Amherst College

Final Proposal:
Do gut microbiota-produced short-chain fatty acids mediate cocaine relapse?

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1. Introduction

In recent years, there has been explosive growth in research on the gastrointestinal microbiota composition and how disruptions to its natural composition (dysbiosis) relate to the pathogenesis of diseases and disorders, such as obesity, mood-anxiety disorders, and autism[1]. However, despite the high rates of comorbidity between mood-anxiety and substance use disorders in humans[2], little work has explored the connection between gut microbiota alterations and the rewarding properties of drugs of abuse. In the US and beyond, cocaine use is highly prevalent and induces significant self and social harm[3]. Notably, the psychostimulant has significant relapse rates and treatments remain unsuccessful. Therefore, this paper will integrate current knowledge on cocaine addiction and relapse with the growing body of work on the microbiota-gut-brain axis to propose methods to assess whether gut dysbiosis augments relapse likelihood and whether this behavior may be mediated by the bacterial metabolite short-chain fatty acid.

Mechanisms Underlying Cocaine Addiction and Relapse

Drugs of abuse, including cocaine, primarily act on the mesocorticolimbic, or reward, pathway. This dopaminergic pathway connects the ventral tegmental area (VTA) to the forebrain structures nucleus accumbens (NAc) and the prefrontal cortex (PFC) and regulates incentive salience to direct goal-driven behavior such as feeding and sex[4,5]. Furthermore, it is now clear that cocaine generates an unnaturally large build-up of dopamine (DA) to act on the NAc's DA receptors and induces plasticity changes in the pathway's structures involved in the development of addiction and relapse tendencies [4,6–8]. First, the neurophysiological transition between cocaine social use to addiction—and vulnerability to relapse—is believed to be mediated by a signaling cascade that precipitates from DA D1-like receptor activation to induce changes in gene

transcription and chromatin remodeling[6] (Figure 1). Mechanistic studies have shown that NAc D1 receptor stimulation increases cyclic adenosine monophosphate (cAMP), cAMP-dependent protein kinase (PKA), and cAMP response element-binding protein (CREB), which facilitates the transcription of a plethora of genes implicated in cocaine abuse (*Arc, cfos, Homer*, and Δ*FosB*)[6,9–11].

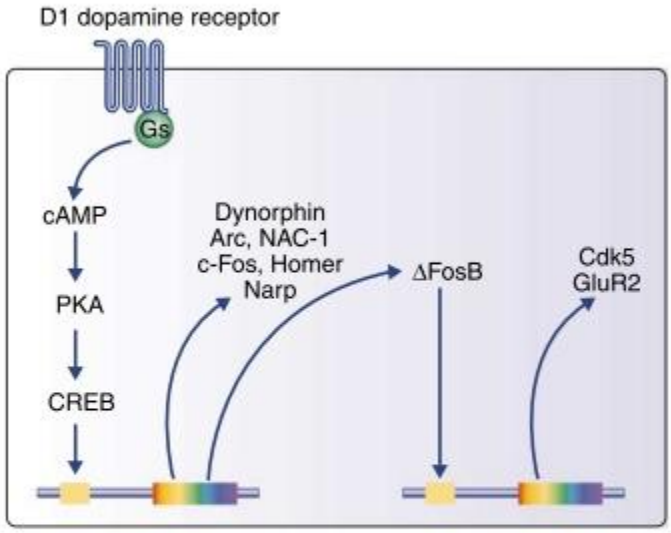


Figure 1: Dopamine D1 receptor-dependent signaling in the NAc. Activation of transcriptional regulator CREB stimulates several transcriptional regulators that induce a cascade of protein synthesis thought to contribute to long-term cocaine-induced neuroplasticity.

Of these CREB-regulated immediate early gene (IEG) products in the NAc, transcription factor ΔFosB is particularly influential and is believed to be an important molecular "switch" in the shift from drug abuse to addiction because 1) unlike the other IEGs, ΔFosB remains in the striatum's DA terminal fields for up to 8 weeks[6,10,11], 2) elevated ΔFosB is correlated with addiction-like behaviors[10], and 3) is responsible for over 25% of all chronic cocaine-induced gene expression changes and these changes become more ΔFosB-dependent (over CREB) over longer use[12]. However, because ΔFosB is relatively transient and decreases over abstinence[6], the lasting regulation of neuroplasticity is thought to be, in part, mediated by transcriptional targets of ΔFosB, such as the neuronal migration-regulator cyclin-dependent kinase 5 (Cdk5)[13,14]. Indeed, past studies have shown in mice that increases in NAc-Cdk5 induced by chronic cocaine

administration and by ΔFosB upregulation both increased the NAc shell's medium spiny neurons' dendritic spine density and that Cdk5-inhibitor treatment had the opposite effect[15,16]. However, there are conflicting reports regarding the relative permanence of spine density increases after an attenuation phase over 30 days and whether they predict behavioral responses[17]. Regardless, in the long-term, NAc-Cdk5 exhibits a compensatory role, as relapse studies pairing cocaine with Cdk5 inhibitors led to mice exhibiting stronger locomotor stimulation after a 10-day withdrawal, but not without this withdrawal period.

The second IEG particularly important in establishing the long-term neuroplasticity related to cocaine addiction is brain-derived neurotrophic factor (BDNF)[6]. BDNF is thought to be specifically salient in drug-seeking during withdrawal because unlike ΔFosB-regulated genes and other activity-dependent genes upregulated by cocaine, BDNF levels in the VTA, NAc, and amygdala (Amy) progressively increase during abstinence[18,19]. Furthermore, BDNF levels have been implicated in cue-induced relapse behavior to cocaine in rodents. Grimm *et al*. (2003) found that rats given cocaine addictions developed significantly greater levels of BDNF in these regions relative to sucrose controls after 30 and 90 days of withdrawal, and that these days parallel cue-induced cocaine craving behavior[18]. Furthermore, cocaine self-administration paired with intra-VTA BDNF infusions during training led to increased cue-induced cocaine-seeking after 30 days of withdrawal, and this effect was reversed by inhibiting the MAPK pathway, suggesting that BDNF-induced potentiation of cocaine-seeking is mediated by the MAPK pathway[20]. BDNF's role in sensitization is further supported by neurophysiological work: using midbrain slices from rats, Pu *et al*. (2006) found that BDNF mediated the enduring weak presynaptic stimulation (WPS)-induced potentiation onto VTA DA neurons elicited by chronic cocaine use followed by withdrawal[21]. Importantly, all these studies found that BDNF had no

effect on the first few days of withdrawal, substantiating its role in the incubation of craving. Furthermore, in chronic cocaine exposure, BDNF signaling has been shown to be necessary for the induction and reinforcement of NAc dendritic spine density[22]. However, whether this structural change is related to relapse behavior is not understood. Taken together, these results show that modulation of CDK5 and BDNF are well-characterized, produce physiological changes, and are implicated in the addictive and relapse-like behaviors from long-term cocaine use.

The Gut-Microbiota-Brain Axis

The human gut microbiota includes a diverse mixture of microorganisms that form a commensal relationship with the host in the gastrointestinal tract[23]. The gut microbes provide the host protection from pathogens, contribute to immune responsivity, and metabolize indigestible fibers[24]. In return, the host provides the microbes the protection and nutrients necessary to sustain the microbiological system[4,25]. Although a majority of the gut microbiota consists of two phyla (Firmicutes and Bacteroidetes)[23], perturbations to the ratio between them or to the highly diverse population of microorganisms have been implicated in the pathogenesis of disorders, such as anxiety, depression, and autism in both clinical and animal studies[1,4,24].

Despite the anatomical distance between the gastrointestinal tract and brain, there are several mechanisms through which the gut microbiota and brain maintain communication, including the vagus nerve, enteroendocrine cell inflammatory signaling, and bacterial metabolites that enter the blood circulation[4,26]. Of particular interest in the study of how the gut microbiota may influence addiction are short-chain fatty acids (SCFAs): the primary by-product of deity fiber fermentation. Although these molecules are primarily characterized as host energy sources, they are also the most extensively studied molecule involved in microbiota-CNS

communication[4,27]. Unlike catecholamines produced by gut microbes, the three most commonly produced SCFAs (butyrate, propionate, and acetate) can cross the blood-brain barrier (BBB) to directly modulate brain functions[4,28]. Of particular interest in studying addiction-induced brain changes, is the ability for SCFAs to act as histone deacetylase inhibitors (HDACi's) in the CNS[24]. In short, histone deacetylases (HDACs) remove the acetyl group from DNA histones to induce a compacted—transcriptionally repressive—chromatin conformation[29]. Because histone acetylation modifications regulate innumerable cellular functions, including CREB transcription, the functional impact of HDACi's in epigenetic regulation of downstream transcription has been difficult to characterize. Furthermore, there is currently very little understanding of where in the brain SCFAs are localized and would be able to directly modulate transcription.

Clues Connecting the Gut Microbiota to Addiction

There is growing evidence that human gut health is directly related to physical and emotional health. In fact, human fecal transplantation from healthy donors is currently the most effective treatment in recurrent *Clostridium difficile* infection and has quickly become a standard of care[28]. Furthermore, there is expanding literature assessing the role of the gut-microbiota-brain axis in mood-anxiety disorders. In the landmark Bercik et al. (2011) study, gut microbiota were transplanted between innately anxious and non-anxious mice and resulted in robust changes in anxiety-like behaviors[30]. They found that inherently anxious mice treated with the non-anxious mouse's microbiota exhibited less anxious behavior and increased HPC BDNF concentrations. Although no work has assessed dysbiosis' effect on NAc BDNF, it has been shown to alter BDNF levels in various regions in different directions[30–32]. Most behavioral research on the gut-microbiota-brain axis has been heavily focused on the microbiota's influence on depression, stress, and cognition[26]. A plethora of work now shows that microbiota transplantations from

human and animal models with depression, obesity, Parkinson's disease, and schizophrenia induced matching abnormal behaviors[8,26,28,31,32]. Although there is some evidence that dysbiosis-induced depression and the hallmark physiology of decreased dopaminergic transmission to the NAc may be mediated by SCFA neuromodulation, hyperactivate HPA-axis, or vagus nerve stimulation,[5,31–33] there is no consensus on the dominant mechanism. This is due, in part, to the complex interactions of the microbial-gut-brain axis; for example, in addition to directly crossing the BBB, SCFAs can bind to and stimulate the vagus nerve and maintain BBB integrity[34].

Despite the high rates of comorbidity and similarly dysregulated neurochemistry between mood-anxiety and substance use disorders[2], there is a lack of publications that experimentally alter the microbiota and assess changes to the rewarding properties and relapse-like behaviors associated with drugs of abuse. Although the gut microbiota's role in substance use disorder is implicated by the altered microbial profiles in individuals addicted to drugs of abuse[4,28,35–37], including cocaine[38], these studies do not discern whether the drug intake or altered microbiota came first.

Furthermore, work relating cocaine use and the gut microbiota is even more scarce. However, recent work has shown that chronic cocaine use is associated with an increase in *Bacteroidetes* and a decrease in *Firmicutes* in humans[38], and reduced microbial diversity in rats[39]. However, too little evidence and too many correlated confounders with human cocaine use—namely alcohol, nicotine, and diet—exist to causate specific microbes with behavioral responses to cocaine[24,40].

To our knowledge, there is only one paper that assesses whether gut microbiota manipulations directly alter behavioral responses to cocaine. Kiraly *et al.* (2016) treated mice to

an oral antibiotic cocktail and assessed both behavioral and transcriptional responses to cocaine[41]. They found that at a dose known to induce conditioned place preference (CPP) in control mice (10 mg/kg), antibiotic-treated (ABX) and control mice both developed strong, equivalent place preferences and locomotor sensitizations. However, at non-CPP-forming doses (5 mg/kg), ABX mice exhibited significantly stronger place preferences and developed greater sensitization over time. Strikingly, replenishing the SCFAs lost in ABX mice resulted in behavioral responses equivalent to that of control mice and SCFA supplementation alone. These results strongly suggest that microbiota depletion enhances sensitivity to the rewarding and sensitizing properties of cocaine use and that SCFAs are critical in this behavioral response. Notably, they confirmed that ABX treatment had no effects on baseline physiology and behavior, including cocaine metabolism and corticosterone. Additionally, they found that after 7 days, ABX-cocaine mice had higher *Bdnf* and *Drd1* (D1-like receptors) and lower *Ntrk2* (BDNF receptor) transcript levels in the NAc than cocaine mice.

Although Kiraly *et al*. (2016) assessed behavioral responses to cocaine without a withdrawal period, their findings provide insight into possible long-term mechanisms that bacterial SCFAs may alter reward circuitry. The behavioral consequences of SCFA depletion and their role as broad HDACi's in promoting genes that are underexpressed in various neurological diseases[27,29,42–44] make them an interesting candidate as mediators in the microbiota's role in addiction. Indeed, chronic cocaine use in rats has been associated with increased microbiome expression of genes involved in SCFA butyrate production[39]. Furthermore, histone acetylation has been implicated in the development of long-term reward circuitry alterations induced by cocaine. For example, chronic HDACi administration reduced cue-induced cocaine-seeking behavior after a 3-week withdrawal[45], indicating that decreases in mesocorticolimbic HDACi's,

such as SCFAs, may contribute to increased relapse risk. Furthermore, HDAC's have been implicated in cocaine-induced alterations of NAc CDK5 and BDNF transcripts[46–48]. Altogether, these findings present an opportunity to explore whether gut-derived SCFAs directly modulate neural circuitry implicated in cocaine relapse behavior.

To test the hypothesis that SCFAs produced by the microbiota can influence relapse-like behaviors, we will first explore whether SCFAs cross the BBB to act on the mesocorticolimbic pathway. Although characterization of SCFA concentrations throughout the brain is sparse[27], given that SCFAs have been shown to directly modulate brain activity in various regions, including the PFC and HPC, it is hypothesized that they will circulate to the NAc. Second, we will assess whether gut-derived SCFAs influence behavioral and physiological responses associated with long-term cocaine use and relapse. We anticipate that SCFA reductions due to gut dysbiosis will produce enhanced long-term relapse-like behavior and its associated molecular changes.

2. Methods & Materials:

Animals and drug treatments

Male C57BL/6J mice will be purchased from Jackson Laboratories and housed under specific-pathogen-free, *ad libitum* food and water conditions on a 12:12 light cycle. All protocols will follow the National Institutes of Health Guide for Care and Use of Laboratory animals. The antibiotic cocktail will be administered through drinking water and is based on doses previously shown to substantially deplete the gut microbiota (Bacitracin 0.5 mg/ml, Neomycin 2 mg/ml, Vancomycin 0.2 mg/ml and Pimaricin 1.2 μ g/ml)[41]. Notably, antibiotics not absorbed by the intestine will be used to minimize off-target effects due to systemic circulation.

Supplementation of SCFAs administered through water reflects concentrations found endogenously in the gut (67.5 mM acetate, 40 mM butyrate, 25.9 mM propionate). Catheterized intracerebroventricular SCFA administration dosage is based on past literature indicating that around 3% of intestinal acetate is taken up by the brain[42] and endogenous gut SCFA concentrations[41]. 2.0 mM acetate, 1.2 mM butyrate, and 0.8 mM of propionate will be administered to the NAc daily.

In part 2, mice will remain on their ABX/SCFA treatment throughout the experiment.

Experimental Procedure

Study 1: Are SCFA's traversing to the mesocorticolimbic pathway?

Study 1 seeks to confirm whether gut-derived SCFA's are directly modulating behavioral responses to cocaine by altering gut microbiota and SCFAs and assessing SCFA concentrations in the NAc. Mice will be randomly divided into four treatment groups of equal size (Table 1). Group 1 will be a control group with no changes in microbiota composition or SCFAs, group 2 will be treated with an antibiotic cocktail (ABX) through their water, group 3 will have water supplemented with ABX and SCFAs (ABX-oSCFA), and group 4 will be treated with ABX and daily intracerebroventricular (icv) injections of SCFAs (ABX-iSCFA). Immediately after the seven-day period, mice will be sacrificed and their NAc-SCFA levels will be assessed via gas chromatography.

Group	Treatment
1	Healthy microbiota (control)
2	ABX
3	ABX + oral SCFA
4	ABX + intracerebroventricular SCFA

Table 1: Treatment groups to assess SCFA concentrations in NAc.

Guide cannula implantation and intracerebroventricular SCFA injections

As described in Frost *et al*. (2014)[42]**,** mice will be anesthetized with 0.5-2.5% isoflurane anesthesia and fixed in a stereotaxic apparatus. Proper anesthesia will be assessed via the jaw reflex. A 20-gauge guide cannula will be implanted into the NAc (Bregma of AP+1.6, ML1.5, DV -4.5)[49] with the T bar set to 8° and held in place by dental cement. SCFAs will be infused into the NAc via electronic syringe pumps daily at consistent times. The injected SCFAs will be infused together over a 5-min period.

Quantification of SCFAs in the NAc

SCFA quantification will be conducted using gas chromatography, as described in Bachmann *et al*. (1978)[50]. Immediately after the seven-day treatment period, mice will be sacrificed and their NAc will be dissected, stored in 5% FBS-L15 media on ice, and homogenized[51]. Five-hundred mg of the homogenate will be suspended in 10 mol/l KOH and be centrifuged (12,000 g, 10 min). The supernatant will be extracted and treated with 3 mol/l sulfuric acid and after centrifugation (38,000 g, 10 min), 1.5 ml of the supernatant is added to 2.5 g of silicic acid. The mixture will be vortexed, transferred to a glass column, and treated with standard gas chromatography reagents[52]. One µl of the solution plugged with phosphoric acid-treated glass wool will be injected into a gas chromatograph (injector, 2-m, 2-mm ID) with an injector and flame ionization detection temperature 175 ℃ and oven temperature of 165 ℃. Nitrogen (45 ml/min) will serve as the carrier gas. Peak heights will be compared to internal standards to quantify butyrate, acetate, and propionate concentrations.

Study 2: Do gut microbiota-SCFAs reduce relapse-like behavior?

To assess whether the changes in behavioral responses to cocaine can be extended to long-term adaptations of relapse-like behavior, methods will be adapted from Massart *et al*. (2015)[53] and

Kiraly *et al*. (2016)[41]. Mice will be treated with either ABX, ABX-oSCFA, ABX-iSCFA, or water (control) for 10 days and will be trained to self-administer cocaine or saline and associate reward with a light cue for 10 days, resulting in eight treatment groups (Table 2). Afterward, mice will be subjected to a 30-day withdrawal period. We will then use the extinction test to assess cue-induced relapse behavior by placing rats into self-administration chambers and assessing seeking behavior. Immediately following the extinction test, mice will be sacrificed to assess whether microbiota depletion modulates neurological changes associated with relapse behavior. Specifically, differences in cocaine-induced changes to NAc medium spiny neuron density and *Bdnf* and *Cdk5* transcription will be determined.

Group	Treatments
1	Cocaine + H_2O
2	Cocaine + ABX
3	Cocaine + ABX + oral SCFA
4	Cocaine + ABX + intracerebroventricular SCFA
5	Saline + H_2O
6	Saline + ABX
7	Saline + ABX + oral SCFA
8	Saliner + ABX + intracerebroventricular SCFA

Table 2: Treatment groups to assess long term behavioral changes to incubated cue-induced cocaine craving.

Jugular vein catheterization

Mice will be anesthetized in the same manner as in the cannula implantation from study 1. As described in Massart *et al* (2015), mice will be implanted with intravenous Silastic catheters into the right jugular vein that connects to a cannula above the head. The cannula will be securely mounted by dental cement and will administer cocaine (groups 1-4) or saline (groups 5-8).

Self-administration, withdrawal (forced abstinence), and extinction test

Rats will be trained to self-administer cocaine for 6 h / day for 10 d. The self-administration chambers train cue association by presenting an active and inactive lever. Active lever presses will administer (0.75 mg/kg, 0.13 ml, 5 s/infusion) and activate a light above the lever for 40 s. Active lever presses during the last 35 s of the light cue will not lead to additional cocaine infusion. Inactive lever presses do not activate cocaine infusion or the light. Lever responses will be recorded for every session. A cohort of rats will undergo extinction tests after 1 d withdrawal to assess how craving incubates. During the 30 d withdrawal period, mice will be housed in their home cage. Extinction tests will be conducted in the self-administration chambers and active lever presses will be quantified. Incubated cue-induced cocaine-seeking will be quantified by taking calculating the difference between active lever presses after 30 d and 1 d.

Dendritic Spine Analysis

As explained in Pulipparacharuvil *et al*. (2008) and in part 1, NAc will be dissected from sacrificed mice and prepared for Golgi-Cox staining. Tissue sections will be immersed in solutions containing mercuric chloride and treated with sodium carbonate at 37℃. NAc neurons will be imaged using a 100x oil-immersion lens on a confocal microscope. Spine density will be quantified by counting the number of spines along 30-100 μm segments of secondary dendrites. The effect of ABX and SCFAs on cocaine response will be assessed by comparing the differences between each treatment's cocaine and saline densities.

Transcript analysis

To quantify changes in genes involved in cocaine-induced dendritic spine density increases, we will use methods previously described by Kiraly *et al*. (2016). NAc tissue will be rapidly dissected and placed on dry ice. qPCR using SYBER green master mix (Quanta) will be carried

out with an Applied Biosystems 7900HT system with the following parameters: 2 min at 95 °C; 40 cycles of 95 °C for 15 s, 59 °C for 30 s, 72 °C for 33 s; and graded heating to 95 °C to generate dissociation curves to confirm amplification of single PCR products. Data will be analyzed by comparing C(t) values of conditions tested to controls using the ΔΔC(t) method. The primers are listed in Table 4. Fold-difference will be assessed by dividing transcription of cocaine treatments by their correlated saline treatments (i.e. 1 - group 1 / group 2).

Target	Forward	Reverse
Bdnf	CCATAAAGGACGCGGACTTGTACA	AGACATGTTTGCGGCATCCAG
Cdk5	GCTGCCAGACTATAAGCCCTAC	TGGGGGACAGAAGTCAGAGAA

Table 3: Primers for qPCR reactions.

3. Anticipated Results & Discussion

The proposed experiment would significantly build on the sparse literature assessing the gut microbiota's role in altering behavioral responses to cocaine. First, it would answer the question of whether gut-microbiota dysbiosis induced the previously reported cocaine sensitization effects[41] through SCFAs that directly act in the reward pathway, rather than through other indirect avenues. Secondly, it would elucidate whether gut dysbiosis—and its associated SCFA deficit—alter relapse-like behaviors after withdrawal and if so, what mechanisms of neuroplasticity they affect.

Although the pioneering work by Kiraly *et al.* (2016) showed that gut dysbiosis increased short-term behavioral sensitivity to cocaine and that this effect is reversed by oral SCFA supplementation[41], they do not assess whether SCFAs are directly acting on the brain's reward pathway. Therefore, we will first compare SCFA concentrations in the NAc of mice with alterations to gut-synthesized SCFAs. Given the evidence that HDACi treatment has broad

applications in reducing cocaine-induced behavioral responses that are similar to SCFAs' effects[41,48], it is hypothesized that NAc-SCFA levels will be significantly reduced in ABX, but not ABX+oSCFA and ABX+iSCFA groups, relative to the healthy microbiota control. These results would further support the notion that gut-derived SCFAs' effect on behavioral responses to cocaine may be due to direct SCFA localization in the reward pathway, as suggested by their role as HDACi's implicated in addiction. Furthermore, these results will add to the currently sparse literature characterizing SCFA localization in the brain and help standardize ivc SCFA administration. The ivc SCFA administration dosage was derived from the sparse information available regarding SCFA absorption in the brain and to our knowledge, no previous study has attempted to co-administer these three SCFAs into the brain. In particular, we can adjust the ivc SCFA dosage to induce NAc-SCFA concentrations that reflect those found in mice with healthy microbiota and ABX mice given oral SCFAs for study 2.

Furthermore, although Kiraly *et al*. (2016) found that ABX-mice exhibited increased cocaine sensitivity in short-term behavioral assays[41], they did not assess the long-term changes involved in relapse behavior. In humans, the incubation of craving increases relapse rates, and treatments to attenuate relapse are ineffective[10,20,53]. However, there is currently no work relating gut dysbiosis to incubation of cue-induced cocaine-seeking after withdrawal. Study 2 aims to address this gap by allowing mice to self-administer cocaine in the presence of a light cue and assessing behavioral and physiological differences in craving incubation in mice with antibiotics-induced gut dysbiosis. Furthermore, because oral SCFAs were found to be important in modulating brain activity associated with short-term responses to cocaine[41], oral and intracerebroventricular SCFA supplementation will be added to determine whether SCFAs play a

role in the longer-term responses associated with relapse and if they do, what gut-microbiota-brain axis communication pathway they utilize to achieve this.

In the self-administration training, we expect that all cocaine-treated mice will demonstrate reliable cocaine administration: greater active lever presses than inactive lever presses. Of the cocaine treatment groups, cue-induced cocaine-seeking after the 30-day withdrawal period is hypothesized to be the greatest in the ABX treatment, followed by equivalent responses between the water control and ABX-oSCFA and ABX-iSCFA groups (Figure 2). In both behavioral assays, the saline control is not expected to induce active lever preference. Because microbiota-synthesized SCFAs can act as broad HDACi's, which have been implicated in regulating epigenetic changes in the reward pathway related to relapse-like behavior[19,21,46,53], we hypothesize that ABX mice will continue their pattern of increased sensitivity to cocaine, even after a withdrawal period. Alternatively, if SCFA supplementation does not reduce cue-induced responses, this would imply that although dysbiosis induces increased long-term relapse behavior, SCFAs are not responsible for this change.

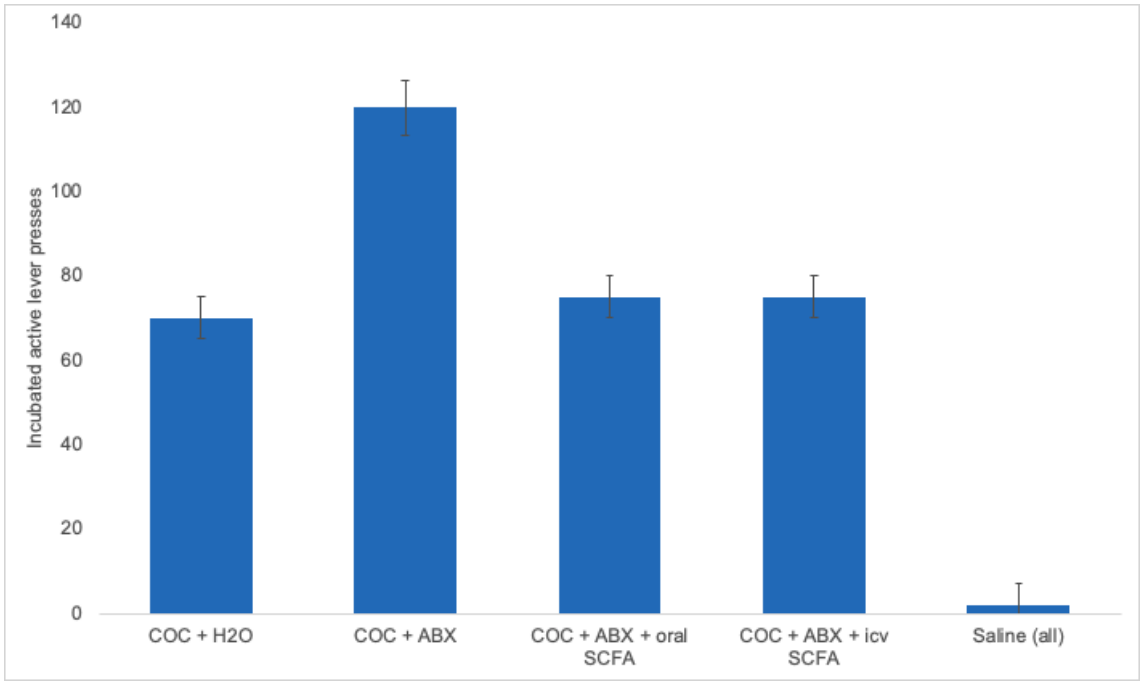
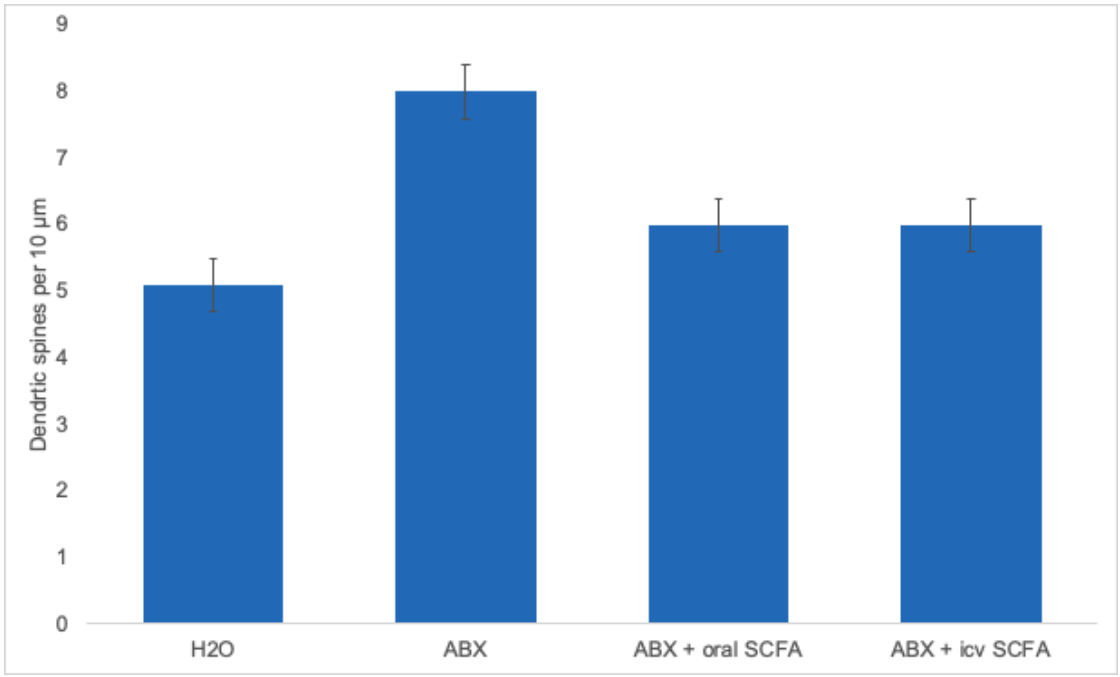


Figure 2: Incubated cue-induced active lever presses after 30-day withdrawal. For each treatment, differences between active lever presses at 30-days and a baseline group at 1-day were plotted. ABX treatment increases incubated presses and this effect is eliminated by SCFA supplementations.

Because chronic cocaine treatment has been shown to increase NAc *Bdnf* transcripts and that BDNF is necessary for dendritic spine density growth of NAc medium spiny neurons, we predict that ABX mice will have a higher density and *Bdnf* transcript level than other treatments (Figure 3A, B). However, whether SCFAs can mediate this effect in the NAc remains unexplored and differential *Bdnf* responses to SCFAs have been reported in various other brain regions[27,29,42]. Given the link between increased BDNF and long-term sensitization, we expect SCFA supplementation to ameliorate ABX-induced changes. We expect notably less dense NAc neurons in saline treatments. Similarly, if SCFAs from the microbiota are responsible for the expected long-term behavioral differences to cocaine, we expect similar NAc *Cdk5* transcription levels as *Bdnf* (Figure 3B).

A)



B)

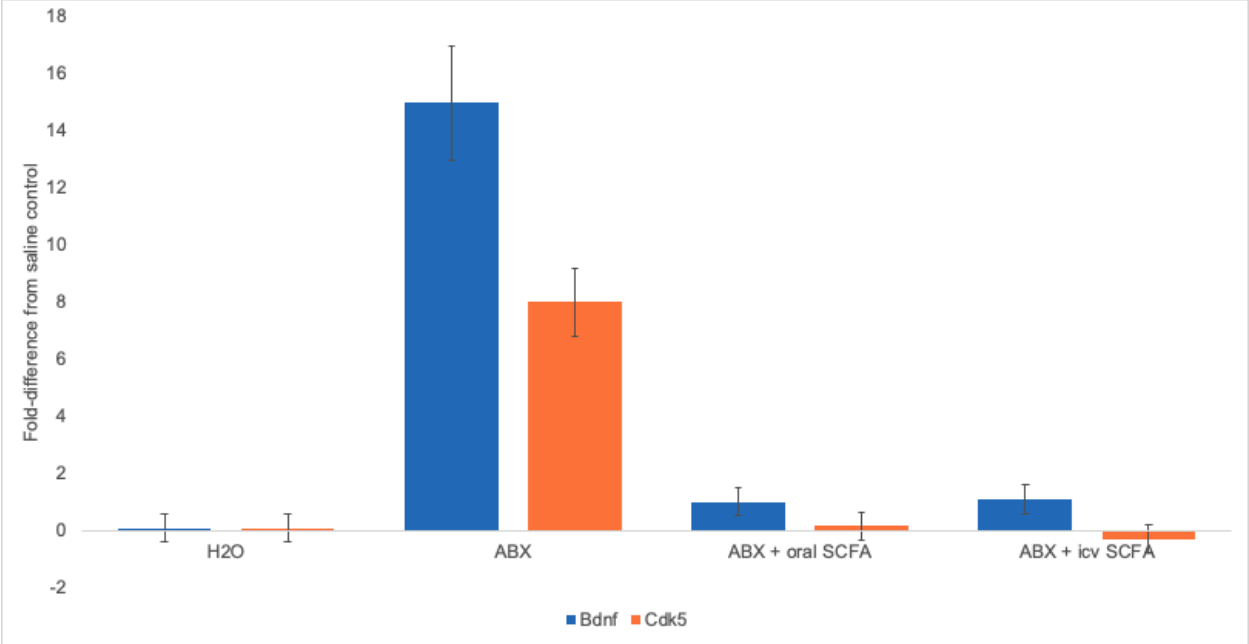


Figure 3: Molecular responses to cocaine withdrawal collected after extinction tests. A) Cocaine-treatment induced dendritic spine densities of the medium spiny neurons in the NAc. Differences between cocaine and saline treatment densities are plotted for each group. B) Cocaine-treatment induced changes in *Bdnf* and *Cdk5* transcripts. Fold differences were determined by comparing cocaine and saline treatments for each group.

Together, these results would be the first to provide evidence that the microbiota, specifically their SCFAs, alter incubated responses to cocaine-seeking and that their impact may be through direct interaction with the CNS. First, similar localization of SCFAs in oral and ivc treatments (study 1) would substantiate the previous claim that short-term changes to behavioral responses in ABX are due to SCFAs directly affecting NAc activity. Additionally, this study would be the first to inject SCFAs of the gut-microbiota into the brain and would provide important insight into proper dosages to reflect concentrations found endogenously. Future work

to assess this treatment should assess whether the icv SCFA dosage reported here induces equivalent behavioral responses as oral SCFA administration.

The hypothesized differences in incubated response to cocaine would strongly suggest that depletion of SCFAs produced by healthy gut microbiota increase relapse behavior by directly modulating brain activity. Transcript analysis of genes strongly implicated in relapse further substantiates that depleted microbiota-produced-SCFAs increase relapse-like behavior because they induce well-characterized NAc transcript changes seen in relapse studies. Equivalent responses to oral and ivc SCFA treatments would validate that the behavioral responses are due to SCFAs directly modulating brain activity, likely as HDACi's. If ivc SCFA supplementation produces results between those of ABX-only and oral SCFA supplementations, SCFAs' impact may be through several different avenues, such as vagus nerve stimulation. Furthermore, even if transcript changes do not reflect those previously shown, this study will be the first to assess how dysbiosis-induced SCFA depletion alters transcriptional responses, which may prove useful for various other reward-related studies.

Cdk5 and *Bdnf* expression are known to be modulated by histone modifications in cocaine addiction, suggesting microbiota-produced-SCFA's role as broad HDACi's may underlie this mechanism. Future ABX studies should include treatments that modify or mimic HDACi activity to assess whether SCFAs modulate histone acetylation to alter behavioral responses to cocaine. Limitations of this study include a lack of physiology and behavioral controls to SCFA and ABX treatments. Although Kiraly *et al*. (2016) found they induced no differences in drinking, locomotion, corticosterone, and cocaine metabolism, future work should assess whether this remains true over longer periods.

The primary goal of this study was to assess whether gut dysbiosis can increase relapse behavior which, despite being highly comorbid in humans, remained untested. Because relapse is highly prevalent and difficult to predict or prevent in cocaine users[10,20,53], results from this study would make probiotic or SCFA treatments highly attractive. Furthermore, these results may spur clinical trials that assess microbiota health and metabolites as biomarkers of relapse risk in humans. Lastly, the novel approaches used here are broadly applicable to the growing field of gut-microbiota-brain axis systems, such as mood-anxiety disorders.

4. References

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