index to discriminate cocaine seeking. **c.** Taxa summarization performed at the phylum level in animals after long access self-administration using the escalation index to discriminate cocaine seeking.

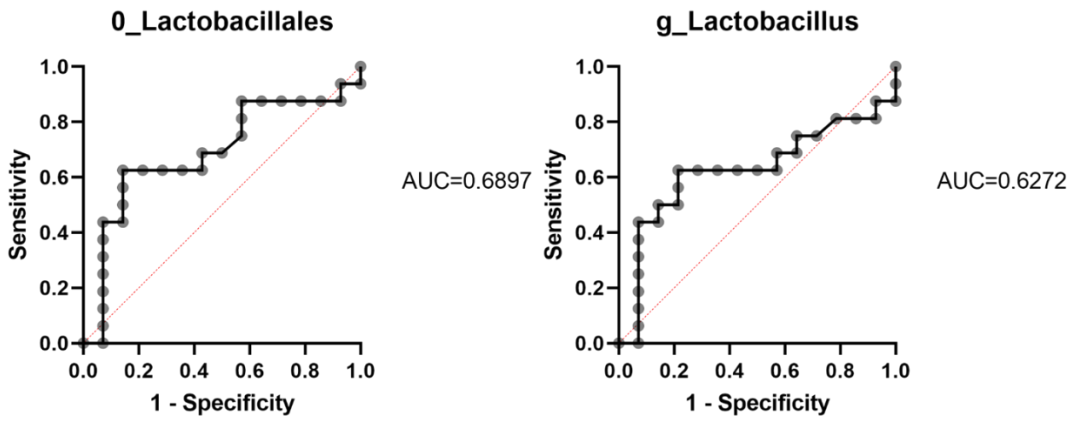




Supplemental Figure 4.5: Characterization of microbiota at baseline predicts future cocaine phenotypes
a. α-diversity in fecal samples at baseline looking at observed OTUs. **b.** Linear discriminant analysis effect size was used to investigate bacterial members that drive differences animals that become high and low cocaine responders. Individual bacteria groups appear responsible for future drug taking phenotype. **c.** Taxa summarization performed at the phylum level. At baseline there is a shift in relative phylum abundance between animals that become low cocaine responders and high cocaine responders.



Supplemental Figure 4.6: Using bacterial taxa associated with obesity and gut related disorders to predict future drug use.
Receiver operator curve (ROC curve) showed that **a.** the phyla Firmicutes (AUC=0.6429) **b.** and Bacteriodetes (AUC=0.6027) did a poor job predicting future drug taking phenotypes. **c.** The ratio of these two phyla, which has previously shown to be associated with gastrointestinal disorders, including obesity, also was poor at predicting future drug taking behavior (AUC=0.625).



Supplemental Figure 4.7: Using bacterial taxa associated with neuropsychiatric disease to predict future drug use.
Receiver operator curve (ROC curve) showed that a. the order *Lactobacillales* (AUC=0.6897) b. and genus Lactobacillus (AUC=0.6272) did a fair/poor job predicting future drug taking phenotypes. These bacterial taxa have previously been associated with neuropsychiatric conditions such as depression, anxiety, and schizophrenia.

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5 MODULATION OF THE GUT MICROBIOTA BY ANTIBIOTICS AND PROBIOTICS ALTERS COCAINE-SEEKING AFTER ABSINENCE IN ADOLESCENT AND ADULT MALE RATS

5.1 Abstract

Research on the gut-brain axis has revealed that gut dysbiosis is associated with several psychiatric disorders including substance abuse. We hypothesize that adolescent vulnerability to drug-related reward and reinforcement might also be related to gut dysbiosis. Using a cocktail of antibiotics in the drinking water of Wistar rats, we sought to determine whether reduced abundance of bacteria in the gut heightens intravenous cocaine self-administration. Moreover, probiotics appear to exert beneficial effects in the context of psychiatric disorders and the possibility exists that probiotic administration may reverse antibiotic-induced gut microbial depletion, restoring normal cocaine-related behaviors. Thus, a subset of rats received a commercial probiotic preparation (Probio' Stick) via syringe feeding during a recess from cocaine self-administration. Adolescent and adult male rats were given two weeks of antibiotic exposure, fecal samples were collected, DNA was extracted, and qPCR was conducted to assess changes gut bacterial abundance. Simultaneously, animals acquired lever-pressing in operant conditioning chambers using a white noise training procedure, followed by cocaine self-administration (0.37 mg/kg per infusion) on a fixed ratio 1 schedule of reinforcement for 8 sessions, 2-hr each, over 10 days. Following 30 days of forced abstinence, animals underwent extinction and cue-induced reinstatement testing. A separate subgroup was tested on a progressive ratio (PR) schedule of reinforcement for two sessions after the 8 FR sessions. At sacrifice, peripheral organs were extracted to investigate gross anatomy and gut inflammation. Body mass and water intake were normal across treatment groups. Gut depletion was similar in

both age groups but adolescents and not adults recovered to baseline abundance levels after a thirty day recovery period. Cecum mass and size were greater in all antibiotic-treated animals, compared to age-matched controls, and a further increase was associated with cocaine intake in adults but not adolescents. No elevation in markers of inflammation was observed. Probiotics tended to restore normal cecum mass. Although gut dysbiosis did not alter cocaine self-administration on fixed or progressive ratio schedules of reinforcement, it did increase reinstatement responding in adults but not adolescent-onset groups. Probiotics during abstinence restored reinstatement to baseline levels in adults but not adolescents. Additional 16S rRNA sequencing of bacterial genes from fecal samples allowed bioinformatic analysis of gut bacterial communities across treatment groups and time, demonstrating that antibiotic consumption and cocaine administration altered microbiota diversity in an age dependent manner. Together these results suggest that gut dysbiosis may be a factor in addiction vulnerability and that probiotics warrant investigation as adjunctive therapies especially in adult patients.

Note: Brett Daniel, Jillian Dawson, Jennysue Kasiah, Natalie Brock, Benjamin Anthony, Sami Hatcher, Bonnie Williams, Benoit Chassaing, and Kyle Frantz all contributed to these experiments.

5.2 Introduction

Substance abuse is a major problem in the United States, with over 25 million Americans using illicit substances each month (SAHMSA, 2016) It is estimated that over 600 billion dollars per year goes towards the treatment and cost of drug addiction (Chung et al. 2006). One main issue in tackling these challenges is a long-lasting vulnerability to relapse that can persist years after abstinence has begun (Cregler and Mark, 1986). Cocaine in particular is the second most abused illicit substance behind marijuana (Quality, 2015). To date, therapies for treating cocaine

addiction and attenuating likelihood of cocaine relapse are generally ineffective (Indave et al., 2016).

Recent evidence suggests that changes in the human gut environment can influence diseases of the central nervous system (CNS) (Cryan and Dinan, 2012). The gut microbiome is a collection of bacteria, fungi, viruses, and protists that inhabit the gastrointestinal tract in commensal, symbiotic, or pathogenic relationships with their host and comprise substantial populations, with trillions of individual organisms (Turnbaugh et al., 2007; Bäckhed et al., 2005; Gill et al. 2006). Gut dysbiosis, defined as loss of overall microbial diversity, expansion of harmful microorganisms, and/or loss of beneficial microbes that combat the severity and prevalence of disease (Petersen and Round, 2014) has been implicated in many gastrointestinal diseases, including inflammatory bowel disease (Dalal et al. 2014; Devkota et al. 2013), Crohn's disease (Khanna et al. 2017; Zuo et al. 2018), obesity (Sun et al. 2018; Wolf et al. 2012), and diabetes (Aw et al. 2018; Burcelin et al. 2011). With regard to CNS diseases and dysfunction, individuals with bi-polar disorder have distinct microbial populations compared to healthy controls (Jiang et al., 2015). Multiple sclerosis patients show altered microbiome diversity and react differently to probiotics compared to healthy controls (Tankou et al. 2018; Jangi et al. 2016). Those with Alzheimer's disease show shifts in microbiota functionalities (Vogt et al., 2017). Moreover, patients with Autism Spectrum Disorder (ASD) take more antibiotics, have altered microbiota diversity, report more abdominal pain and irregular bowel movements, and show increased intestinal permeability and lower short chain fatty acid (SCFA) levels, compared to healthy controls (Wang et al., 2012; Ludvigsson et al., 2013; Yassour et al., 2016). Finally, patients diagnosed with schizophrenia have higher relative abundance levels of *Lactobacillus* bacteria compared to controls (Castro-Nallar et al. 2015; Yolken et al. 2015).

In animal models of psychiatric conditions, mice show greater levels of anxiety- and depression-like behaviors after rearing in germ-free conditions (Bercik et al., 2011), along with altered microbial diversity, motor activity, and inflammatory cytokine profiles, compared to conventionally colonized mice (S. Simpson, 2018; Diaz Heijtz et al., 2011; Bercik et al., 2010). Restoration of a compromised gut microbiome via probiotics results in less depressive phenotypes in a mouse model of depression (Desbonnet et al. 2010). These studies and others support a role for bidirectional communication between the gut and brain, i.e. the "gut-brain axis" in neurological and mental disorders (Dinan and Cryan, 2017).

Due in part to the high comorbidity between neuropsychiatric disorders and Substance Use Disorder (SUD) (Regier, 1990; Thaipisuttikul et al. 2014; Ross et al. 2012; Kelly et al. 2013), the gut-brain axis has recently been implicated in SUD. Both clinical and animal studies in this nascent field support this concept. Chronic cocaine users have distinct microbial populations compared to healthy controls (Volpe et al., 2014), while heroin, methamphetamine, and/or ephedrine users show altered functional diversity of their microbiota compared to healthy controls, with underrepresentation of microbes likely to regulate cellular processes and signaling, as well as metabolism (Xu et al., 2017). Mice given a cocktail of non-absorbable antibiotics in drinking water had higher sensitivity to cocaine conditioned place preference than controls, an effect ameliorated with exogenous administration of a cocktail of SCFAs (Kiraly et al., 2016). Rats that developed methamphetamine conditioned place preference showed increased microbiota diversity compared to methamphetamine-naïve controls (Ning et al., 2017), along with a decrease in bacterial taxa responsible for synthesis of the SCFA propionate and less propionate in fecal samples. Propionate and other SCFAs are produced via metabolic fermentation of the diet by the microbiota (Stilling et al., 2016) and play critical roles in

neurological disease (Koh et al., 2016). Despite this clear evidence that drug reward is associated with gut microbial function, intravenous (i.v.) cocaine self-administration and subsequent extinction and reinstatement of cocaine-seeking after abstinence remain unexplored. The present study addressed this gap by allowing male rats to self-administer cocaine while receiving antibiotics in the drinking water, then testing extinction responding and cue-induced reinstatement of cocaine-seeking after 30 days of recess from both antibiotics and cocaine. We predicted that antibiotic treatment would decrease the abundance of gut microbes and increase the reinforcing effects of cocaine and/or cocaine-related cues.

While antibiotics are used medicinally to treat pathogenic bacteria and experimentally to induce gut dysbiosis, probiotics are live microorganisms intended to have health benefits when consumed (Ciorba et al. 2012). Probiotic treatments might relieve symptoms of neuropsychiatric disorders (Cenit et al. 2017, Scriven et al. 2018). They reduce depressive-like behaviors in mice (Desbonnet et al. 2010) and depression symptoms and psychological outcomes in humans (Paineau et al. 2008; Girard et al. 2009; Messaoudi et al. 2010; Kazemi et al. 2018). Research investigating probiotic cocktails as a possible therapy for schizophrenia is already underway (Dickerson et al. 2014). The present experiment extended this work to test the hypothesis that probiotic treatment during a 30-day drug recess would restore normal (lower) levels of extinction and/or reinstatement responding in rats that had received antibiotics before and during cocaine self-administration.

Adolescence is a transitional life-stage, marked by vast changes in neuronal circuitry (Pattwell et al., 2016) and often associated with high risk-taking behavior and experimentation with drugs of abuse (Poudel and Gautam, 2017). Most adult drug addicts report adolescent-onset of drug use (Prescott and Kendler, 1999; Brown et al., 2004; Patton et al., 2004). Yet, in our

laboratory, adolescent male rats reinstate less to drug seeking after abstinence, compared to adult counterparts (Li et al. 2009, Doherty et al. 2012, Doherty et al. 2013), suggesting that adolescence may be associated with resistance to some enduring effects of drugs, including cocaine, morphine, and heroin. Similarly, perturbations to the microbiome in early life are associated with behavioral alterations in adulthood (Rodríguez et al. 2015), effects attributed to a highly dynamic gut environment during adolescence (Borre et al.2014; Greenhalgh et al., 2016; Hollister et al., 2015), but direct comparisons between adolescent and adult perturbations in microbial communities are lacking. Therefore, this study included adolescent-onset age groups of male rats in explorations of both antibiotic and probiotic effects on cocaine-related behaviors. We predicted that adolescents are resistant to perturbations in the microbiota and to associated changes in drug-seeking behavior, compared with adult counterparts.

5.3 Materials and Methods

5.3.1 Subjects

Adolescent and adult male rats (Charles River Laboratories, Inc, Raleigh, NC, USA) arrived at Georgia State University's animal housing facility at postnatal day (PND) 22 and 70-74, respectively. Animals acclimated to pair-housing in humidity and temperature-controlled ACS cages (Optirat Gen II by Animal Care Systems; Centennial, CO) on a reverse light cycle (12:12 hr, lights on at 19:00 hr) for three days prior to catheter surgery. Animals were given *ad libitum* access to food in water while in the home cage, except as described below for antibiotic treatments, were assessed daily for general health, and weighed periodically. All procedures were conducted in adherence to the Principles of Laboratory Animal Care and the National Institute of Health Guide for the Care and Use of Laboratory Animals (8^{th} edition, 2011) and approved by Georgia State University's Institutional Animal Care and Use Committee.

5.3.2 Surgery

Intravenous catheters were assembled as previously described (Roberts and Koob, 1982) with minor modifications including a shorter length of tubing inserted into the jugular vein for adolescents compared with adults (2 vs. 4 cm)(Shahbazi et al., 2008). As described previously (Suess et al. 2020, under revision, Chapter 3 present document), incisions were made at midscapular and ventral neck locations, the right jugular vein was isolated, and drug delivery tubing was inserted into the incision in the vein. A catheter portal and backplate were implanted subcutaneously at the midscapular location, about 5 cm from the base of the neck. Catheter patency was maintained by intravenous (i.v.) administration of Timentin (ticarillin disodium and clavulnate potassium; 100 mg/ml), heparinized saline (100 USP units / 1ml) twice daily for three days post-operative, then daily for the rest of self-administration, using approximately 0.2 ml of each solution for adults and 0.1 ml for adolescents. Previous veterinarian consultation informed us that i.v. antibiotics would not influence intestinal flora.

5.3.3 Gut microbial depletion

After three days of surgical recovery, half the animals received an antibiotic (abx) cocktail in the drinking water: Bacitracin (0.5 mg/ml), Neomycin (2 mg/ml), and Ampicillin (1 mg/ml), as adapted from prior reports (Kanhere et al. 2018; Kiraly et al. 2016), based on preliminary experimentation in this laboratory. These antibiotics have broad mechanisms of action and target bacterial groups that are highly abundant in the gastrointestinal tract. The solution was prepared and changed out every 48 hours. Total solution intake per cage was calculated based on change in bottle mass over two days, with intake per animal estimated by dividing total intake by the number of animals per cage Controls received H_2O only in their bottles. Experimental animals received abx during the 4 days of lever-press training (see below)

and throughout cocaine self-administration, but were switched back to H_2O only at the start of forced abstinence. An experimental timeline for antibiotic intake is included in Figure 5.1.

5.3.4 Gut microbial restoration

A separate cohort of animals received antibiotics as above, but then received either probiotics or a placebo preparation throughout the 30 days of forced abstinence. Probio' Stick (Lallemand Inc., Montreal, CN) consisted of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175, along with malic acid and xylitol, and was mixed with phosphate buffered saline (PBS) to form a suspension that contained 10 $x10^9$ colony forming units (CFUs). Probiotic and placebo were made daily and administered to subjects at a volume of 0.5 ml at 09:00 hr, per previous reports (Tillmann et al., 2018) with some alterations. Briefly, subjects were lightly restrained with a cloth towel in red-light illumination during their dark phase. A syringe containing the preparation was presented at the animal's mouth with the tip just touching the animal. If the animal did not consume the solution after a few seconds, the tip was inserted into the animal's mouth behind the teeth. Contents were expelled over approximately 10 sec. As animals habituated over 3-10 days to the preparations and procedures, each of them transitioned to approaching the syringe tip in home cages consume freely. Any remaining probiotic or placebo preparation was recorded but negligible. An experimental timeline for probiotic intake is included in Figure 5.1.

5.3.5 Feces collection, water intake, and body mass

To collect fecal samples, rats were held by the base of the tail so that the front paws were on a solid surface and the back paws were gently lifted up and down off of the surface until a fresh fecal pellet was released. When animals were in operant conditioning chambers, fecal pellets were simply collected from the sterile tray under the animal. Samples were placed in

collection tubes on ice then moved to a -80 ^{o}C freezer for storage. Water intake was assessed during training and self-administration, and every three days during forced abstinence Body mass was assessed daily during testing, then twice weekly during abstinence.

5.3.6 Cocaine self-administration

Cocaine self-administration procedures were adapted from previously experiments (Suess et al. 2020, in revision). Briefly, animals acquired lever pressing behavior over four days via a negative reinforcement procedure (contingent removal of an aversive white noise stimulus) in daily 2-hr sessions in operant conditioning chambers (Med Associates, Inc., St. Albans, VT, USA), after which cocaine infusions (0.36 mg/kg/ infusion) were paired with white noise removal for two sessions, then lever-pressing resulted in cocaine infusions only for eight additional sessions conducted over ten days (with a two-day weekend recess). During cocaine sessions (but not the first four white noise training sessions), the catheter portal was attached to polyethylene tubing that led to a variable-speed syringe pump (Med Associates, Inc., St. Albans, VT, USA) which delivered drug solution via a stainless-steel swivel (Instech Laboratories, Inc., Plymouth Meeting, PA, USA). Sessions were conducted under a fixed ratio (FR)-1 schedule of reinforcement with a timeout after each drug infusion (20 sec). Reinforced lever-presses also triggered a cue-light above the active lever to come on for 2 sec, and a house light to go off for 20 sec. Responses on a separate inactive lever were recorded but had no scheduled consequences. A subset of control rats went through the same procedures but always had saline in the syringes, rather than cocaine.

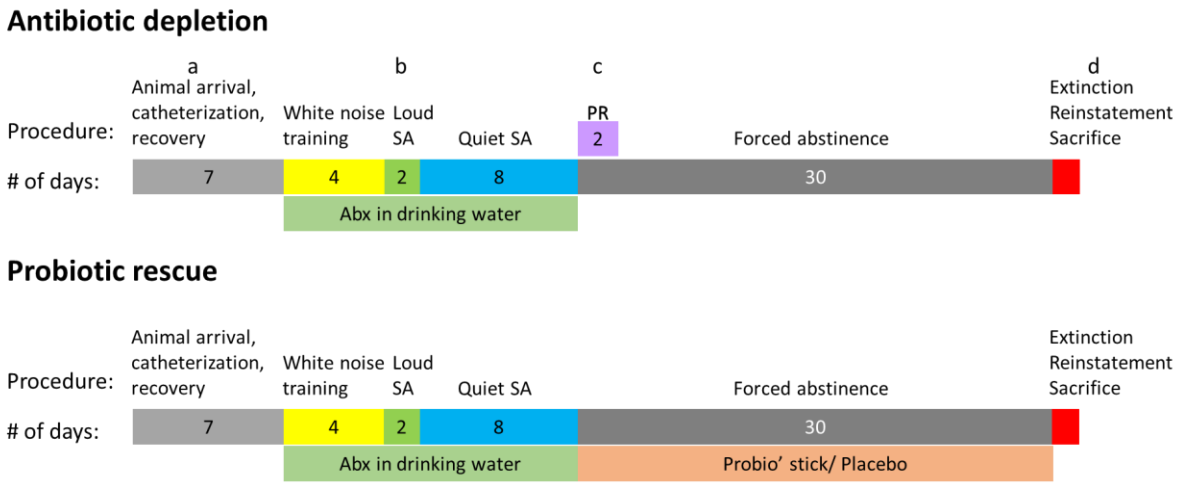


Figure 5.1 Experimental design for administering antibiotics and probiotics to alter cocaine related behaviors

a. Adolescent (PND 22) and adult (PND 70-74) male Wistar rats arrive at Georgia State University. Baseline measurements of weight, drinking behavior, and fecal microbiota are collected. Animals are implanted with indwelling jugular vein catheters for drug self-administration. **b.** Animals acquire lever pressing behavior through a previously validated white noise training paradigm (4 days). Animals subsequently self-administer cocaine with white noise acting as a negative reinforcer (2 days) followed by cocaine self-administration with no noise (8 days). **c.** Animals then underwent two days of progressive ratio self-administration or entered 30 days of forced abstinence. During abstinence, animals had water bottles switched to tap water and did not have access to the drug-taking chambers. A separate cohort of animals received either probiotics (Probio' stick) or placebo via oral syringe during forced abstinence. **d.** Animals that completed forced abstinence underwent extinction and cu-induced reinstatement followed by sacrifice.

5.3.7 Progressive ratio testing

At the conclusion of self-administration, a subset of animals continued to self-administer cocaine for two days under a progressive schedule of reinforcement (PR), in which the number of active lever presses required for successive cocaine infusions increased based on a geometric progression (Roberts et al. 1993). The end of the session was achieved when animals went one hour without a drug infusion or a maximum of six hours. Two days of PR sessions were conducted on consecutive days.

5.3.8 Abstinence, extinction, and cue-induced reinstatement

At the conclusion of self-administration, a separate subset of animals entered a 30-day forced abstinence period during which they were confined to their home cages. Subsequently, animals underwent extinction testing in which the animals were placed back into their chambers for five consecutive 1-hr sessions with no drug-paired cues or drug available. Immediately afterwards, animals underwent a single 1-hr reinstatement test, in which presses on the active lever yielded drug-paired cues including activation of the syringe pump, although no syringe was loaded and cocaine was still not available.

5.3.9 Sacrifice and organ extraction

At the end of either progressive ratio or reinstatement, animals were anesthetized with 0.1-0.2 mls of Sodium Pentobarbital (Somnasol: Henry Schein Animal Health, Melville, NY) and then rapidly decapitated. Trunk blood was collected in EDTA blood collection tubes (BD microtainer, Franklin Lakes, NJ) then spun at14,000 RPM for 20 minutes to separate out serum then stored at -20 °C for downstream analysis. Brains were flash frozen in chilled isopentane on dry ice then stored at -80 °C for downstream applications. Abdominal organs including cecum, distal colon (1 cm closest to rectum), spleen, and liver were dissected and transferred to dry ice then to -80 °C for downstream analysis. Mass and photographs of abdominal organs were also collected.

5.3.10 DNA extraction and qPCR for bacterial DNA

Identification of changes in total bacterial load was conducted as previously described (Kanhere et al. 2018). In brief, fecal samples were homogenized and DNA extracted according to the Earth Microbiome Project (www.earthmicrobiome.org/emp-standard-protocols). DNA was isolated using the PowerSoil DNA Isolation Kit (Qiagen, Germantown, MD). The V4 region of

the 16S rRNA genes was amplified with quantitative polymerase chain reaction (qPCR). Specific procedures, forward and reverse primers were adapted from previous work (Kanhere et al. 2018) with minor adjustments. Fold change in total bacterial load is calculated by normalizing to DNA content as described previously (Caporaso et al. 2012). Samples were taken before behavioral testing (Pre), at the conclusion of cocaine self-administration and/or antibiotic intake (Peak), and after 30 days of abstinence (Post).

5.3.11 Microbiota composition analysis via 16S rRNA sequencing.

Fecal samples from the timeline listed above were processed to identify microbiota composition, as previously described (Chassaing et al., 2015; Suess et al. 2020b). Briefly, DNA from fecal samples were extracted using QIAamp 96 Powerfecal QIAcube HT Kit (Quiagen, San Diego, CA) with mechanical disruption (bead-beating). 16S rRNA genes, region V4, from each sample were PCR amplified using a composite reverse primer and a forward primer containing a unique 12-base barcode used to tag PCR products from respective samples. PCR products were purified with magnetic beads (Beckman Coulter, Brea, CA), visualized by gel electrophoresis and quantified using Epoch Microplate Spectrophotometer (Biotek, Winooski, VT). A master DNA pool was generated and sequenced using a Illumina MiSeq (2*250 bp, paired end) at Cornell University.

5.3.12 Fecal Lipocalin-2

Inflammation in fecal samples was assessed as previously described (Chassaing et al. 2012). Briefly, frozen fecal samples were reconstituted with PBS/0.1% Tween 20 then vortexed to get homogenous solution. After centrifugation for 10 mins at 12,000 rpm, clear supernatants were collected for analysis. Supernatants were used as input for the Duoset rat Lcn-2 ELISA kit (R&D Systems, Minneapolis, MN) and followed according to manufacturer’s instructions.

Quantification of lipocalin was assessed using a spectrophotometer and plotting absorbances against a standard curve.

5.3.13 16S rRNA sequences analysis

Sequencing outputs were analyzed at Georgia State University, as previously described (Chassaing et al., 2015; Suess et al. 2020b). Briefly, sequence outputs were demultiplexed, quality filtered using the Quantitative Insights Into Microbial Ecology (QIIME) software package (Caporaso et al., 2012), and forward and reverse Illumina reads were joined using the fastq-join method (http://code.google.com/p/ea-utils) (Aronesty, 2013). QIIME default parameters were used for quality filtering and sequences were assigned to OTUs using the UCLUST algorithm (Edgar, 2010) with a 97% threshold of pairwise identity and classified taxonomically using the Greengenes reference database (McDonald et al., 2012). Principal coordinate analysis of the unweighted UniFrac distances was used to assess the variation between samples (beta diversity) (Lozupone et al., 2011). Beta diversity was further visualized via jackknife-supported PCA plots. Alpha diversity and rarefaction curves were calculated and displayed using Observed OTUs as through the use of QIIME.

5.3.14 Statistics

Self-administration behavior was analyzed via a mixed-model analysis of variance (ANOVA) in a 2 x 2 x 2 design with age (adolescents vs adults), drug (cocaine vs saline), and antibiotic treatment (abx vs H_2O) as between-subject variables and active lever-presses or number of infusions as the primary dependent measures. Sham controls and saline controls were collapsed into a single control group. Comparison of total cocaine intake (mg/kg) was conducted using independent samples t-tests (two-tailed). PR responding was compared using number of infusions as the dependent measure. Extinction and reinstatement analyses were split into

separate tests. Results from animals receiving probiotics or placebo during abstinence were analyzed similarly but separately, using univariate ANOVA, with presses on the previously active lever presses as the dependent measure and age, probiotic treatment, and session as independent variables. Fecal lipocalin-2 was analyzed using a mixed-model ANOVA, with age (adolescents vs adults), drug (cocaine vs saline), and antibiotic treatment (abx vs H_2O) as between-subjects variables and time point (Pre, Peak, and Post) as the within-subjects factor. Alpha was set at .05 in all cases. Any post-hoc comparisons were conducted using Tukey's Range Test.

Fecal qPCR abundance results are presented as change in cycle threshold (ΔCT), normalized to an animal's pretreatment phase (Pre), on a logarithmic scale. Abundance differences were assessed using a similar ANOVA test as behavior but inserting sample timepoint as a repeated measure. For between-subject microbiota diversity, differences in distinct clustering in PCA plots was assessed via PERMANOVA method using vegan R-package through QIIME.

All data were analyzed using SPSS v.23 (SPSS, Chicago, IL). Behavioral data, lipocalin-2, and qPCR graphs were generated in PRISM 7/ GraphPad software. Between and within groups diversity graphs were generated via QIMME. LEfSe and cladograms were generated using the online Galaxy tool (http://huttenhower.sph.harvard.edu/galaxy/).

5.4 Results

5.4.1 Gut microbial depletion alters host physiology

Neither cocaine or antibiotics in the drinking water altered body mass (Supplemental Figure 5.1) or fluid intake (Supplemental Figure 5.2) during the course of the experiment. Neither cocaine or antibiotics influenced spleen, liver, or intestinal mass at sacrifice (data not

show). Animals in the antibiotic treatment group had larger ceca compared to water controls, regardless of age. In adults, the effect of cocaine in antibiotic treated rats further increased cecum mass ($F=28.96$, $p<0.001$), an effect not observed in adolescent counterparts (Figure 5.2 a). Furthermore, differences in the cecum were visually apparent between antibiotic- and water-treated groups (Figure 5.2 b). Specifically, antibiotic treated animals had longer and wider ceca (and a more leathery texture) compared to water controls. Ceca also appeared darker and appeared to contain more fecal material. Inflammation was not altered by cocaine or antibiotics experience, as measured using the fecal lipocalin-2 biomarker (Supplemental Figure 5.3).

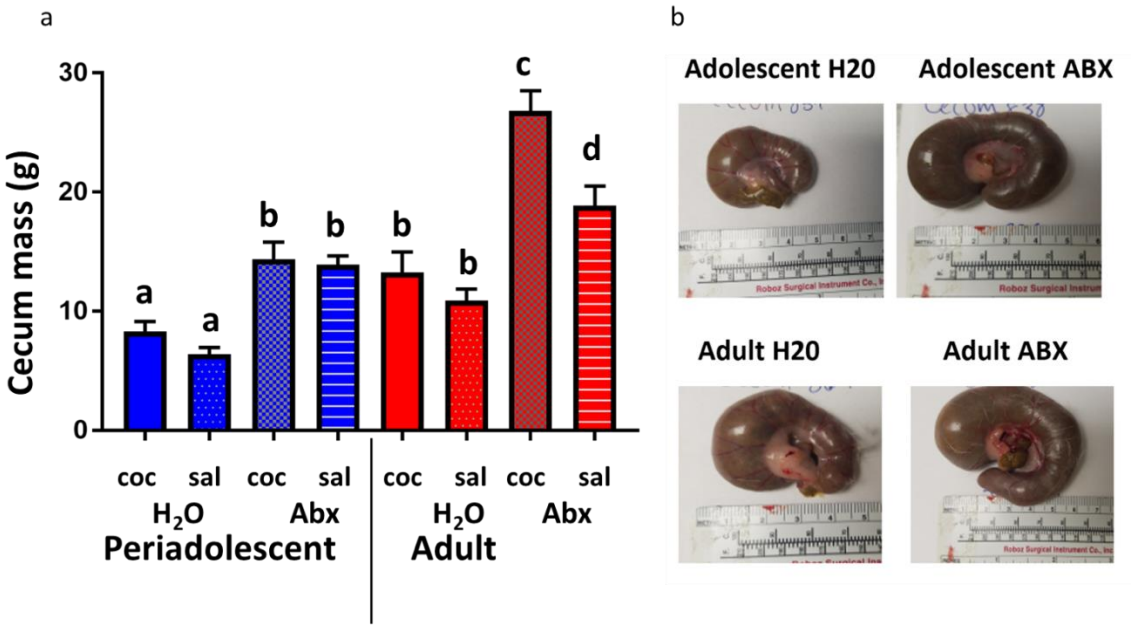


Figure 5.2 The influence of antibiotics and cocaine on cecum mass and size
a. Total cecum massed assessed at sacrifice. Antibiotic intake was associated with increased cecum weight in both adolescents and adults. Cocaine further increased cecum weight in adults but not adolescents. **b.** Photographs illustrating the effect of antibiotics on cecum size. All error bars are ±S.E.M.

5.4.2 Gut microbial depletion does not alter self-administration behavior

During self-administration, neither age nor antibiotic treatment altered rates of active lever presses (Figure 5.3 a) or cocaine infusions (Figure 5.3 b). Cocaine animals had more active lever presses and total cocaine infusions during periods when drugs were available compared to saline controls. In cocaine treated animals, neither age nor antibiotic treatment altered total cocaine intake (Figure 5.3. c). In animals that subsequently underwent PR self-administration, no age or antibiotic effects on cocaine infusions were observed on either test day (Supplemental Figure 5.4 a) and day 2 (Supplemental Figure 5.4 b). Furthermore, neither age nor antibiotic treatment altered lever pressing during PR (Supplemental Figure 5.4).

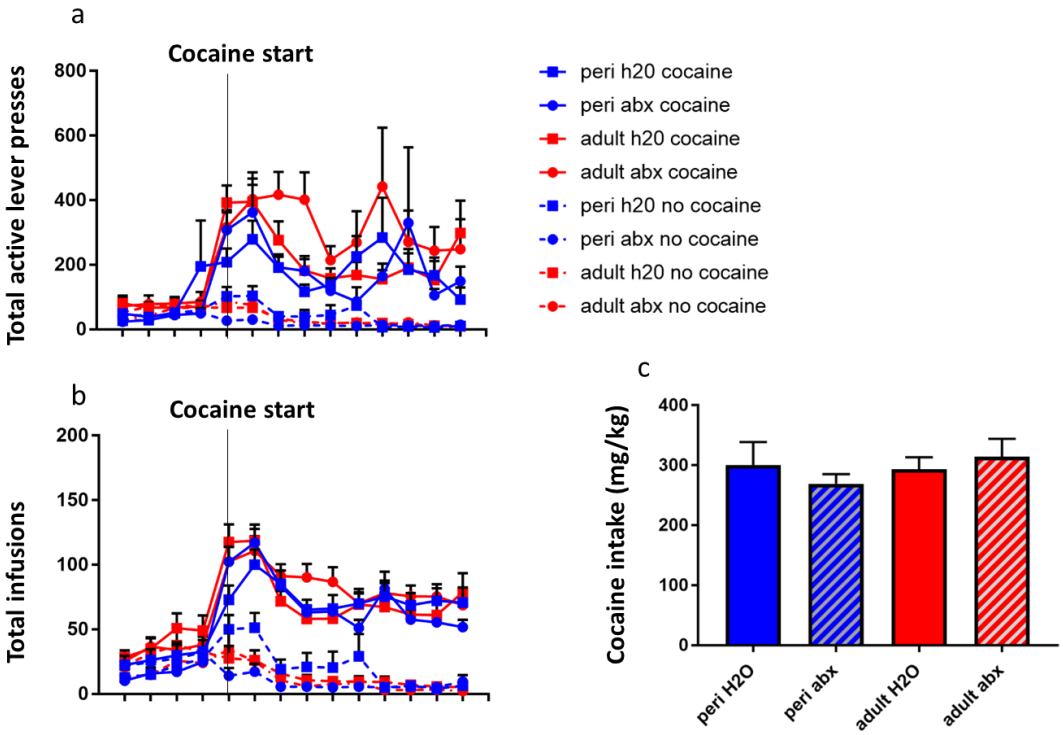


Figure 5.3 Antibiotic influence on cocaine self-administration
a. Animals that were administered cocaine had higher levels of active lever presses compared to no cocaine controls. Neither age nor antibiotic treatment altered total active lever presses. **b.** Animals taking cocaine earned more infusions than no-cocaine controls, but neither age nor antibiotic treatment altered total infusions. **c.** Neither age nor antibiotic treatment altered total cocaine intake. Vertical lines show onset of cocaine availability. All error bars are ± S.E.M.

5.4.3 Gut microbial depletion exacerbates cocaine-seeking behavior in adults but not adolescents

Cocaine experienced animals had higher rates of active lever-pressing during extinction and cue-induced reinstatement, compared with saline controls (F=4.94, p=0.004) (Figure 5.4). Adult cocaine animals had more presses during the reinstatement if they had received antibiotics during self-administration compared to their age-matched water treated counterparts (F=21.92, p=0.02) (Figure 5.4 b), an effect not observed in adolescent counterparts. Adult cocaine groups that had received antibiotics also trended toward higher extinction responding, but this was not statically significant.

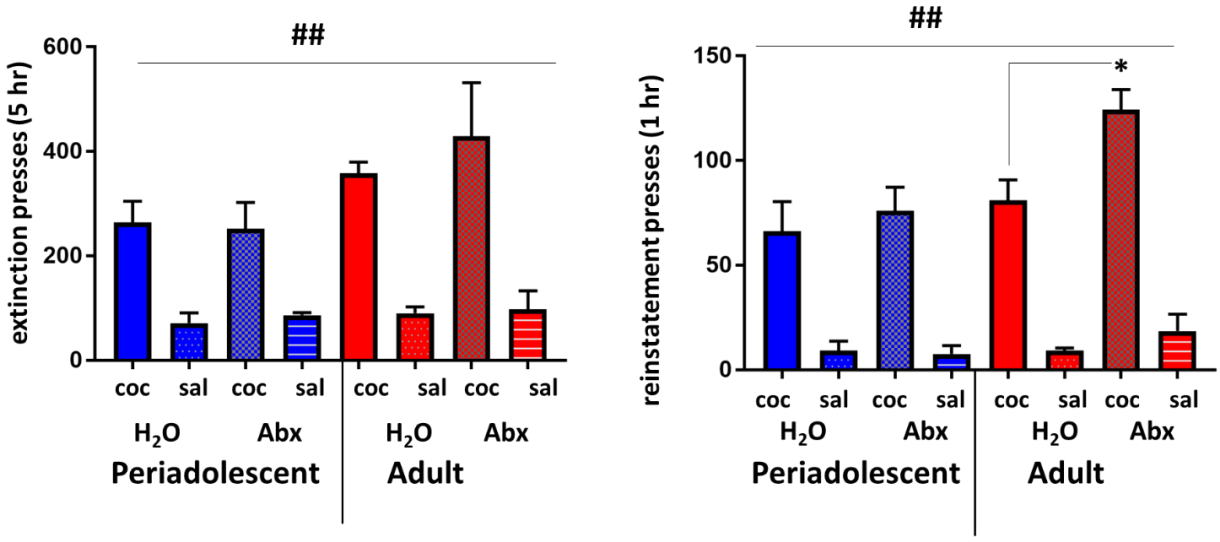


Figure 5.4 How inducing gut dysbiosis via antibiotics alters extinction responding and cue-induced reinstatement
Animals that self-administered cocaine had higher levels of total extinction presses compared to no cocaine counterparts (##) (**left panel**). Neither age nor antibiotics influenced extinction behavior. Animals that self-administered cocaine had higher levels of reinstatement presses compared to no cocaine counterparts (##) (**right panel)**. Adults that experienced dysbiosis via antibiotics had higher levels of reinstatement compared to no antibiotic counterparts (*) **(right panel)**. This effect was not observed in adolescent counterparts. All error bars are ±S.E.M.

5.4.4 Antibiotics alter the microbiota's abundance in an age specific manner

The microbiota was sampled at three different time points in the experiment (Figure 5.5a). Animals of both age groups with antibiotic experience showed a nearly 100 fold decrease in bacterial abundance compared to baseline levels, an effect not seen in water controls (Figure 5.5 b,c). Yet adolescent subjects with antibiotic experience recovered to baseline abundance levels after the 30 days of abstinence whereas adults remained dysbiotic after 30 days (Figure 5.5 b,c). Cocaine exposure did not influence these effects of antibiotics on bacterial abundance.

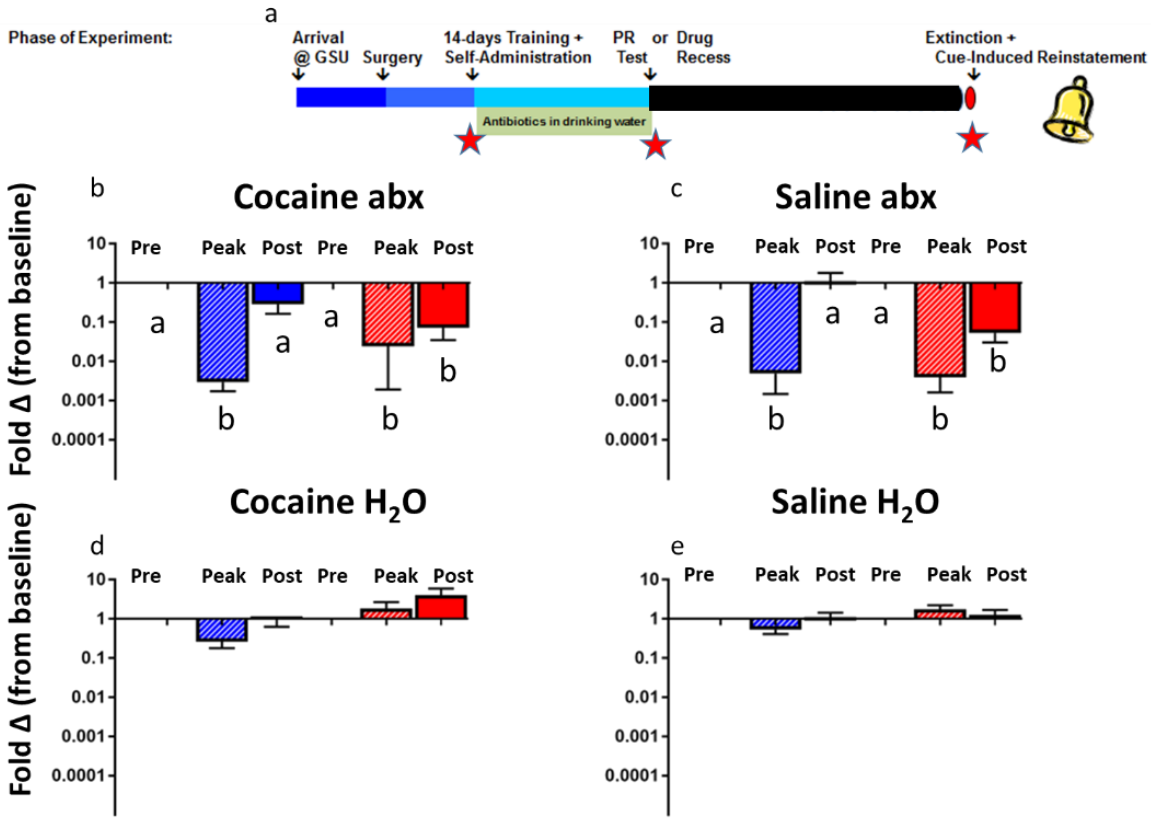


Figure 5.5 The influence of antibiotics and cocaine on gut microbiota abundance
a. Diagram of the experimental timeline with stars representing the key timepoints of fecal collection. Samples taken before cocaine/ abx administration (pre), samples taken right after 14 days of cocaine intake and antibiotic intake (peak), and samples taken after 30 days of abstinence from cocaine and antibiotics (post). **b.** In animals that have cocaine, antibiotics illicit a sharp decrease in gut microbial abundance. Adolescent animals recover to near baseline levels after the 30 day washout, but adult animals still have a dysbiotic gut after 30 days. **c.** Saline animals show a similar effect, demonstrating that antibiotics are the primary facilitator of this effect. Both

cocaine **d**. and saline **e**. animals that were not exposed to antibiotics do not show a significant decrease in gut microbial abundance (although a small decrease was observed in adolescent animals at the peak timepoint). All error bars are ±S.E.M.

5.4.5 Microbiota diversity and richness is altered by antibiotics and cocaine in a time dependent manner

In adult subjects we observed that the microbiota profile was altered by cocaine when comparing baseline (pre) samples with end of experiment (post) samples (p=0.003) (Figure 5.6; left panel). Irreversible microbiota changes are also observed in antibiotic treated vs water treated animals at pre and post (p=0.002) (Figure 5.6; right panel). While alpha diversity is unchanged in adults when looking at our perturbations (Supplemental Figure 5.5 a and c), we observe that taxa which contribute to microbiota profiles are different between adult control and adult cocaine animals as well as adult H_2O and adult abx animals (Supplemental Figure 5.5 b and d).

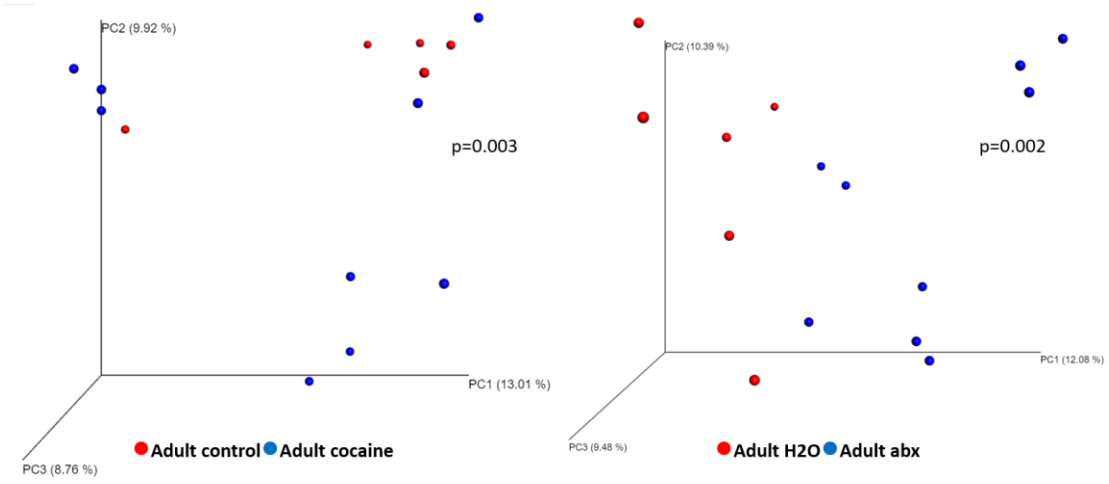


Figure 5.6 Gut microbiota changes in adults
Among adult rats, cocaine self-administration shifted microbiota diversity **(left)**, as did abx intake **(right)**.

When investigating the microbiota composition of adolescent subjects, we also see a shift due to cocaine intake between baseline and post (p=0.005) (Figure 5.7;left). When looking at

antibiotic's contribution to the adolescent microbiota we see that antibiotic and non-antibiotic subjects have similar diversity profiles between pre and post (p=0.567) (Figure 5.7; right). While alpha diversity was unchanged in adolescents (Supplemental Figure 5.6 a and c), we do observe that taxa which contribute to microbiota profiles are different between adolescent control and adolescent cocaine animals (Supplemental Figure 5.7 b). Even though adolescent H_2O and adolescent abx animals have no differences in microbiota diversity, they do show robust differences in individual taxa when assessed via LEfSe (Supplemental Figure 5.6 d).

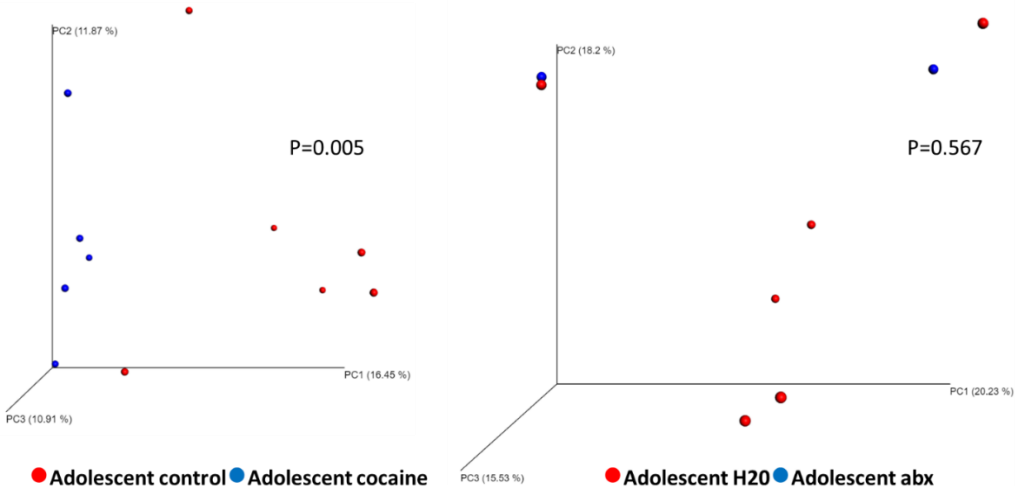


Figure 5.7 Gut microbiota changes in adolescents
Among adolescent-onset groups, cocaine self-administration shifted microbiota diversity **(left**), but abx intake did not (**right**).

5.4.6 Probiotic administration affected host physiology

Neither probiotic nor placebo administration during abstinence altered normal body mass and fluid intake compared to prior cohorts of animals that did take cocaine during abx treatment but were not syringe fed during abstinence (data not shown). Furthermore, neither probiotics nor placebo altered the mass of spleen, liver, or distal colon (data not shown). Although difficult to

analyze due to small group size and missing data for a no-cocaine, placebo-control group, it appeared as though adolescent-onset groups taking cocaine had smaller cecum mass than cocaine-naïve controls, an effect that was not altered by probx treatment. Among adults, cocaine self-administration did not appear to alter cecum mass, but probx treatment did decrease cecum mass, with significant effects in the cocaine-experienced animals. Non-significant outcomes in the saline-treated controls is due to low n (=1) for the saline-probx treatment group. (figure 5.8).

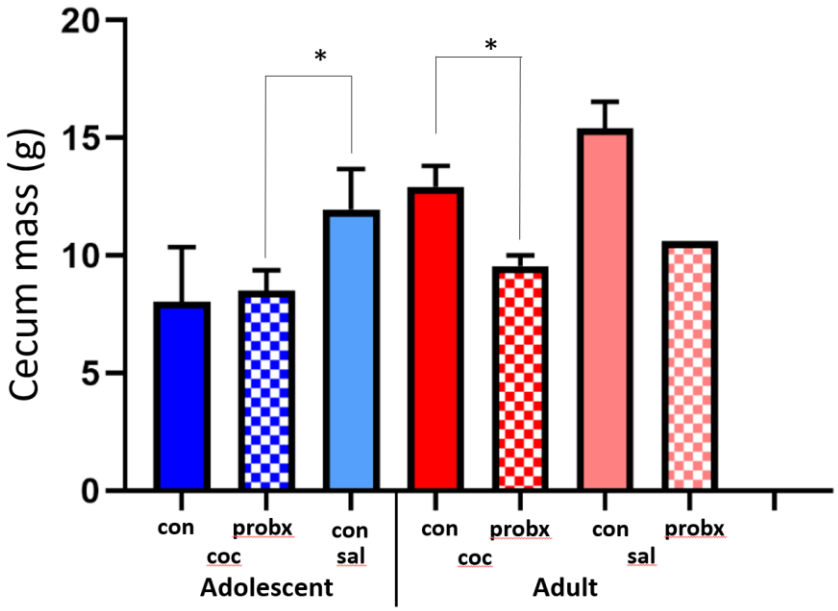


Figure 5.8 Probiotics attenuate cecum size
Adolescent animals that had probiotics and cocaine had a smaller cecum mass compared to saline placebo counterparts. In adults that took cocaine, probiotics resulted in a smaller cecum mass compared to placebo counterparts. All error bars are ±S.E.M.

5.4.7 Probiotics alter cocaine seeking in an age dependent manner

In tests of extinction and reinstatement of cocaine-seeking after 30 days of abstinence and probiotic (or placebo) treatment, animals that took cocaine had higher extinction ($t(24)=3.14$, $p=0.004$) and reinstatement responding compared to no cocaine controls, as expected ($t(24)=2.20$, $p=0.038$); Figure 5.9 a, b). Among adults, probiotic treatment decreased rates of

reinstatement responding among cocaine-experienced rats (t(9)=3.76, p=0.005) but this effect was not significant in adolescent-onset groups (figure 5.9 c). To account for failure among some rats to extinguish their lever-pressing during the five extinction tests, we calculated a Change Score by subtracting the number of active lever-presses during last extinction session (ext5) from the number of presses during the single reinstatement test session. Again, cocaine-experienced rats showed higher change scores than no-cocaine controls (t(24)=2.24, p=0.0349), and the probiotic treatment decreased change scores in adults (t(9)=7.08, p<0.001), but not adolescent-onset groups.

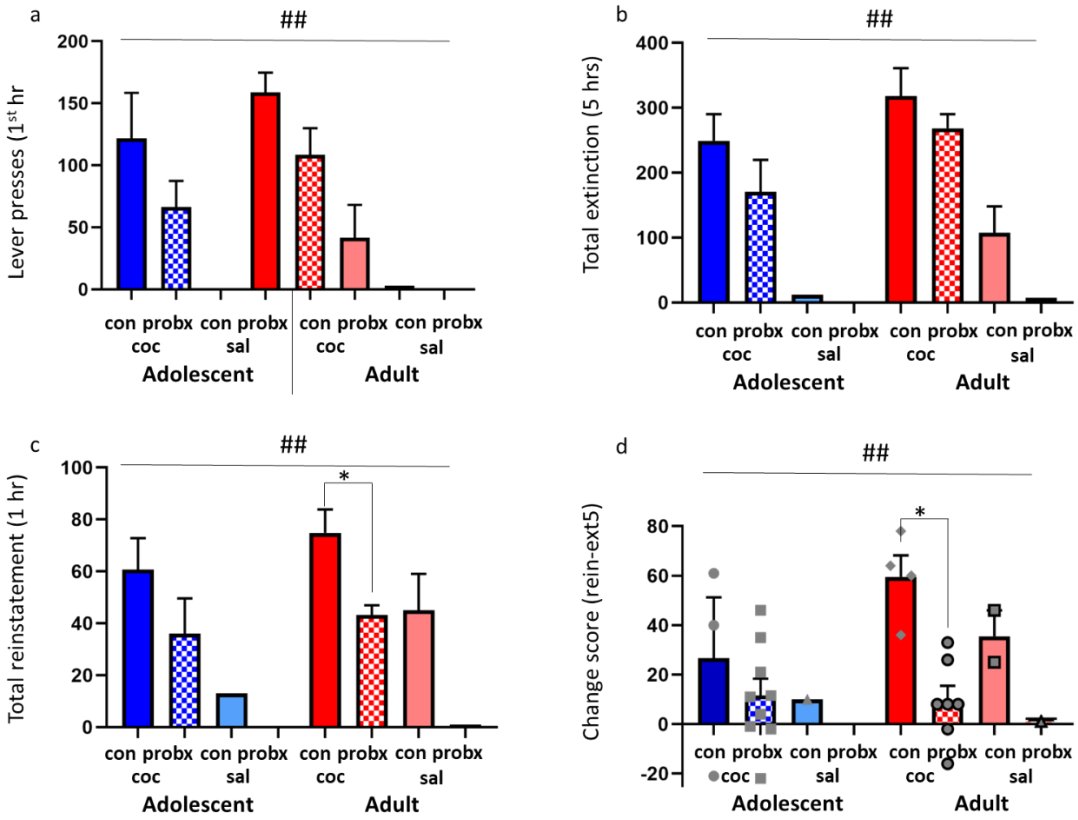


Figure 5.9 Probiotics modulate cocaine-seeking

a.Neither age nor probiotic treatment altered extinction responding in the first 1-hr session, but cocaine-experienced animals pressed more on the previously active lever than controls (##). b. Neither age nor probiotic treatment altered extinction responding over all extinction sessions combined, but cocaine-experienced animals pressed more on the previously active lever than controls (##). c. Cocaine-experienced animals pressed more on the previously active lever than controls (##) during the 1-hr reinstatement test, and probiotics attenuated reinstatement

compared to placebo controls in adults but not adolescents (*). d. Similarly, cocaine-experienced animals showed a greater change between the last extinction session and 1-hr reinstatement session, compared with controls (##), and probiotics reduced the change score compared to age-matched placebo controls in adults but not adolescents (*). All error bars are ±S.E.M.

5.5 Discussion

The present experiment tested the hypothesis that antibiotic-induced microbial depletion in the gut would exacerbate cocaine reward and reinforcement in adult male rats, with similar or reduced effects among adolescent male rats. This hypothesis was partially supported in that rates of cocaine self-administration were not affected by antibiotic treatment in either age group, but enduring effects of cocaine were indeed exacerbated as measured by rates of cue-induced reinstatement of cocaine-seeking after 30 days of forced abstinence, an effect observed in adults but not adolescent-onset treatment groups. Moreover, preliminary results included in this experiment lend support for the additional hypothesis that probiotic treatment during forced abstinence could rescue and/or further reduce normal levels of cue-induced reinstatement, such that adults receiving probiotics reinstated to lower levels than their placebo-treated, age-matched counterparts. As with antibiotics, the probiotic effects were not robust among adolescent-onset groups, although this portion of the experiment is hampered by low numbers of animals in each treatment group.

The antibiotic cocktail provided to rats in this study was effective at depleting the gut of the normal robust bacterial communities, as demonstrated by qPCR estimating overall bacterial abundance and confirming prior reports with a similar cocktail provided to adult male mice (Kiraly et al. 2016). All groups administered antibiotics for 14 days had their microbiota significantly depleted by over 100 fold. After 30 days of washout, adolescents had their microbiota return to baseline levels, whereas adults remained dysbiotic. On the other hand, cocaine self-administration alone did not reduce overall gut microbial abundance in either age

group. With regard to age differences in the timeline of recovery from the antibiotic insult, the microbiota may reach a stable state and become somewhat difficult to reshape in adulthood (Lozupone et al. 2013), such that only extremely stressful stimuli can cause a shift (stress, antibiotics, diet, etc). While perhaps beneficial under normal conditions associated with maintenance of homeostasis (Arumugam et al. 2011), this quality may prevent adults from recovering their microbiota over a 30-day "washout" after a 14-day antibiotic treatment. Faster recovery to baseline among younger animals could relate to greater fluctuation during development (Mohammadkhah et al. 2018; Tanaka et al. 2017; Borre et al. 2017) and reports that adolescent microbiota are still in unstable states, distinct from adults (Neufeld et al. 2011, Agans et al. 2011, Derrien et al. 2019).

Adolescence is a stage of rapid developmental with many physiological changes occurring including alterations in brain circuitry, hormones, and behavior. Researchers are now starting to piece together that these developmental changes occur on a similar timepoint as gut microbe maturation (Borre et al. 2014, Jasarevic et al. 2016). While many reports classify adolescence as a period of vulnerability, there are many instances when these rapid developmental changes can confer resiliency. Changes in physiology are thought to vital for adolescent vitality as they allow for adaptation to novel environments away from their parents. The combination of risk taking behavior and qualities that conifer resiliency are adaptive in allowing animals to seek out environments, individuals, and habitats that will allow them to be fit adults (Schramm-Sapyta et al. 2006). Drugs of abuse and antibiotics both have aversive qualities, throwing the body out of homeostasis. The above evidence would suggest that alterations in animal behavior through these aversive behaviors would be less robust in adolescents compared to adults due to resiliency.

The present antibiotic treatment exerted additional physiological impact in the gut. For example, antibiotics increased cecum mass regardless of age, an effect that was exacerbated by cocaine self-administration in adults, but not adolescents. Both antibiotics and germ-free conditions have previously been shown to increase cecum mass and size in mice (Kiraly et al. 2016; Grover et al. 2015). Although antibiotics and cocaine both induce inflammation in the gastrointestinal tract (Sun et al. 2019; Knoop et al. 2016; Chievero et al. 2019; Volpe et al. 2014; Linder et al. 2000), elevated cecum mass in the present experiment was not associated with elevated levels of the inflammatory marker, Lipocalin-2. Indeed, Lcn-2 levels were at or below the threshold of detection when using the same kit with mice (Chassaing et al. 2012,). It is possible that Lcn-2 is too limited as a marker of inflammation, as it is mainly expressed in neutrophils (Chassaing et al. 2012; Kjeldsen et al. 2000), but it is also possible that the increase in cecum mass could result instead from reduced gastric motility in the absence of bacteria that aid in digestion (Raja et al. 2018). Regardless, it seems that probiotic treatment may restore normal cecum mass, at least in adults. It could be the case that probiotics also keep cecum mass low in adolescent-onset groups, given a trend toward lower cecum mass in the probiotic-treated adolescent-onset group, compared to the no-cocaine, placebo controls. Yet the placebo-treated cocaine self-administration group did not show elevated cecum size either and the present dataset is too small to draw strong conclusions.

Gut microbial depletion was not associated with changes in acute cocaine-related reward and reinforcement, as measured by rates of acquisition and maintenance of cocaine self-administration on a schedule of reinforcement incurring low behavioral cost, i.e. FR1, 2-hr daily sessions, or on a schedule of reinforcement that measures acute reinforcing efficacy, i.e. PR, maximum 6-hr sessions. The overall trends in acquisition of lever-pressing using white noise

negative reinforcement, the transition to cocaine self-administration, and lack of age differences in overall cocaine intake replicates previous reports from our group (Suess et al. 2020a, Li et al. 2009). The lack of antibiotic impact on cocaine reward and reinforcement could be related to the present timeline of experimentation, i.e. gut bacterial populations may not yet have been depleted sufficiently by the time the rats were associating lever-pressing with cocaine reward. Alternatively, parameters of cocaine self-administration that incur higher behavioral cost such as higher FR requirements, second-order schedules, repeated PR testing, or models of compulsive drug-seeking in the face of aversive stimuli such as shock or aversive white noise, may be required to expose the short-term impact of gut-brain signaling on self-administration behavior. It is also possible that alterations in the microbiota do not play a role in the operant conditioning associated with cocaine self-administration, even though it did alter the classical conditioning required to demonstrate cocaine conditioned place preference in mice (Kiraly et al 2016).

The major finding in this report is that antibiotic treatment during cocaine self-administration was associated with higher rates of cue-induced reinstatement after 30 days of forced abstinence, but probiotic treatment during abstinence rescued lower levels of reinstatement, among adult rats. In the first phase of experimentation, antibiotic treatment increased reinstatement by approximately 33%, whereas in the second phase of experimentation, probiotic treatment decreased reinstatement by about 50%. Notably the levels of reinstatement among antibiotic treated rats was different across experimental phases, i.e. average 120 presses in the first phase vs. only 80 presses in the second phase, but nonetheless, the probiotics in the second phase still reduced reinstatement responding further down to average 40 presses. The absence of effect of gut manipulations on extinction responding is not unexpected, as the effect of age on enduring impact of cocaine, heroin, or morphine has varied across experiments from

our group to include both, one, or the other (Li et al. 2009; Doherty et al. 2009; Doherty et al. 2013). Furthermore, a trend toward probiotic effect on extinction responding among adults could become more robust when complete treatment groups are examined. The absence of effect of gut manipulations on extinction and reinstatement among adolescents could be related to the shorter timeline of gut dysbiosis in the younger rats; as noted above, overall bacterial abundance appears to have returned to baseline by the time of extinction and reinstatement testing. These findings support previous evidence that adolescent animals are resilient and protected from several aversive stimuli, particularly drugs of abuse (Ator and Griffiths 2003; Frantz et al. 2006; Schramm-Sapyta et al. 2009; Shram et al. 2008; Doherty and Frantz 2013; Hodgson et al. 2009; O'Dell et al. 2007; Schramm-Sapyta et al. 2006; Silveri Marisa et al. 2006). It remains possible that the probiotic could further decrease extinction and/or reinstatement responding among adolescent-onset groups, as a trend toward reduction is observed at present, despite the small size of treatment groups to date.

Beyond microbial abundance, we profiled the bacterial communities from fecal samples in order to test for differences in diversity between or within treatment groups that might relate to age or cocaine experience. Perhaps not surprisingly, the microbiome was significantly different across age groups at the pre-antibiotic/pre-cocaine baseline timepoint. This expands on previous reports that adolescents and adults have distinct gut profiles (Agans et al. 2011) to reveal several microbial groups that have higher relative abundance among adolescents than adults, and vice versa. We also observed that cocaine was associated with a shift in microbial composition in both age groups that persists through 30 days of washout, despite the lack of effect of cocaine alone on bacterial abundance and despite a return to baseline abundance levels among

adolescents by that time point. In the context of antibiotic effects, measures of diversity shifted with antibiotic exposure and recovery among adults, but did not change among adolescents..

The LEfSe analysis reveals bacterial groups of potential interest for future investigation. For example, the genus *Prevotella* was robustly expressed in adult cocaine vs. control subjects and appears related to peripheral inflammation (Larsen et al. 2017). Interestingly, the genus *Coproccus* was robustly expressed in adolescent cocaine groups and adult antibiotic groups and is linked to depression (Valles-Colomer et al. 2019). On the other hand, taxa observed with higher relative abundance among adolescent cocaine-experienced rats vs. their age-matched controls have been associated with mental health (Valles-Colomer et al. 2019) and SCFA production (Machiels. et al 2014). This comes as a surprise given that SCFA supplementation has been shown to attenuate cocaine related behaviors (Kiraly et al. 2016). It is possible that SCFAs do not alter reward sensitivity in adults as they do in adolescents. Further studies should test whether SCFAs are altered in an age dependent manner in response to antibiotics and/or cocaine.

The mechanisms through which cocaine might change gut microbial communities remain to be explored, but several possibilities exist. For example, cocaine certainly activates the sympathetic nervous system and hypothalamo-pituitary axis, which exerts significant impact on gut function. Cocaine also changes compromises pulmonary function (Maceira et al. 2014), triggers inflammatory responses (Fox et al. 2012), and damages the mucosal membrane around the gut (Chivero et al., 2019), inducing colitis or other gut related disorders (Linder et al. 2009), or actually perforating the duodenum or other gastric tissue (Feliciano et al. 1999; Uzzaman. et al. 2010). Indirectly, cocaine alters dietary regimens among users (Ersche et al. 2013) and

decreases in body weight (Cochrane et al. 1998). These cocaine effects alter the gut milieu, with potential to promote or inhibit growth of specific bacterial populations.

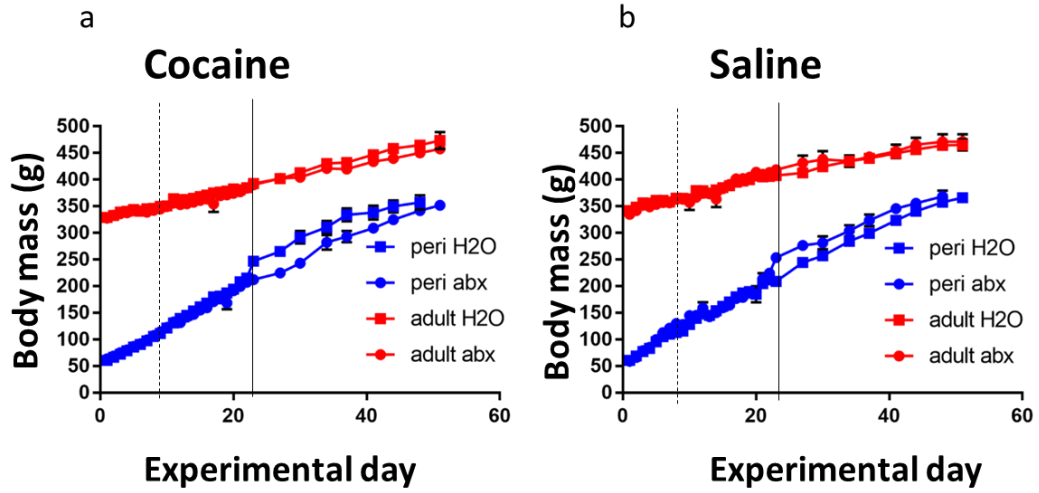
Mechanisms through which the gut milieu influences behavioral reward and reinforcement also warrant investigation. Microbial depletion that enhanced cocaine reward was also associated with abnormal levels of reward-related transcripts in the nucleus accumbens (Kiraly et al. 2016), providing an example of brain changes after gut manipulations. Gut microbes may influence brain function via vagal signaling, as microbial metabolites appear to activate the vagus nerve, potentially reaching limbic system and striatal targets through a synapse in the nucleus tractus solitarius (NTS) (Han et al. 2018). In fact, vagotomy eliminates beneficial probiotic effects through GABA receptors (Bravo et al. 2011). Moreover, gut dysbiosis may result in changes in peripheral cytokines, transmitter precursors, SCFA, and other neuroactive molecules that normally reach the brain via circulation (Cryan et al. 2012).

While this report provides details on the relationship between the gut microbiota and substance use disorder, there are some limitations that should be addressed in future studies. The National Institutes of Health (NIH) and other government-funded agencies have initiated mandates to use both sexes for research designs, analysis, and reporting unless due justification is claimed. Sex differences in drug related behaviors have been extensively reviewed with men using illicit drugs more frequently (Abuse et al. 2013) but women escalating drug use more quickly than men (Lynch et al. 2002; Brady et al. 1999). Sex differences in the gut microbiota composition have also been reported (Org et al. 2016; Kim et al. 2020). We fully support subsequent experiments that investigate our findings in females. This study also provides promising results using probiotics to attenuate drug-seeking behavior. Yet it only utilizes a small group of animals; follow-up experiments will increase the power of analysis. Furthermore, this

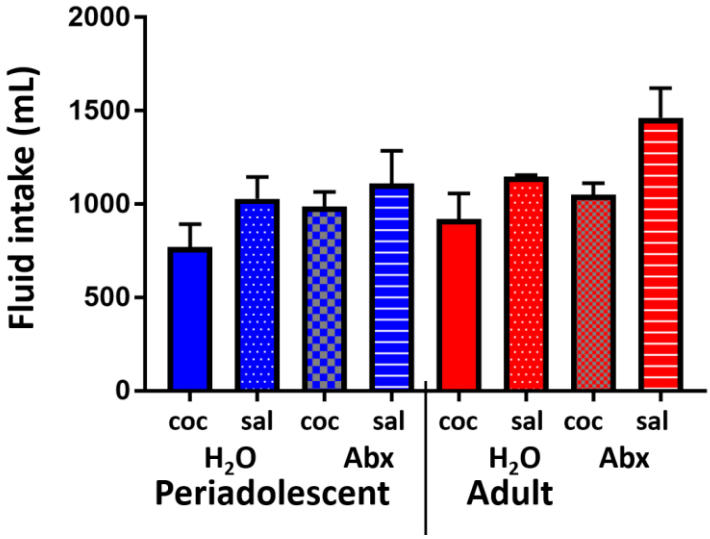
report does not investigate brain adaptations in response to microbial alterations. Analysis of reward related brain regions and examination of dysregulation of reward related transcripts/ proteins, or synapse activation or function are a logical next-step identifying gut to brain interactions. Expanding to examine whether similar gut-behavior interactions exist with other commonly abused substances such as alcohol, heroin, or amphetamines is also important.

In conclusion, the present experiment suggests a critical role for gut health in attenuating cocaine reward and reinforcement, at least among adult subjects. Whereas antibiotics significantly altered gut bacterial communities and elevated reinstatement of cocaine-seeking after abstinence among adult male rats, probiotic treatment during abstinence brought reinstatement levels back down below control levels. Although these effects of gut microbial depletion were not robust in adolescent male rats, the probiotic treatment might still be associated with lower levels of reinstatement of cocaine-seeking in younger subjects, based on a trend in the current results with relatively few animals per treatment group. These datasets suggest that probiotics may serve as an affordable, accessible, and effective adjunct treatment for cocaine abuse.

5.6 Supplemental Figures

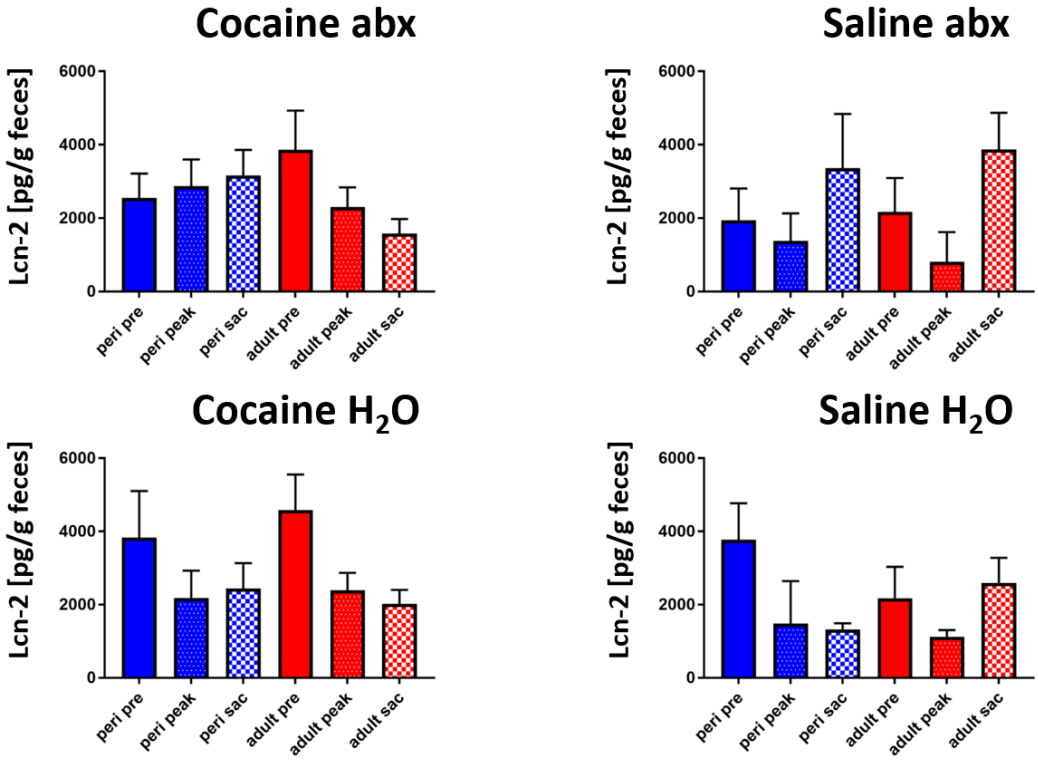


Supplemental Figure 5.1: The influence of antibiotic and cocaine on body mass throughout the experiment.
Progression of body weight factoring age (Peri vs adult), antibiotics (abx vs H_2O), and drug (**a.** cocaine vs. **b.** saline). Dashed line represents onset of antibiotics and solid line represents cessation of antibiotics. All error bars are ±S.E.*M.*

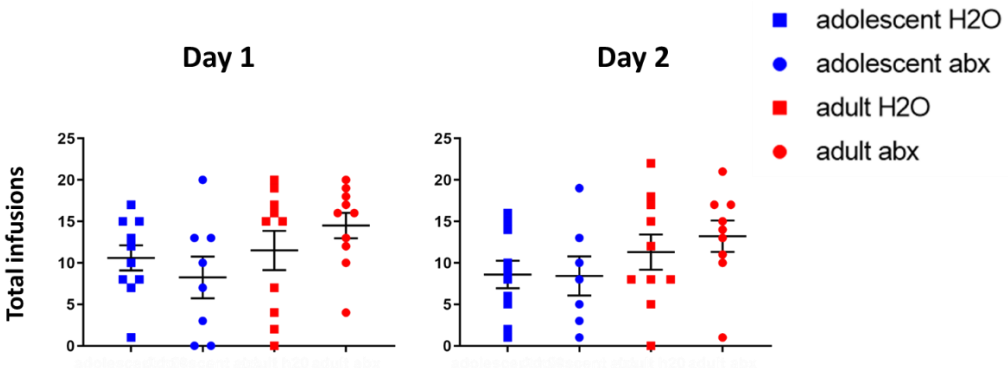


Supplemental Figure 5.2: Home-cage fluid intake throughout the experiment

No differences by age, abx treatment, or cocaine experience were observed. All error bars are ±S.E.M.

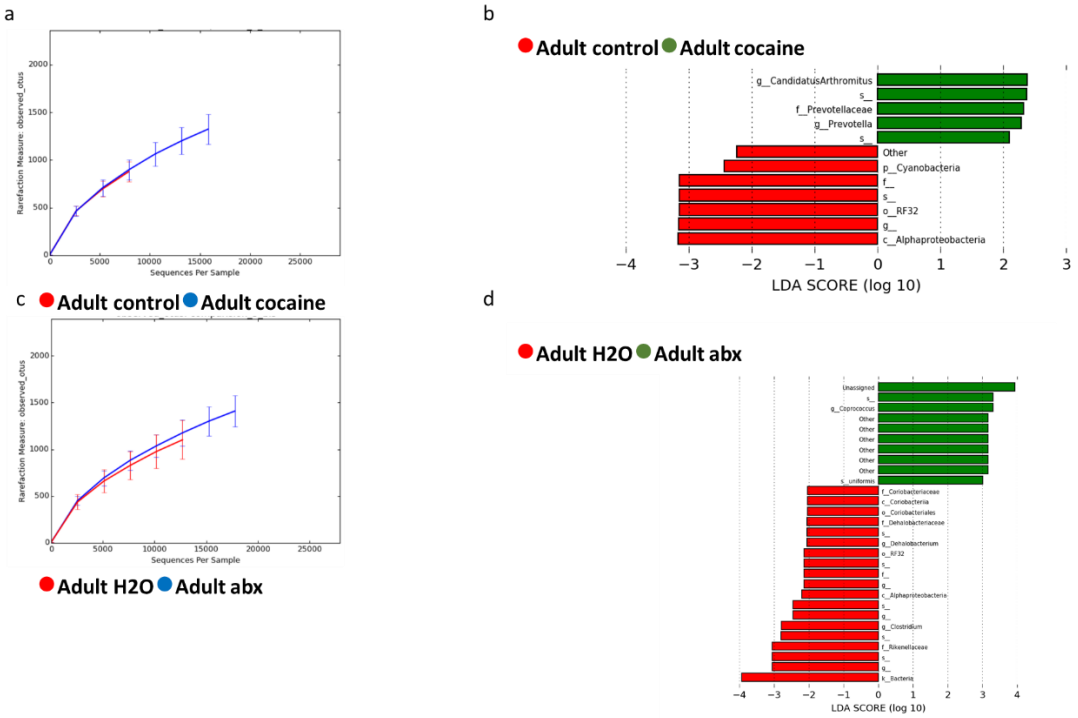


Supplemental Figure 5.3: Peripheral gut inflammation measured by fecal lipocalin-2. Using the marker fecal lipocalin-2 (Lcn-2) we observe that neither cocaine nor antibiotics alter the inflammatory response. All error bars are ±S.E.M.



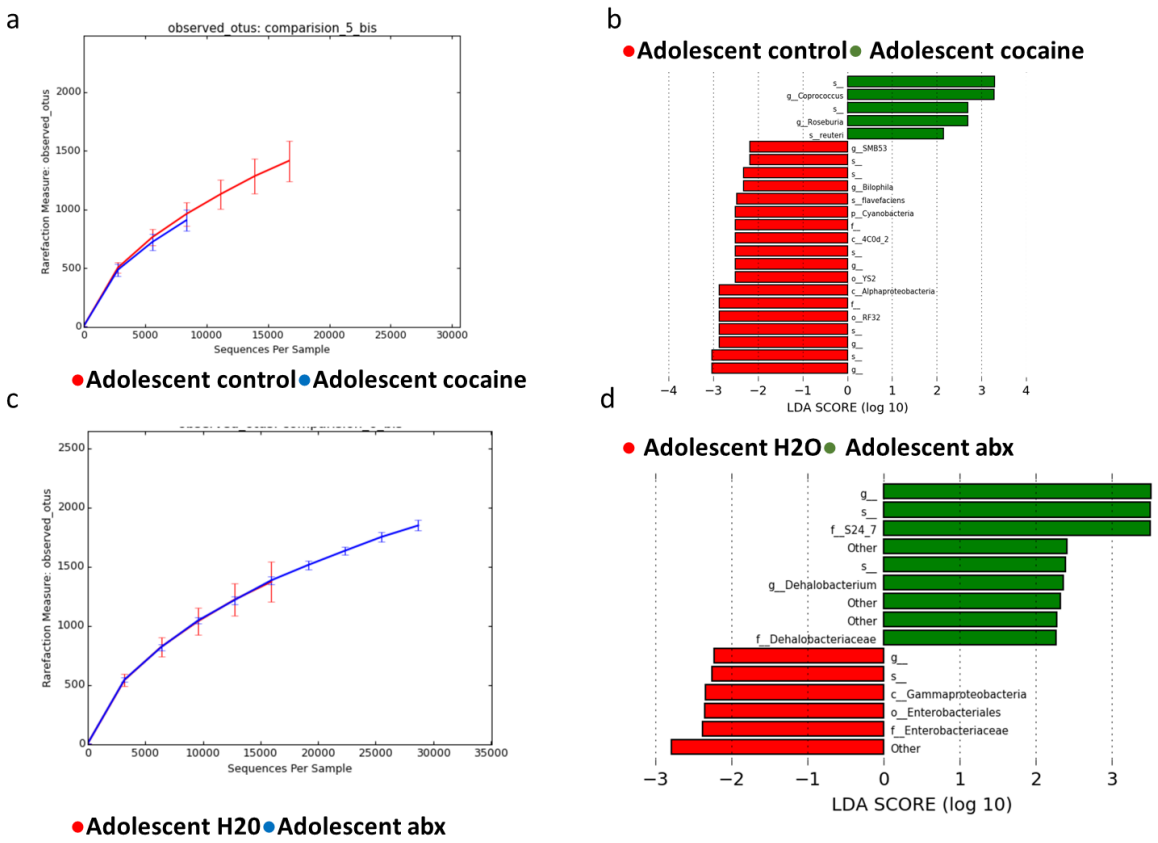
Supplemental Figure 5.4: How inducing gut dysbiosis via antibiotics affects the motivation to seek out cocaine through a progressive ratio schedule.

Number of infusions were not significantly different between **a.** day 1 and **b.** day 2 of progressive ratio self-administration. Neither age, nor antibiotics altered active lever presses during progressive ratio testing. All error bars are ±S.E.M.



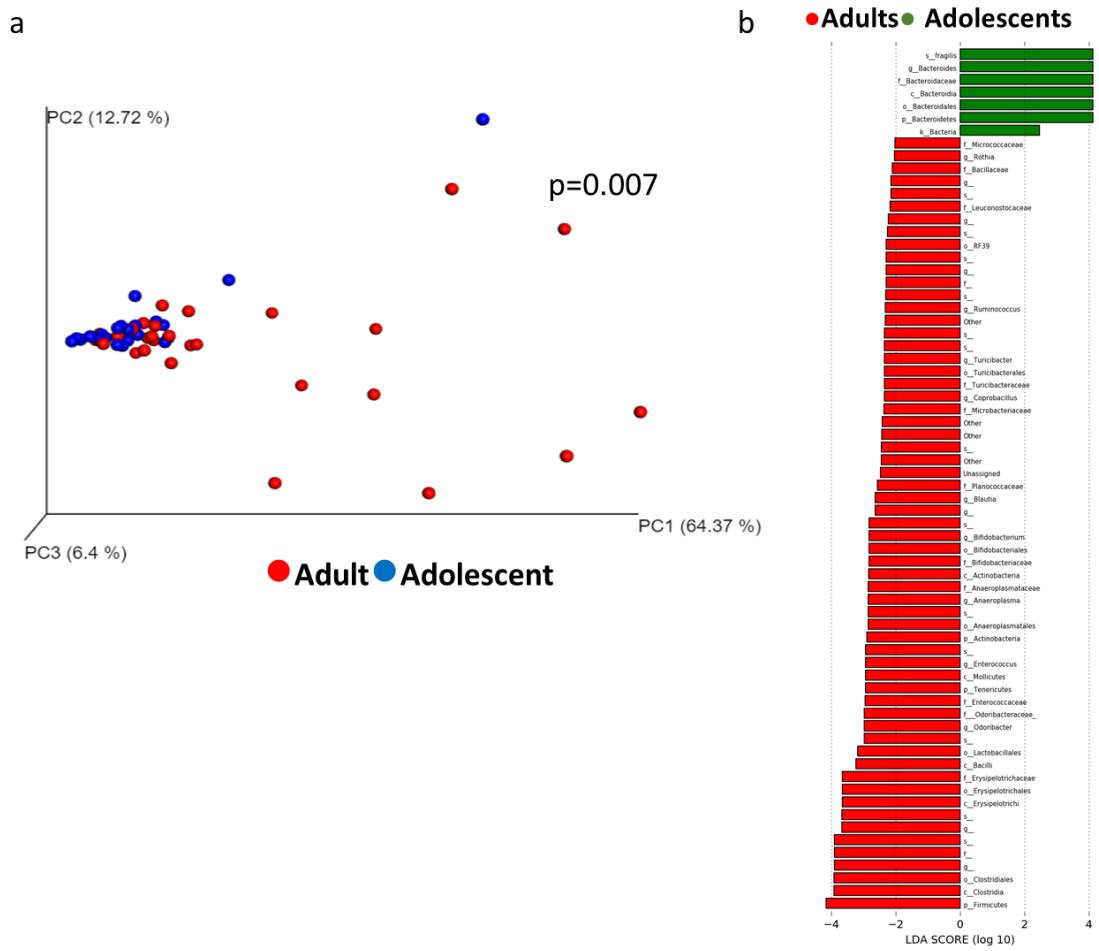
Supplemental Figure 5.5 Characterization of the adult microbiota after cocaine and antibiotic intake

a. Alpha diversity plots show no difference between cocaine and non-cocaine adults at sacrifice. **b.** However, several bacterial groups are in higher relative abundance among cocaine or control groups (green or red, respectively). **c.** Alpha diversity plots show no difference between H_2O and antibiotic adults at sacrifice, **d.** However, several bacterial groups are in higher relative abundance among antibiotic or control groups (green or red, respectively).

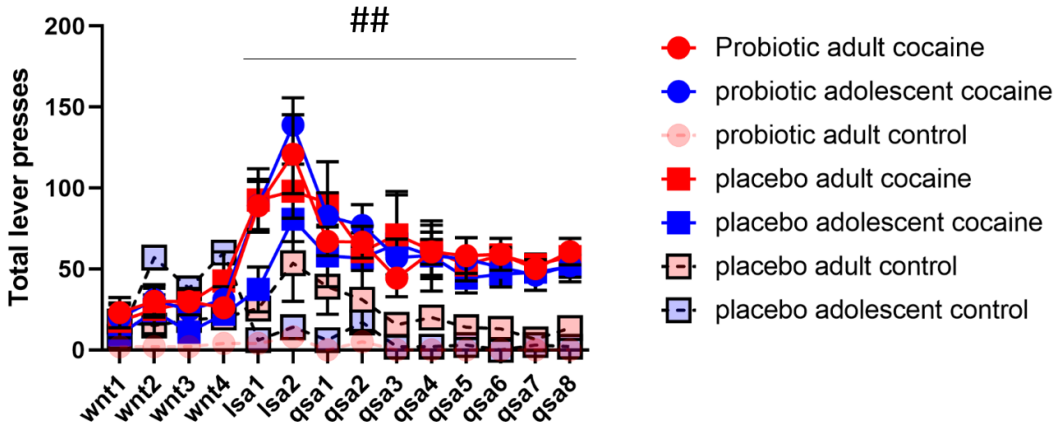


Supplemental Figure 5.6 Characterization of the adolescent microbiota after cocaine and antibiotic intake

a. Alpha diversity plots show no difference between cocaine and non-cocaine adolescents at sacrifice. **b.** However, several bacterial groups are in higher relative abundance among cocaine or control groups (green or red, respectively). **c.** Alpha diversity plots show no difference between H_2O and antibiotic adolescents at sacrifice. **d.** However, several bacterial groups are in higher relative abundance among antibiotic or control groups (green or red, respectively).



Supplemental Figure 5.7: Adolescent and adult microbiota profiles are distinct
a. Using PCA beta diversity analysis we find that adolescents (sampled at PND 25) and adults (sampled at PND 73-77) cluster distinctly. **b.** LEfSe shows that several bacterial groups are in higher relative abundance among adolescents or adults (green or red, respectively).



Supplemental Figure 5.8 Active lever-presses during cocaine self-administration before probiotic treatment.
Animals taking cocaine had higher levels of active lever presses compared to no-cocaine controls. Age groups did not differ in active lever presses and no differences at baseline existed between future probiotic vs. placebo treatment groups. All error bars are ±S.E.M.

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6 CONCLUSIONS

6.1 Summary of findings

Adolescence is a period of development in which risk-taking, novelty-seeking, and exploration are heightened (Spear et al. 2000). While these qualities may predispose adolescents towards initial drug use and abuse, adolescents also possess resilience factors that appear protect them from aversive stimuli (Schramm-Sapyta et al. 2006; Shram et al 2008; Doherty et al. 2013; O'Dell et al. 2007). A major goal of this research is to identify what makes adolescents and adults different in their drug seeking behavior. While evidence has shown that under maturation of prefrontal cortex, limbic system, and other reward related brain regions may be responsible for heighted drug use and poor judgement (Spear et al. 2000), the role of an equally underdeveloped gut microbiota is vastly underexplored.

We have several lines of evidence show that adolescent rodents are less sensitive to aversive stimuli, both in terms of how these stimuli modulate behavior and the gut microbiota. Through the use of a white noise stimuli as a negative reinforcer we are able to facilitate lever pressing in operant conditioning chambers in both adolescent and adult male rats. This method can be extended to multiple different types of research designs. White noise generators are easy to install, are inexpensive to purchase and maintain, can be fine-tuned by the experimenter based on current needs, and do not cause harm or pain to the subject. White noise used as a positive punisher also attenuates cocaine seeking behavior in adults but not adolescents, again reinforcing the idea that adolescents are less vulnerable or resistant to aversive stimuli.

The relationship between the gut microbiota and substance use disorder is underexplored. We attempted to determine whether the high variability we observe in

cocaine taking behavior was related to specific gut microbiota profiles. Results show that the microbiota in drug naïve individuals is similar but begins to differentiate and become unique throughout cocaine exposure. In addition, the use of particular microbiota taxa at baseline can be used as a diagnostic tool to predict the severity of future drug use. Both of these findings can be used to assist clinicians in diagnosing whether casual drug use will develop into severe addiction or if children have microbiota profiles that may be early indicators of future maladaptive phenotypes.

Illicit drug use possess a huge economic and healthcare burden on society (French et al. 2008; Whiteford et al. 2013; Patel et al. 2016). Tools to predict future drug use as well as medications to attenuate current use are ineffective. Persons who successfully moderate or abstain from drug use tend to fall back into addictive habits or relapse. To that end there are currently no successful therapies that significantly prolong abstinence and prevent relapse in cocaine abuse. Our research sought to examine whether manipulation of the gut microbiota alters the rewarding aspects of cocaine. Adult rats that experienced antibiotic induced gut microbial depletion had increases in reward seeking behavior measured by the cue-induced reinstatement model. These findings were not seen in adolescent counterparts, even though antibiotics resulted in a similar reduction in abundance and diversity in the microbiota. A key finding was that adolescent animals had their microbiota return to baseline levels after thirty days of washout, while adults remained dysbiotic. This resilience shown by adolescent subjects seems to protect them from the enduring effects of antibiotics and cocaine. This resilience to aversive stimuli may protect adolescent subjects from the vicious cycle of drug abuse. Outside stimuli such as diet, stress, and antibiotics alter the microbiota composition. Altered microbiota composition may change gut produced metabolites and neuroactive

molecules, which through either direct signaling pathways or indirect pathways through circulation may alter brain function. Alterations in brain function, particularly in reward related brain regions could result in further maladaptive behavior towards harmful stimuli. While cocaine intake does not alter microbiota abundance it does create a shift in diversity and species richness that persists even through a 30 day period of cocaine abstinence. Cocaine being an aversive stimuli, may perpetuate the vicious cycle of cocaine addiction more severely in adults vs adolescents due to the inability of the microbiota to bounce back to health levels.

One way that increases in drug seeking-behavior could be attenuated is returning the microbiota abundance and diversity back to pre-intake levels. We sought to do this using a probiotic formulation that has shown to alleviate other neuropsychiatric symptoms, such as depression and stress. Preliminary findings show that in adult animals that have experimenter induced gut microbial depletion, probiotic administration reduces cue-induced reinstatement. This finding was not observed in adolescent counterparts. It is possible that probiotics, while beneficial, do possess aversive qualities that the body tends to reject. This would make their efficacy in adolescent subjects less than adult counterparts.

6.2 Future directions & Limitations

While this dissertation synthesizes many new findings there are some limitations that must be reported. The work in this paper is conducted with male subjects only. Due to physiological and neural differences between males and females, as well as females experiencing different severity of disease states compared to males, makes the use of both sexes a mandate in scientific research. This is especially true in our line of work given that drug seeking, intoxication, withdrawal severity, and likelihood to relapse all skew

towards one sex. In fact, it has been reported that females initiate drug use more quickly than men (Lynch et al. 2002; Brady et al. 1999). Furthermore there is preliminary evidence that the microbiota composition is driven by sex (Org et al. 2016; Kim et al. 2020) and that stimuli that may influence the microbiota (diet, environment, stress, etc.) are sex dependent. We acknowledge that the use of male subjects does have its benefits in reducing the number of experimental animals, as previous literature that guided our research was done with male animals. We also acknowledge that previous work with only male subjects does not paint a complete story of gut-brain-cocaine interactions and that females are needed to complete findings. Along with our age dependent results, we encourage all follow up experiments to not only have males and females, but also adolescent and adult counterparts. While little is known on how adolescence contributes to gut related changes in cocaine behavior, less is known when adding in the variable of sex. The use of both age groups and both sexes will advance our understanding of how the microbiota contributes to substance use disorder.

These experiments contribute to previous literature that the gut microbiota is associated with substance use disorder (Kiraly et al. 2016; Ning et al. 2017; Meckel et al. 2019). While it has also been shown that the gut microbiota is associated with several other neuropsychiatric disorders such as schizophrenia (Nguyen et al. 2019), depression (Peirce et al. 2019), and bipolar disorder (Flowers et al. 2020), the mechanisms underlying these relationships are remain unknown. Until the mechanism of this connection is understood, the scope of our findings and these relationships between neuropsychiatric disorders and the microbiota will be limited. Besides the evidence that the vagus nerve and circulatory factors play a part in gut brain communication (Cryan et

al. 2012; Cryan et al. 2019), recent findings are attempting to map out gut-brain neuronal circuitry and vice versa. Optogenetic activation of vagal sensory ganglion results in sustained self-stimulation behavior as well as dopamine release from areas of dorsal striatum. Neuronal labeling follow up experiments revealed glutamatergic neurons of the dorsolateral parabrachial region as a region that mediates gut related signals to the striatum. In summary, these researchers propose that gut vagal afferents synapse on the nodose ganglion of the vagus nerve, sending signals to brain stem then up to areas of midbrain and striatum to facilitate approach and reward behavior (Han et al. 2018). These findings are reinforced in a study that found brainstem neurons are activated via gut microbial depletion. These neurons can then relay information to premotor glutamatergic neurons that feedback to the gut to regulate gut motility (Muller et al. 2020). This reports highlights the idea that the gut brain axis is “bi-directional” and that feedback loops exist in this circuit involving the brain. Future experiments should refine these and other circuits and uncover what are the changes in the brain that occur due to gut dysbiosis. Furthermore, are these changes in the brain long lasting and do they impart long term detriments to the gut.

While understanding how the gut and brain communicate is important, understanding what changes occur in the brain in response to gut dysbiosis is equally vital. Mice that experienced experimenter induced gut microbial depletion were found to have an alteration in reward related transcripts in the nucleus accumbens (Kiraly et al. 2016). It is logical that any change in reward related behavior is not a direct consequence of the microbiota, but rather the microbiota acting on the brain which shapes behavior. Future experiments should investigate the brain in post-mortem subjects to determine

what changes occur in response to gut dysbiosis. Furthermore, examining the brain at different stages of antibiotic administration and different phases of cocaine addiction (intoxication, withdrawal, and relapse) would uncover exactly how experimenter induced gut dysbiosis could affect the brain. Furthermore, examination of brains after probiotic treatment would determine if alterations in reward related brain regions return to normal when the microbiota returns to normal.

A major focus of chapter three in this dissertation was to determine the reinforcing and punishing qualities of a white noise stimuli. The intensity of the white noise used in these experiments was 74-85 dB, which was intended to be aversive, but not painful based on previous findings. While this method was effective at facilitating acquisition to lever pressing and attenuating cocaine seeking in some animals, it failed to influence cocaine taking during self-administration. It is possible that white noise was aversive for some subjects and not for others, as there was consider variability in cocaine taking behaviors. Future studies should titrate white noise per each animal, with the goal of making the noise aversive enough to facilitate lever pressing, but loud enough to attenuate cocaine taking and seeking.

Sampling of the microbiota was done using fecal boli collected from animals at various parts of the experiment. This was advantageous to our design as it allowed us to assess changes in the microbiota across the experiment. Importantly, baseline samples allowed within subject comparisons, a huge advantage since microbiota comparisons to control subjects are difficult given that there is no such thing as a standard microbiota. The decision to sample the microbiota from fecal samples does have some flaws, the biggest of which is loss of several taxa are lost as food moves through the digestive tract

to be excreted. Some researchers have suggested that colon or cecal microbiota sampling is a better method to assess the microbiota, as key species are not lost through digestion, metabolism, or defecation (Stanley et al. 2015;Pang et al. 2012). Combined with findings that the microbiota between humans and rodents contains dissimilarities (Nguyen et al. 2015) consideration for sampling the microbiota in cecum along with running parallel experiments using other animal models (such as *Mus musculus*) are work considering.

The probiotic administration during abstinence leading to attenuation of cocaine seeking during reinstatement is a very promising finding. Unfortunately the power in these results is low due to low subject counts in each experimental groups. There are also some interesting trends, such as probiotics attenuating reinstatement in adolescent animals that are not significant due to high variability and low subject numbers. Follow up experiments will bolster the number of subjects in this group to determine if behavioral findings hold true or if new findings emerge. Follow-up experiments will also need microbiota analysis similar to antibiotic only animals to determine if changes in microbiota abundance and diversity relate to changes in cocaine-seeking behavior. We are also very interested to test whether probiotics can be used as a prophylactic measure to protect against cocaine taking and cocaine-seeking. Future experiments should give probiotics to subjects for the duration of the experiment in the hopes that initial cocaine use will not be robust. The use of these particular probiotics in specific aim three came from previous research that showed attenuation of depression and anxiety like behavior through chronic administration in lab rodents (Messaoudi et al. 2011; Arseneault-Breard et al. 2012; Ait-Belgnaoui et al. 2014). While this probiotic blend has beneficial effects,

future studies should consider targeted probiotic treatment that replace bacteria lost in dysbiotic subjects, with the goal to restore the gut microbiota to baseline levels.

6.3 Final thoughts

This study examines how the gut microbiota is involved in cocaine-taking and cocaine-seeking tasks. An observed variability in cocaine behavior is related to microbiota profiles and shifts in cocaine taking are associate with microbiota changes. Moreover, microbial depletion is associated with higher cocaine seeking, whereas microbial restoration is associated with attenuated cocaine seeking. This study has also shown that adolescents are less affected by aversive stimuli than adults, including a punishing white noise stimulus as well as gut microbial depletion or restoration. Instead of adolescence being thought of as a period of vulnerability, we would like to think of it as a period of resiliency during which dynamic changes in brain and gut physiology can protect the organism from long-term perturbations. We proposed a vicious cycle of drug abuse in which drug seeking behavior can be modulated indirectly via gut-brain axis interactions. Maladaptive stimuli can induce gut dysbiosis resulting in modulation of reward related brain regions. Altered brain functioning can drive addiction like behaviors which perpetuate the cycle. Adolescence is a key period during this cycle as the rewarding properties of drugs and the aversive properties of external stimuli are different compared to adults. When studying addiction and attempting to create therapies to combat substance use disorder, adolescent individuals and the gut brain axis should be considered.

6.4 References

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