UNIVERSITY OF CALIFORNIA SAN DIEGO

Relationship Between Addiction-like Behaviors, Gut Microbiome, and Metabolomics in Alcohol-dependent Rats

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

Mohini Iyer

Committee in charge:

Professor Giordano de Guglielmo, Chair
Professor Cory Root, Co-Chair
Professor Barry Grant

2024

The Thesis of Mohini Iyer is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2024

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

Introduction, Conclusion, in part, contain material as it appears in Doyle, M. R., Dirik, S., Martinez, A. R., Hughes, T. E., Iyer, M. R., Sneddon, E. A., Seo, H., Cohen, S. M., & De Guglielmo, G. (2024). Catechol-O-Methyltransferase inhibition and alcohol use disorder: Evaluating the efficacy of tolcapone in ethanol-dependent rats. Neuropharmacology, 242, 109770. The thesis author was an author of this paper.

Chapter 1, Chapter 2, Chapter 3 in part, contain reprints of material as it appears in Doyle, M. R., Dirik, S., Martinez, A. R., Hughes, T. E., Iyer, M. R., Sneddon, E. A., Seo, H., Cohen, S. M., & De Guglielmo, G. (2024). Catechol-O-Methyltransferase inhibition and alcohol use disorder: Evaluating the efficacy of tolcapone in ethanol-dependent rats. Neuropharmacology, 242, 109770. The thesis author was an author of this paper.

.

ABSTRACT OF THE THESIS

Relationship Between Addiction-like Behaviors, Gut Microbiome, and Metabolomics in Alcohol-dependent Rats

by

Mohini Iyer

Master of Science in Biology

University of California San Diego, 2024

Professor Giordano de Guglielmo, Chair
Professor Cory Root, Co-Chair

Alcohol Use Disorder (AUD) is a significant public health concern, with alcohol-related deaths accounting for approximately 178,000 deaths nationwide and increasing in recent years (White et al., 2022). To better understand the role of the gut microbiome in AUD, a chronic intermittent ethanol (CIE) model was utilized in heterogeneous stock (HS) rats, and gut microbial and metabolic changes were measured.

Differential microbiome and metabolomic features were found between sexes, dependence timepoints, addiction levels, and pharmaceutical treatment responses. Before alcohol dependence, high-addiction females had significantly lower Tenericutes levels than low-addiction females, suggesting a predictive capability of Tenericutes in addiction for females. After dependence, high-addiction rats had higher SR1 abundances than low-addiction rats. Rats responding well to naltrexone treatment had higher abundances of Verrucomicrobia, Fusobacteria, and SR1.

Metabolomic analysis revealed differential levels of linoleic acid between vulnerable and resistant rats at baseline. These differences were hypothesized to be linked to variations in catechol-O-methyltransferase (COMT) activity, an enzyme involved in the degradation of catecholamines associated with addiction (Coker et al., 2020a; Schacht et al., 2022a). A COMT inhibitor was tested in alcohol-dependent rats with promising results.

The results demonstrate that microbial abundance levels can predict addiction-like behaviors in females and response to treatment drugs. Further research on microbial differences between different types of medication could provide valuable insights for optimizing AUD treatment strategies.

INTRODUCTION

Alcohol Use Disorder: A Multifaceted Disease. Alcohol Use Disorder (AUD) is a complex and multifaceted disease characterized by excessive alcohol consumption and mental or chemical dependency on alcohol (Association, 1994; Grant et al., 2016; Koob, 2014; Koob and Volkow, 2010). The Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5) outlines eleven criteria for diagnosing AUD, encompassing various aspects of the disorder, such as impaired control, social impairment, risky use, and pharmacological criteria (Grant et al., 2016). The severity of AUD ranges from mild to severe based on the number of criteria met, highlighting the heterogeneous nature of the disorder (Grant et al., 2016). This heterogeneity poses a significant challenge in developing effective treatments, as individuals with AUD may respond differently to alcohol and therapeutic interventions (Koob, 2024).

Current Treatments for AUD and Their Limitations. Despite the prevalence and severity of AUD, current treatment options are limited to three FDA-approved drugs – acamprosate, disulfiram, and naltrexone – which are typically used in conjunction with therapy (Koob, 2024). However, these treatments have shown inconsistent efficacy due to individual differences in hormonal, neurochemical, and genetic factors (Bahji et al., 2022; Becker and Lopez, 2016; Spanagel, 2017). The heterogeneity of AUD and the varying responses to alcohol and treatment underscore the need for more personalized and targeted therapeutic approaches (Koob, 2024).

Animal Models of AUD: The Need for Genetic Diversity. To better understand the complex nature of AUD and develop more effective treatments, animal models that capture the genetic diversity and heterogeneity found in human populations are essential (Solberg Woods

and Palmer, 2019). Traditional animal models, such as inbred strains, have limitations in modeling the genetic variability observed in humans (Solberg Woods and Palmer, 2019). Heterogeneous stock (HS) rats, on the other hand, are an outbred population that more closely mimics the genetic diversity of human populations (Chitre et al., 2023). By utilizing HS rats in the study of AUD, researchers can better characterize the range of addiction-like behaviors and treatment responses, leading to more translatable findings (Carrette et al., 2021; de Guglielmo et al., 2023a; Kallupi et al., 2020; Sedighim et al., 2021).

Characterizing Addiction-Like Behaviors in HS Rats. To comprehensively assess AUD in HS rats, it is crucial to characterize a range of addiction-like behaviors that parallel the eleven criteria outlined in the DSM-5 (Grant et al., 2016). This approach ensures that the animal model captures the multifaceted nature of AUD and provides a more accurate representation of the disorder (Carrette et al., 2021; de Guglielmo et al., 2023a; Kallupi et al., 2020; Sedighim et al., 2021). By focusing not only on alcohol intake but also on other aspects of addiction-like behavior, such as impaired control, motivation, and withdrawal symptoms, researchers can gain a more comprehensive understanding of the underlying mechanisms and potential therapeutic targets (Carrette et al., 2021; de Guglielmo et al., 2023a; Kallupi et al., 2020; Sedighim et al., 2021).

The Gut Microbiome and Its Role in AUD. In recent years, the gut microbiome has emerged as a potential key player in the development and maintenance of AUD (Simpson et al., 2022). The gut microbiome, a complex community of microorganisms residing in the gastrointestinal tract, has been shown to influence various aspects of human health, including brain function and behavior (Jandhyala et al., 2015). The bidirectional communication between the gut and the brain, known as the gut-brain axis, involves multiple pathways, including neural,

endocrine, and immune signaling (Bercik et al., 2012; Breit et al., 2018). Disruptions in the gut microbiome, such as those caused by excessive alcohol consumption, have been linked to altered brain function and addiction-like behaviors (Bercik et al., 2012).

Metabolomics: A Window into the Mechanisms of AUD. Metabolomics is a comprehensive analytical approach that characterizes the small molecule metabolites within biological systems, offering insights into physiological and pathological states. Utilizing advanced technologies such as mass spectrometry and nuclear magnetic resonance spectroscopy, this field enables the quantitative and qualitative analysis of the metabolome. Through the systematic study of these biochemical compounds, metabolomics provides crucial data for understanding metabolic interactions and disease mechanisms. In this context, metabolomics allows for the investigation of the mechanisms underlying AUD and the identification of potential biomarkers and therapeutic targets (Leclercq et al., 2020; Thistlethwaite et al., 2022). By analyzing the metabolomic profiles of individuals with AUD, insights into the metabolic pathways and signaling molecules involved in the disorder can be elucidated (Leclercq et al., 2020; Thistlethwaite et al., 2022). Metabolomic studies have revealed significant differences between individuals with AUD and healthy controls, as well as between different stages of the disorder (Leclercq et al., 2020; Wang et al., 2022). These findings suggest metabolomics could be used to develop more personalized diagnostic and treatment approaches for AUD.

Identifying Novel Therapeutic Targets: The Role of Linoleic Acid and COMT. Through the integration of behavioral, microbial, and metabolomic data, this study aimed to identify novel therapeutic targets for AUD. One promising target that emerged from the metabolomic analysis was linoleic acid, which was found to be differentially expressed in rats with high and low addiction-like behaviors. Linoleic acid has been shown to modulate the levels

of S-adenosylmethionine (SAM), a key methyl donor involved in the regulation of catechol-O-methyltransferase (COMT) activity (Pita and Delgado, 2000; Zhu, 2002). COMT is an enzyme that plays a crucial role in the degradation of catecholamines, such as dopamine, which are involved in the reward and reinforcement pathways associated with addiction (Schacht et al., 2022a). Based on these findings, targeting COMT activity was hypothesized to be a potential therapeutic strategy for AUD. To test this hypothesis, a separate study investigated the effects of tolcapone, a COMT inhibitor, on alcohol self-administration in dependent and non-dependent rats. Tolcapone has been shown to improve cognitive function in patients with Parkinson's disease by regulating cortical dopaminergic signaling (Apud et al., 2007). Recent studies have also suggested that tolcapone may be effective in reducing alcohol consumption and impulsive behaviors associated with AUD (Coker et al., 2020a; Schacht et al., 2022a).

Study Objectives and Significance. The primary objective of this thesis was to investigate the relationship between addiction-like behaviors, gut microbiome composition, and metabolomic profiles in a genetically diverse population of HS rats. The chronic intermittent ethanol vapor exposure (CIE) model to induce alcohol dependence and characterizing a range of addiction-like behaviors, was used to identify microbial and metabolomic signatures associated with AUD vulnerability and treatment response. Furthermore, the potential of linoleic acid and COMT as novel therapeutic targets for AUD was explored through targeted pharmacological interventions. The significance of this work lies in its comprehensive approach to understanding the complex nature of AUD and its potential to inform the development of more personalized and effective treatment strategies. By integrating behavioral, microbiome, and metabolomic data from a genetically diverse animal model, the findings provide valuable insights into the underlying mechanisms of AUD and highlight the importance of considering individual

variability in the development of therapeutic interventions. Moreover, the identification of novel therapeutic targets, such as linoleic acid and COMT, opens new avenues for the treatment of AUD and may ultimately lead to improved outcomes for individuals struggling with this debilitating disorder.

Acknowledgements

Introduction, in part, contains material as it appears in Doyle, M. R., Dirik, S., Martinez, A. R., Hughes, T. E., Iyer, M. R., Sneddon, E. A., Seo, H., Cohen, S. M., & De Guglielmo, G. (2024). Catechol-O-Methyltransferase inhibition and alcohol use disorder: Evaluating the efficacy of tolcapone in ethanol-dependent rats. Neuropharmacology, 242, 109770. The thesis author was an author of this paper.

Chapter 1 METHODS

Subjects

Adult Heterogenous stock (HS) rats (n = 48, half males and half females) were obtained from Dr. Leah Solberg Woods at Wake Forest University (Solberg Woods and Palmer, 2019). Rats were 3-4 weeks old upon arrival at UCSD and were quarantined for 2 weeks before experiments began. Heterogenous stock rats were used due to the high genetic diversity in their population, which mimics the genetic diversity in human populations (Woods & Mott, 2016). For the COMT study, adult Wistar rats (n = 44, 20 males and 24 females) were obtained from Charles River. Experiments were initiated when rats were 10–12 weeks old. All rats were housed in pairs on a on a 12 h/12 h reversed light/dark cycle in a temperature (20-22°C) and humidity (45-55%) controlled vivarium with ad libitum access to water and food pellets (PJ Noyes Company, Lancaster, NH, USA). All the procedures were performed in accordance with the ARRIVE guidelines (Kilkenny et al., 2010), adhered National Research Council's Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of the University of California, San Diego.

Drugs

The ethanol solution (10% v/v) was 95% ethanol mixed with tap water and delivered at a volume of 0.1 ml/reinforcer.

Naltrexone (Sigma Aldrich) was dissolved in sterile saline (0.9%) and injected intraperitoneally (IP) at the dose of 3 mg/kg 30 minutes before the test session.

Tolcapone ((3,4-dihydroxy-5-nitrophenyl)(p-tolyl)methanone)) was purchased from Combi-Blocks (San Diego, CA, USA). It was dissolved in 10% DMSO, 10% Tween-20, and 80% 0.9% sterile saline and generally administered IP at a volume of 0.1 ml/kg. The vehicle was

also 10% DMSO, 10% Tween-20 and 85% sterile saline and administered at a volume of 0.1 ml/kg. All pretreatments were administered IP 60-min before self-administration sessions began.

Saccharin (0.04% w/v) was purchased from Sigma-Aldrich (Burlington, MA), mixed with tap water, and delivered at a volume of 0.1 ml/reinforcer.

Operant ethanol self-administration

Standard operant conditioning chambers (29 cm × 24 cm × 19.5 cm; Med Associates, St. Albans, VT, USA) were used for ethanol self-administration sessions. Chambers were enclosed in lit, sound-attenuating, ventilated environmental cubicles. The front door and back wall of the chambers were made of transparent plastic, while the other walls were made of opaque metal. The opaque metal on the right side of the chamber was the front panel. Each chamber had two retractable levers on the front panel that would extend at the start of each session. Two drinking spouts were also on the front panel and were connected to infusion pumps. Activation of the right (active) lever released a 10% v/v ethanol solution (0.1 ml/reinforcer) over 6 seconds into the spout followed by a 20-second timeout period during which the cue light above the active lever was on and any active lever presses did not result in additional infusions. Activation of the left lever delivered water (0.1 ml/reinforcer). Responses for both levers were recorded on a computer with MED-PC IV software installed, which also controlled the fluid delivery.

The HS and Wistar rats from the COMT were trained on how to self-administer ethanol for three weeks. They first self-administered water (0.1 ml/reinforcer) in a 16-hour session on a fixed-ratio 1 (FR1) reinforcement schedule. Food was available ad libitum. They then self-administered a 10% v/v ethanol solution (0.1 ml/reinforcer) in another 16-hour session on FR1. Next, they were able to self-administer ethanol for three 30-minute FR1 sessions, and finally,

could self-administer either ethanol (right lever) or water (left lever) on FR1 during daily 30-minute sessions (0.1 ml/reinforcer). Tolcapone testing for a subset of the COMT rats began after 14 daily sessions.

Ethanol dependence

After three weeks, the HS rats were made dependent using the chronic intermittent ethanol vapor exposure (CIE) model (Fig. 1) (Kononoff et al., 2018; Vendruscolo and Roberts, 2014). They were placed in a vapor chamber and were exposed to ethanol for 14 hours, with 10 hours of abstinence. This was done for two weeks to make them dependent. The rats were then allowed to drink during their abstinence via self-administration and their drinking patterns were measured for four weeks. Blood alcohol levels (BALs) were also measured every three days via tail vein bleeds and quantified via gas chromatography during both sessions (de Guglielmo et al., 2023b). Behavioral testing was conducted during acute withdrawal, 6-8 hours after the vapor is turned off, every three days.

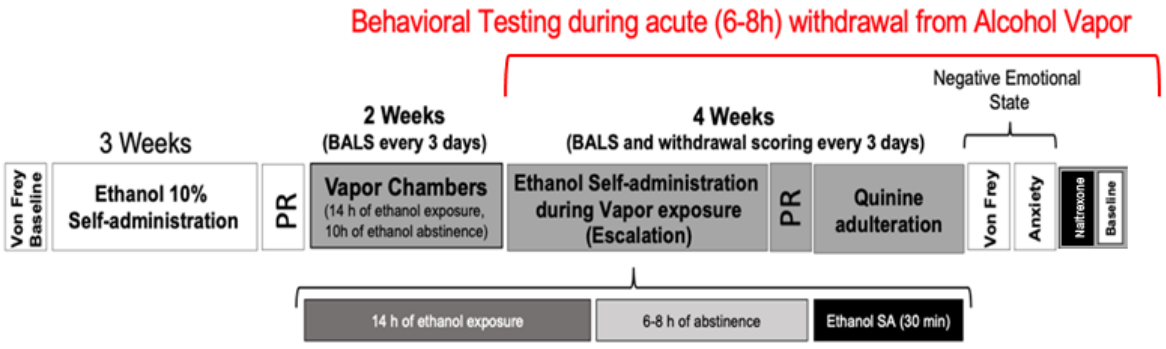

Figure 1. Timeline of experiment. PR stands for progressive ratio testing. SA refers to self-administration (of ethanol).

COMT rats in their vapor chambers were exposed to ethanol for 16 hours daily. Ethanol vapor levels were titrated to generate blood alcohol levels (BALs) of around 150–225 mg/dl.

BALs were measured weekly using the same methods as the HS rats. Ethanol self-administration sessions started again once BALs hit the target of 200-250 mg/dl. Sessions were on Mondays, Wednesdays, and Fridays during acute withdrawal (6-8 hours after the alcohol vapor was turned off). Tolcapone and vehicle pretreatment began after 14 sessions were completed.

Tolcapone treatment for COMT Rats

Pretreatment took place one hour before sessions. Pretreatment order was determined via a Latin square design with a control session between tests. Rats received injections of vehicle and tolcapone (15 mg/kg and 30 mg/kg).

Estrous cycle in COMT rats

To assess the estrous cycle, female rats were vaginally swabbed immediately after self-administration sessions on test days. The samples were air-dried and stained with a Hema 3 stat pack staining kit (30-s/solution; Fisher Scientific, Pittsburgh, PA). Images of the smears were captured on a BZX700 microscope (Keyence, Itasca, IL), and phase determination was made by a blinded observer.

Behavioral Assays

Progressive ratio testing

Following the establishment of the escalation of intake, rats participated in a single progressive ratio (PR) test to measure the motivation to obtain alcohol. The PR response requirement increased as follows: 1, 1, 2, 2, 3, 3, 4, 4, 5, 5, 6, 6, 7, 7, 8, 8, 9, 9, 10, 10, 11, 12, 13, 14, 15, 16, 17, etc (Kallupi et al., 2020; Schlosburg et al., 2013; Vendruscolo et al., 2012). The session ended after the 30-minute limited hold or 1-hour maximum session duration elapsed, whichever came first.

Hyperalgesia

Paw withdrawal in response to a dynamic plantar aesthesiometer (Ugo Basile, Varese, Italy) was tested for mechanical sensitivity as previously reported (Kallupi et al., 2020). The force required and amount of time to paw retraction were recorded for three measures for each hind paw. Measurements were obtained at baseline (before self-administration began), and during acute withdrawal, after the establishment of alcohol dependence (6-8 h after the vapor was turned off). Data are expressed as force (g) for baseline and as percent change of force (g) from each baseline.

Compulsive-like behavior

Compulsivity was quantified by modifying the alcohol solution with quinine, a bitter compound, and subsequently assessing the intake by rats. This approach aimed to evaluate whether the subjects would endure the aversive taste to obtain the reward, serving as an index of compulsive behavior. The choice of quinine adulteration over foot-shock therapy was based on findings that both methods yield comparable results in demonstrating compulsive-like behaviors, but quinine allows for repeated testing on the same animals without the risk of sensitization (Hopf and Lesscher, 2014). Measurements were conducted before and after the establishment of alcohol dependence.

Elevated-plus maze (withdrawal-induced anxiety)

To assess withdrawal-induced anxiety, an elevated plus maze was employed, wherein the duration that rats spent in the open arms of the maze was recorded. This metric was utilized as anxiety is a prevalent symptom of alcohol withdrawal in individuals with Alcohol Use Disorder (AUD), according to the American Psychiatric Association (Association, 1994). A greater

amount of time spent in the open arms, as opposed to the closed arms, is associated with diminished anxiety, indicative of less withdrawal-related stress (Walf and Frye, 2007). Anxiety measurements were taken after establishing alcohol dependence.

Microbiome and Metabolomic analysis

Fecal Collection and 16S sequencing

Fecal pellets were collected immediately from the animal into a sterile tube at each timepoint (before and after the establishment of alcohol dependence), put on dry ice, and then frozen at -80°C for storage until sequencing. The pellets were then sent for extraction and sequencing by the UCSD Microbiome Sequencing Core. At the time of DNA extraction, feces were thawed and extracted with the Qiagen DNeasy powersoil kit. The samples are then amplified by PCR in triplicate and then pooled. The 16S V4 gene was amplified using universal primers (525F-806R). Each sample was normalized to 240 ng per sample and purified. After purification, the A260/A280 ratio of the final pool was recorded to ensure purity, with a tolerance range of 1.8-2.0. The barcoded amplicons from all of the samples were normalized, pooled to construct the sequencing library, and sequenced using an Illumina MiSeq sequencer.

Qiime2 and PICRUSt2

After sequencing, sequences were demultiplexed, and processed using the Quantitative Insights into Microbial Ecology (QIIME2) software package. The DADA2 plugin in QIIME2 (q2-dada2) was used to generate Amplicon Sequence Variants (ASVs). Principal coordinate analysis (PCoA) of the unweighted UniFrac distances was used to assess the variation between samples (beta diversity) (Bolyen et al., 2019). Beta diversity was further visualized via PCoA plots – weighted and unweighted unifrac distances were generated. Taxonomy assignment and

rarefaction were performed using QIITA and Qiime2 (Gonzalez et al., 2018). ANCOM-BC was run for each timepoint and distinct group. Alpha diversity was measured using both the Shannon diversity index and Chao1 index and compared between baseline and treatment conditions. The PICRUSt2 pipeline was used with ASVs to infer predicted sample pathway abundances (Douglas et al., 2020).

Metabolomics

The UPLC-MS/MS platform includes a variety of chromatographic methods and mass spectrometry ionization modes that allow for broad coverage of compounds that are separated by different chemical properties. Metabolites were identified through a comparison of the ion features to a reference library of standards that include molecular weight (M/Z), retention time, and the MS/MS data, which was cross-referenced in the GNPS database using molecular networking (Aron et al., 2020). Features were batch normalized, Log transformed (base 10), and auto-scaled (mean-centered and divided by the standard deviation of each variable), zeros or missing values were imputed, and standard statistical analyses were performed. Welch's two-sample t-test, PCA, PLSDA, and a one-way ANOVA were used for parametric data with Tukey's post-hoc, and Kruskal Wallis was used for non-parametric data. PLS-DA is a supervised analytic method that uses multivariate regression techniques to extract features that differentiate class membership. PLS-DA is performed using Metaboanalyst (Xia et al., 2009) a Java-based GUI for R and Bioconductor packages [44] Classification and 10-fold cross-validation are performed using the corresponding wrapper in the caret package (Ritchie et al., 2015). P values were adjusted for multiple comparisons using the Benjamini-Hochberg false discovery rate (FDR). Correlation heatmaps were generated using Pearson r distance measures.

Behavior Statistical analysis

Data were analyzed using Prism 9.0 software (GraphPad, San Diego, CA, USA). Paired t-tests were used for baseline ethanol intake, water reinforcers earned, and ethanol preference between the last three sessions of responding before tolcapone pretreatment in non-dependent or dependent rats (or last three sessions before the start of ethanol vapor exposure, in rats not tested in a non-dependent state). Ethanol preference was calculated by dividing the number of ethanol reinforcers by the number of total reinforcers (ethanol + water) and multiplying by 100. A three-way, repeated measures ANOVA was used to analyze the number of ethanol and water reinforcers earned in rats before and during dependence across the 14 sessions. A two-way, repeated measures ANOVA was used to analyze ethanol intake in male and female rats across the 28 sessions. A one-way, repeated measures ANOVA with Dunnett's post-hoc was used to evaluate the effects of tolcapone in each dependent rats self-administering ethanol, non-dependent rats self-administering ethanol, and rats self-administering saccharin. A two-way, repeated measures ANOVA, with Dunnett's post-hoc, was used to evaluate the effects of tolcapone in male and female rats.

Acknowledgements

Chapter 1, in part, contains reprints of materials as they appear in Doyle, M. R., Dirik, S., Martinez, A. R., Hughes, T. E., Iyer, M. R., Sneddon, E. A., Seo, H., Cohen, S. M., & De Guglielmo, G. (2024). Catechol-O-Methyltransferase inhibition and alcohol use disorder: Evaluating the efficacy of tolcapone in ethanol-dependent rats. Neuropharmacology, 242, 109770. The thesis author was an author of this paper.

Chapter 2 RESULTS

Behavior

Robust inter-individual differences in alcohol escalation were observed in both males and females, reflected by a higher between-subjects coefficient of variation (SD/mean) compared with within-subjects in the Escalation Index (Fig. 2A). The high correlation coefficient between days and the low coefficient of variation within-subjects demonstrate that the standard operating procedures used maximized the detection of individual phenotypes and minimize nonspecific effects that are caused by the experimenter or environment.

To further identify subjects that are vulnerable vs resistant to compulsive alcohol intake, an Addiction Index was computed (Carrette et al., 2021; de Guglielmo et al., 2023a; Kallupi et al., 2020; Sedighim et al., 2021) by averaging normalized responses (Z-scores) for three behavioral domains: escalation of intake, motivation and compulsivity (Fig. 2B). Individuals with High Addiction Index (HA) showed increased escalation under an FR schedule (Fig. 2C), increased responding under a PR schedule (Fig. 2D), increased responding despite adverse consequences (Fig. 2E), increased withdrawal-induced anxiety (Fig. 2F, only males) and withdrawal-induced pain thresholds (Fig. 2G) compared to rats with Low Addiction Index (LA). These results demonstrate that the animal model and experimental design will measure several addiction-like behaviors and identify individuals (males or females) who are vulnerable or resistant to compulsive alcohol use and the adverse effects of chronic use on withdrawal anxiety and hyperalgesia.

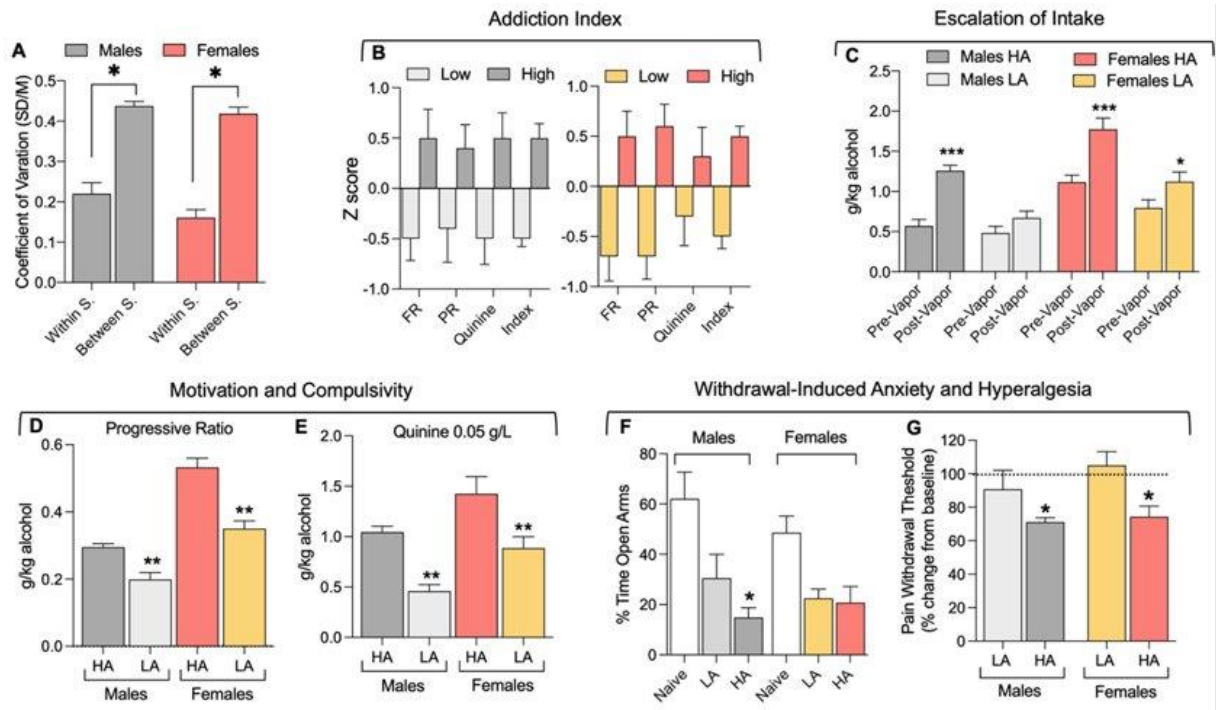

Figure 2. Behavioral differences between sexes and addiction levels. Behavioral assays were conducted on male and female rats post alcohol dependence. HA refers to high-addiction rats; LA, low-addiction. A) Variation between subjects and within subjects for both males and females. B) Addiction Index differentiating between high and low addiction-like behaviors in rats. The addiction index was determined by calculating the Z score of behavioral domains. C) Escalation in alcohol intake after vapor sessions, separated by sex and addiction index. D) Measurement of motivation through progressive ratio (PR) schedule for rats during the self-administration phase. E) Measurement of compulsivity through quinine adulteration of alcohol solution during the self-administration phase. F) Anxiety was measured through time spent in the open arms of an elevated plus maze. G) Hyperalgesia was measured via Von-Frey testing. *p<0.05, **p<0.01

Microbes

The possibility of a relationship between gut microbial composition and alcohol dependence was tested by collecting fecal samples from all the rats and using 16s sequencing on the samples. Conducting a Welch's t-test on the microbial abundance levels, found an overall significant increase ($p<0.05$) in Firmicutes, Verrucomicrobia, Actinobacteria, Tenericutes, Elusimicrobia, Fusobacteria, TM7, and significant decrease ($p<0.05$) in Bacteroidetes, Proteobacteria, Spirochaetes, Cyanobacteria, Deferribacteres (Table 1).

Table 1. Significant metabolic changes after alcohol dependence, between sexes, and addiction levels. Relative microbial abundance measured via microbial analysis of fecal samples taken before and after alcohol dependence. NS refers to $p>0.05$.

Phylum	Baseline		Dependent		Pre- vs. Post-Dependence
	High Addiction vs. Low Addiction	Males vs. Females	High Addiction vs. Low Addiction	Males vs. Females	
Bacteroidetes	NS	NS	NS	↑	↓
Firmicutes	NS	NS	NS	↓	↑
Proteobacteria	NS	↓	NS	NS	↓
Spirochaetes	NS	NS	NS	NS	↓
Verrucomicrobia	NS	NS	NS	NS	↑
Actinobacteria	NS	NS	NS	NS	↑
Tenericutes	NS	NS	NS	NS	↑
Cyanobacteria	NS	↓	NS	↓	↓
Elusimicrobia	NS	NS	NS	↓	↑
Deferribacteres	NS	NS	NS	NS	↓
Fusobacteria	NS	↓	NS	NS	↑
TM7	NS	↑	NS	NS	↑
SR1	NS	NS	↓	NS	NS
Synergistetes	NS	NS	NS	NS	NS
Thermi	NS	↓	NS	NS	NS

Microbiome composition before and after dependence was measured. The difference in each microbe's abundance was compared and a closer look was taken specifically for sexes to see whether microbial changes for each sex matched overall microbial changes. Microbial changes were categorized as significant increases, significant decreases, or no significant difference (Table 2). Table 2 shows that microbial abundance changes for SR1, Synergistetes, Thermi, GN02, and OD1 differed in the type of change that occurred post-alcohol dependence, when comparing against overall changes and/or between sexes. These differences suggested sex-specific responses to alcohol and prompted further analysis of sex differences in microbial and metabolomic abundances.

Table 2. Differences in microbial changes between sexes compared to overall changes after alcohol dependence. NS refers to no significant changes found after alcohol dependence. Significance was determined using unpaired Welch's t-test, comparing abundance levels for each group before and after dependence. The direction of change was determined by comparing the mean abundances of each group before and after dependence.

Phylum	Pre- vs. Post- Dependence		
	Overall	Males	Females
Bacteroidetes	↓	↓	↓
Firmicutes	↑	↑	↑
Proteobacteria	↓	↓	↓
Spirochaetes	↓	↓	↓
Verrucomicrobia	↑	↑	↑
Actinobacteria	↑	↑	↑
Tenericutes	↑	↑	↑
Cyanobacteria	↓	↓	↓
Elusimicrobia	↑	↑	↑
Deferribacteres	↓	↓	↓
Fusobacteria	↑	↑	↑
TM7	↑	↑	↑
SR1	NS	↓	↓
Synergistetes	NS	↑	↓
Thermi	NS	↑	↓
Planctomycetes	NS	NS	NS
GN02	NS	↓	↓
OD1	NS	NS	↓

Figure 3 shows relatively similar Bacteroidetes and Firmicutes abundances between sexes, but significantly higher concentrations of Proteobacteria and Cyanobacteria in females. After alcohol dependence, both males and females see a decrease in Bacteroidetes and a subsequent increase in Firmicutes levels, as these two microbes are negatively correlated (Fig. 3). Both sexes also see an increase in Verrucomicrobia and Actinobacteria, alongside a decrease in Proteobacteria, Cyanobacteria, and Spirochaetes (Fig. 3). These results support the hypothesis that excessive alcohol use causes a change in gut microbiome composition.

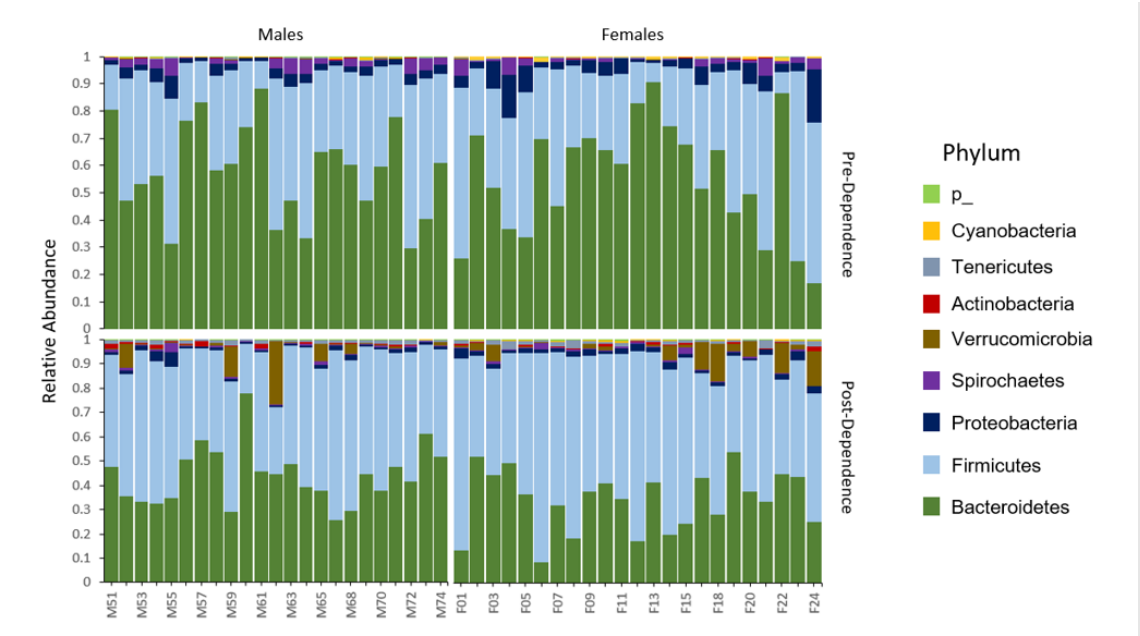

Figure 3. Gut microbiome composition differs between sexes and with alcohol dependence. Phylum-level abundances of microbes by sex, both pre- and post-dependence. Microbiome composition was analyzed via 16s sequencing of fecal samples. Phyla with a relative abundance <0.05 were summed up and combined into the "p_" category.

Between sexes, there was a significantly higher abundance of Proteobacteria and Cyanobacteria in female rats compared to males (Fig. 4A). Both sexes were further broken down by their ethanol intake during their self-administration sessions. Female rats with low ethanol intake had a significantly higher Synergistetes abundance than higher intake rats (Fig. 4C). Male rats had higher SR1 but lower Fusobacteria in low-intake rats compared to high-intake rats (Fig. 4B).

Both sexes were also separated by high and low addiction index values. Before alcohol dependence, the only microbe that had a significant difference between high addiction and low addiction rats was Tenericutes in females (Fig. 4E). No microbial differences post-dependence were found in either sex. The addiction index was also used to look at individual differences in the cohort of HS rats. Sexes were not separated for this analysis. Pre-alcohol dependence

microbes had no significant differences in microbial composition between high and low-addiction rats. Post-alcohol dependence, only SR1 differed between addiction levels (Fig. 4D).

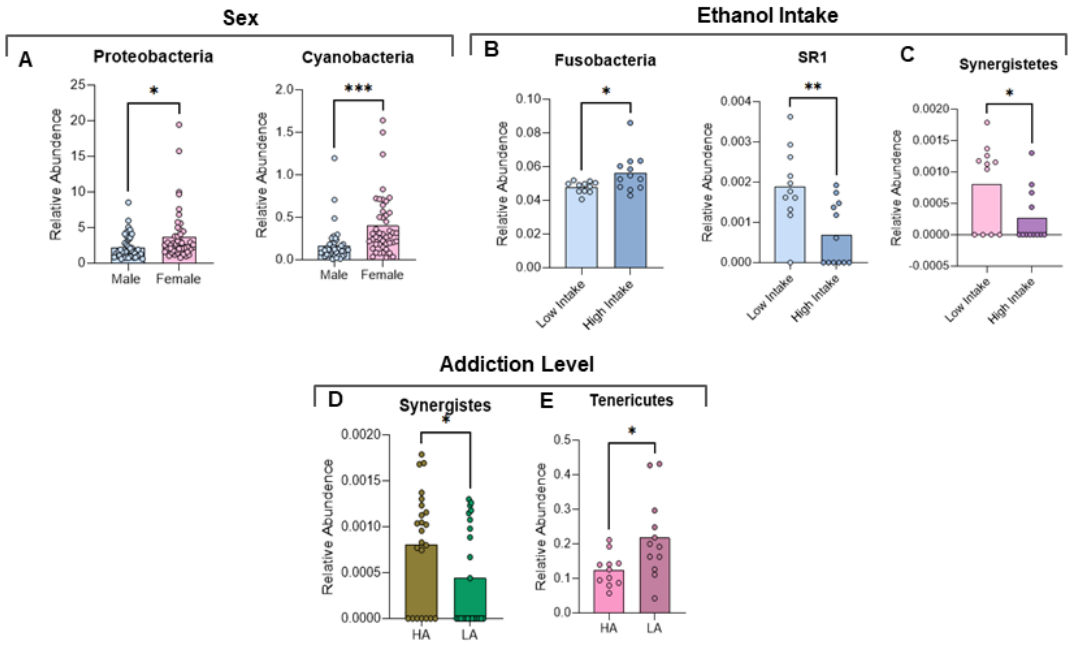

Figure 4. Relative abundance of microbes differs between sexes and ethanol intake levels. Abundance levels for sexes were compiled across both timepoints. A) Relative abundance of Proteobacteria and Cyanobacteria in male and female rats B) Relative abundance of Fusobacteria and SR1 in male rats, separated by ethanol intake levels. C) Relative abundance of Synergistes in female rats, separated by ethanol intake levels. D) Relative abundance of Synergistes after alcohol dependence. HA refers to high addiction, LA refers to low addiction. E) Relative abundance of Tenericutes in female rats before alcohol dependence. *p<0.05, **p<0.01, ***p<0.001

Treatment

After establishing alcohol dependence in HS rats, the efficacy of naltrexone was tested, an FDA-approved opioid antagonist, for the treatment of AUD.

Ethanol self-administration and intake decreased in HS rats following naltrexone treatment when compared to vehicle levels (Fig. 5A). Interestingly, individual differences were identified in response to naltrexone, with some animals responding very well to the treatment (over 75% decrease in intake) and others showing no change or even increased intake (Fig. 5A).

To further investigate these differences, the rats were divided into two groups based on their response to naltrexone: high responders and low responders. Assignments to these groups were based on the percent change in ethanol intake from vehicle levels. The microbial composition between the response groups was then analyzed and compared. Notably, rats that responded well to naltrexone treatment (high responders) had significantly higher abundances of Verrucomicrobia and Fusobacteria before dependence and higher levels of SR1 after dependence compared to their low-responding counterparts (Fig. 5B,C).

These findings suggest that the gut microbiome may play a role in individual responses to naltrexone treatment for AUD. The identification of specific microbial taxa associated with treatment response could potentially serve as biomarkers to predict the effectiveness of naltrexone in individual patients and guide personalized treatment strategies.

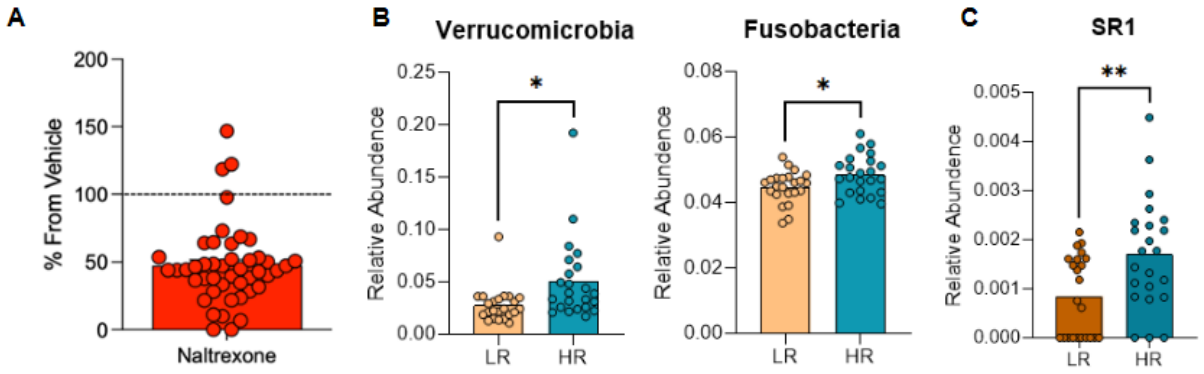

Figure 5. Microbial differences found between response to Naltrexone A) Ethanol intake post naltrexone treatment versus vehicle B) Pre-dependence abundance levels of Verrucomicrobia (left) and Fusobacteria (right) were significantly increased in HR rats compared to LR rats. LR refers to rats that showed a low response to Naltrexone; HR, high response. C) SR1 microbial abundance was higher in HR rats compared to LR rats post alcohol dependence. *p<0.05, **p<0.01

Metabolome

Table 3. Changes in metabolic abundance levels by sex and vulnerability. Metabolites identified by the VIP scores plot were measured. ND refers to no significant difference found between groups.

Metabolites	Males vs. Females
PC (O-18:1/21:1)	↓
Progesterone	↑
Glycodeoxycholic acid.4	↓
Cholic acid	↓
AC1L1X1Z	↓
Ursocholic acid	↓
Pantothenate	↓
Nonaethylene glycol	↓
Undecaethylene glycol	↓
Taurohyocholic acid.1	↓
Dioctyl adipate	↑
Heneicosapentaenoic acid	↑
Progesterone	↑
Taurohyodeoxycholic acid	↓
Taurocholic acid.2	↓
Taurocholic acid	↓
STK734268	↓
Eicosanoids	↓
STK734268.1	↓
Rimantadine hydrochloride	↓
PC 25:1;O2	↑
Equol	↓
PC(O-18:0/0:0)	↓
Tauromuricholic acid.1	↓
Taurodeoxycholic acid	↓

Metabolites	Resistant vs. Vunerable
Taurocholic acid	ND
9-Octadecenamide, (Z)	ND
Taurocholic acid.7	↓
Tauroursodeoxycholic acid	ND
Tris(2-butoxyethyl) phosphate	↓
Dibutyl phthalate	↓
Decaethylene glycol	ND
Undecaethylene glycol	ND
Taurocholic acid.2	ND
Leukotoxin A	↓
Taurocholic acid.8	↓
Linoleic acid	↓
Lyso PC (22:6).2	↓
Prostratin	↑
PC(0:0/20:4)	↓

Metabolic analysis of the rats' plasma at baseline was conducted. A Variable Importance in Projection (VIP) plot was created to showcase the top 15-25 most important metabolites identified by Partial Least Squares Discriminant Analysis (PLS-DA) (Fig. 6A, 7A). Any VIP score over two is considered significant to the model. Taurocholic acid and Undecaethylene glycol were listed as important for both sex and addiction level differences (Fig. 6A, 7A). The difference in abundances of listed metabolites was calculated and listed in Table 3. Most metabolites were found to be lower in females and vulnerable (high-addiction) rats (Table 3). However, Progesterone, dioctyl adipate, Heneicosapentaenoic acid, and PC 25:1;O2 were found to be higher in females compared to males (Table 3). Only Prostratin was found to be higher in

the vulnerable population when compared to the resistant (low addicted) group (Table 3). Ursocholic Acid, Phosphatidylcholine (PC), Taurocholic Acid, Glycodeoxycholic Acid, and Linoleic Acid were identified as important in both analyses.

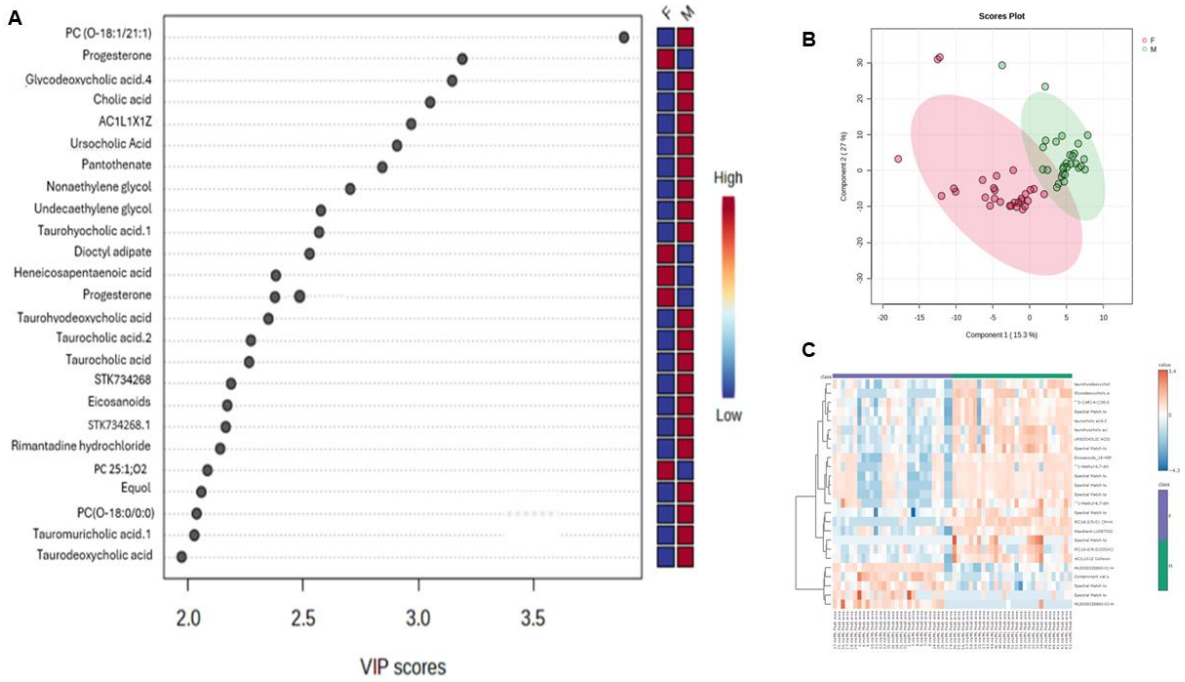

Figure 6. Metabolic differences between sex and addiction levels. The plots were created using Metaboanalyst. A) VIP plot listing top 25 metabolites that are important for the predictive factor of the model. F refers to female rats; M, male rats. Boxes on the right side of the graph indicate the abundance level for the metabolite in each group. B) PCA Scores plot for males and females. C) Heatmap of metabolic composition between males and females.

To visualize the separation between groups, a Principal Component Analysis (PCA) scores plot was generated. A clear separation is seen between the male and female clusters (Fig. 6B), while only a slight differentiation is observed between the resistant and vulnerable clusters (Fig. 7B). It is important to note that PCA is an unsupervised method used to visualize the separation between groups and does not directly prove the predictive capability of the model. The observed separation patterns are further supported by the heatmap of metabolic

compositions, which shows a clear difference in metabolic abundance levels between sexes (Fig. 6C) but not between addiction levels (Fig. 7C).

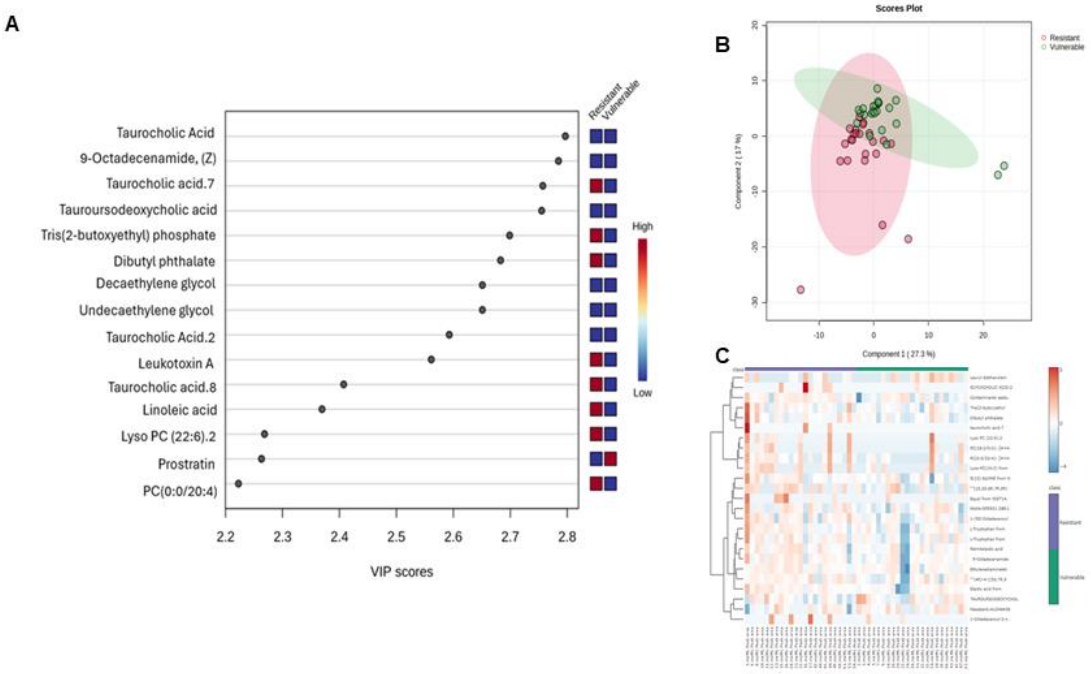

Figure 7. Metabolic differences between addiction levels A) VIP plot listing top 25 metabolites that are important for the predictive factor of the model, regarding addiction level. Boxes on the right side of the graph indicate the abundance level for the metabolite in each group. B) PCA scores plot for resistant and vulnerable rats. C) Heatmap of metabolic composition between alcohol-resistant and alcohol-vulnerable rats.

Effects of Tolcapone on Ethanol and Saccharin Self-Administration in Nondependent and Dependent Rats

The effects of tolcapone pretreatment were evaluated in rats responding for ethanol in a non-dependent state as well as in dependent rats self-administering ethanol during acute withdrawal (Fig. 8). There was a significant effect of dose in dependent rats when measuring both ethanol intake in g/kg ($F(1.738, 48.66) = 6.080$; $p = 0.0062$) and as a percentage of baseline level of responding ($F(1.890, 52.92) = 7.192$; $p = 0.0021$) (Fig. 8A, D). In both cases, rats responded significantly less after receiving a pretreatment of 30 mg/kg tolcapone compared to vehicle (g/kg: $p = 0.0016$; % of baseline: $p = 0.0016$). In contrast, there was no effect of tolcapone dose in non-dependent rats, regardless of whether data were analyzed as ethanol intake

(F (1.784, 30.34) = 0.3876; p = 0.6585) or percentage of baseline (F (1.881, 31.98) = 0.4844; p = 0.6091) (Fig. 8B, E). Tolcapone also did not affect responding maintained by saccharin (reinforcers: F (1.600, 14.40) = 0.9745; p = 0.3824; % of baseline: F (1.663, 14.97) = 0.9452; p = 0.3945) (Fig. 8c, f). There was no change in response for water in any of the groups (dependent: F (1.706, 47.76) = 0.3680; p = 0.6605; non-dependent: F (1.590, 27.04) = 0.4195; p = 0.6157; saccharin: F (1.842, 18.42) = 0.2256; p = 0.7826) (Fig. 8G-I).

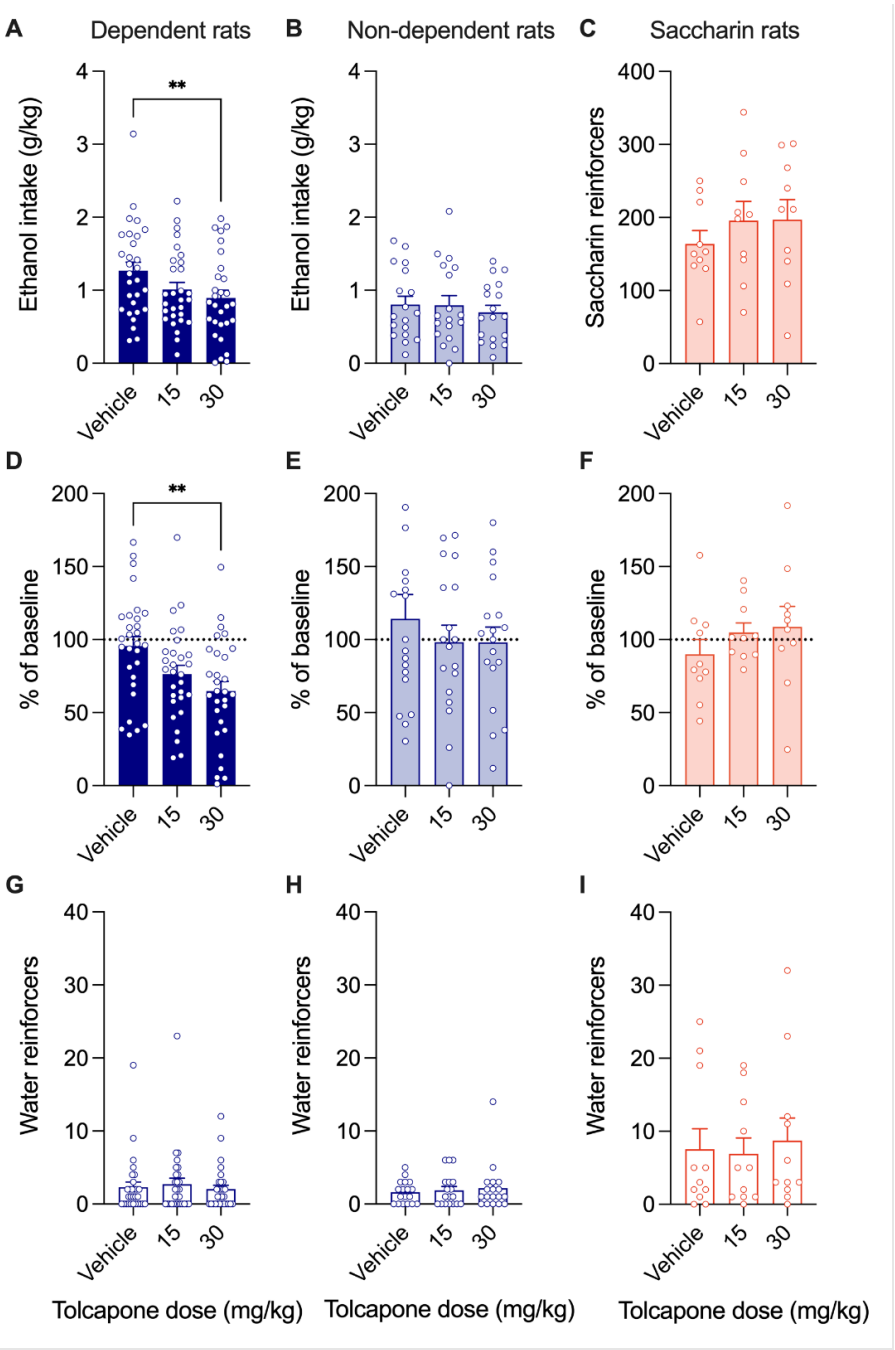

Figure 8. Effects of tolcapone in dependent rats, non-dependent rats, and rats responding for saccharin. Effects of tolcapone pretreatment (10 or 30 mg/kg, i.p.) on ethanol self-administration in dependent (dark blue bars) and non-dependent rats (light blue bars) and dependent rats and on saccharin self-administration (orange bars). Abscissae: vehicle or dose of tolcapone administered 60-min before the session. A, B) Ethanol intake (in g/kg) in dependent (A) and non-dependent (B) rats. C. Number of saccharin reinforcers earned . D, E) Number of ethanol reinforcers earned by dependent (D) and non-dependent rats (E) as a percentage of average responding in the three baseline sessions prior to pretreatments. F. Saccharin reinforcers earned as a percentage of baseline responding. G-I) Number of water reinforcers earned by dependent (G) and non-dependent rats (H) and rats responding for saccharin (I). Bars represent mean and error bars represent SEM. Data points are individual subjects. Symbols indicate statistical significance in a Dunnett's post-hoc comparison where ** is $p < 0.01$ compared to vehicle.

Sex Differences in Ethanol Intake and Response to Tolcapone Treatment

Ethanol intake and effects of tolcapone were also analyzed in male and female subjects (Fig. 9). There was no main effect of sex in the number of reinforcers earned across the 28 self-administration sessions ($F(1, 17) = 0.2306$; $p = 0.6372$) (Fig. 9A). However, there was a sex x session interaction ($F(27, 459) = 1.562$; $p = 0.0374$). There was a main effect of sex on ethanol intake (g/kg) ($F(1, 17) = 19.45$; $p = 0.0004$) where females had higher ethanol intake than males, but no sex x session interaction ($F(27, 459) = 1.399$; $p = 0.0898$). When the last three self-administration sessions before and after dependence were analyzed, there was a main effect of sex ($F(1, 27) = 30.20$; $p < 0.0001$) and dependence state ($F(1, 27) = 33.80$; $p < 0.0001$) where females had higher intake than males and dependent rats had higher intake than non-dependent rats (Fig. 9C).

Regarding tolcapone pretreatments in dependent rats, there was a main effect of dose ($F(2, 54) = 5.761$; $p = 0.0054$) and a main effect of sex ($F(1, 27) = 21.63$; $p < 0.001$) in dependent rats (Fig. 9D). Post-hoc analyses indicated there was a significant decrease in ethanol intake in female rats at both doses (15 mg/kg: $p = 0.0339$; 30 mg/kg: $p = 0.0173$), but only a trend in male rats (30 mg/kg: $p = 0.0801$). However, when data were analyzed as a percentage of baseline responding, there was a significant main effect of dose ($F(2, 54) = 7.145$; $p = 0.0018$) where tolcapone was effective at reducing responding in both female (15 mg/kg: $p = 0.0319$; 30 mg/kg: $p = 0.0207$) and male rats (30 mg/kg: $p = 0.0179$) (Fig. 9G). In contrast to dependent rats, tolcapone was not effective in nondependent rats when analyzed in terms of ethanol intake ($F(2, 32) = 0.4216$; $p = 0.6596$) (Fig. 9E) or percent of baseline responding ($F(2, 32) = 0.7792$; $p = 0.4673$) (Fig. 9H); however, there was still a main effect of sex for ethanol intake ($F(1, 16) = 20.76$; $p = 0.003$) where females had greater ethanol intake than males (Fig. 9E). Finally, there

was no effect of tolcapone (ethanol intake: $F(2, 16) = 1.015$; $p = 0.3844$; % of baseline: $F(2, 18) = 1.704$; $p = 0.2101$) or sex ($F(1, 8) = 0.2452$; $p = 0.6338$), regardless of whether the number of reinforcers (Fig. 9F) or percent of baseline was evaluated (Fig. 9I).

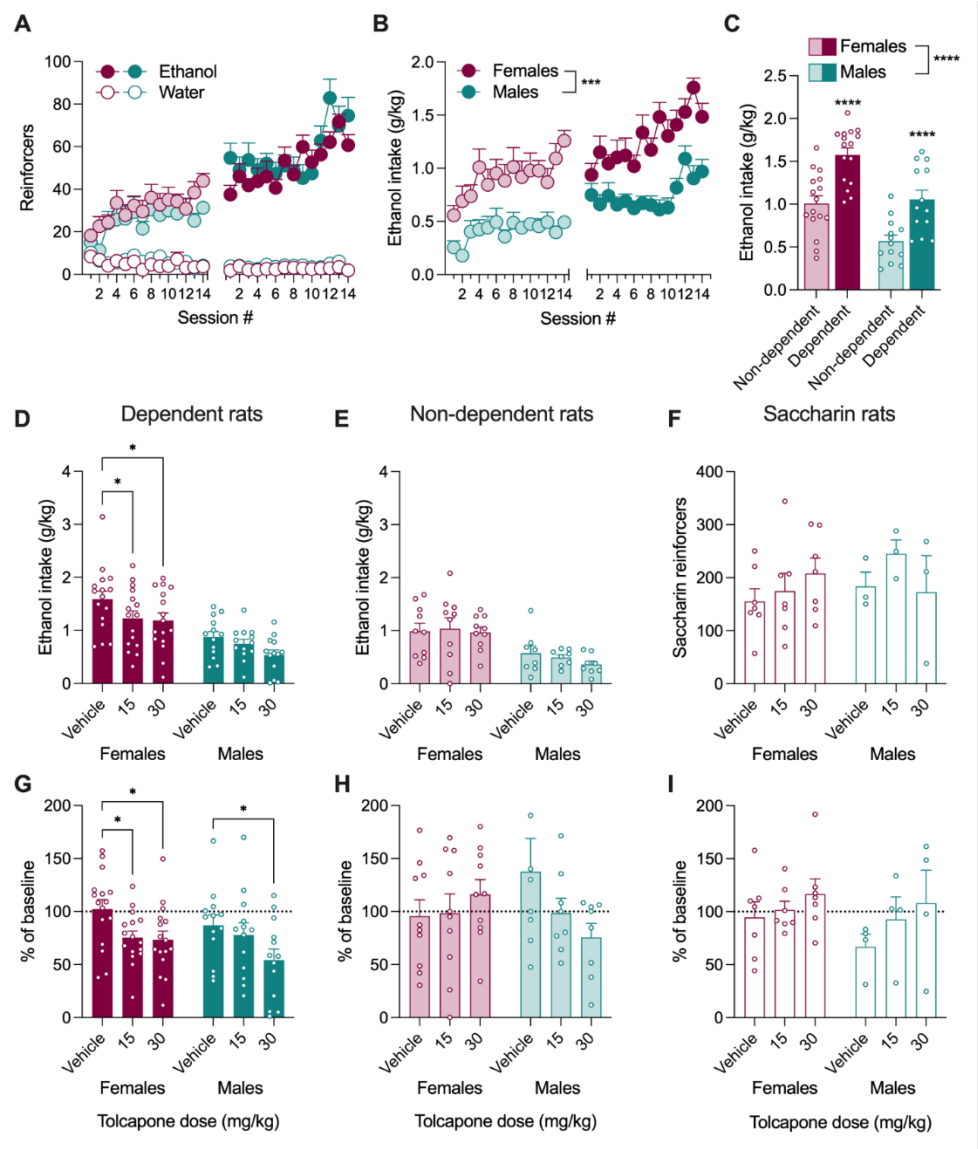

Figure 9. Ethanol self-administration and effects of tolcapone in female and male rats. Ethanol self-administration and effects of tolcapone pretreatment (10 or 30 mg/kg, i.p.) in female and male rats on ethanol self-administration in dependent and non-dependent rats and on saccharin self-administration. A. Reinforcers of ethanol and water earned across 14 non-dependent (left) and 14 dependent (right) sessions. B. Ethanol intake in g/kg corresponding to number of reinforcers earned. C. Baseline ethanol intake (in g/kg) in nondependent and dependent rats prior to tolcapone pretreatments. D, E. Ethanol intake (in g/kg) in dependent (D) and non-dependent rats (E) during sessions in which tolcapone was administered. F. Number of reinforcers earned of 0.04% saccharin solution during sessions in which tolcapone was administered. G-I. Ethanol or saccharin intake as a percentage of average responding in the three baseline sessions prior to pretreatments. Bars represent mean and error bars represent SEM. Data points are individual subjects. Symbols in panel C represent significant main effect, where **** is $p < 0.0001$. Symbols in other panels indicate statistical significance in a Dunnett's post-hoc comparison where * is $p < 0.05$

Tolcapone Effectiveness as a Function of Estrous Phase

The effectiveness of tolcapone as a function of estrous phase was also evaluated in dependent female rats, collapsed across tolcapone dose (Fig. 9). Representative images for each quantified phase are shown in Figure 9. Tolcapone was significantly more effective at reducing ethanol intake (as a percentage of baseline responding) in rats in estrus and metestrus (when estradiol is the lowest; (Goldman et al., 2005), compared to rats in proestrus and diestrus (when estradiol is the highest; $t(20) = 2.441$; $p = 0.0241$) (Fig. 10).

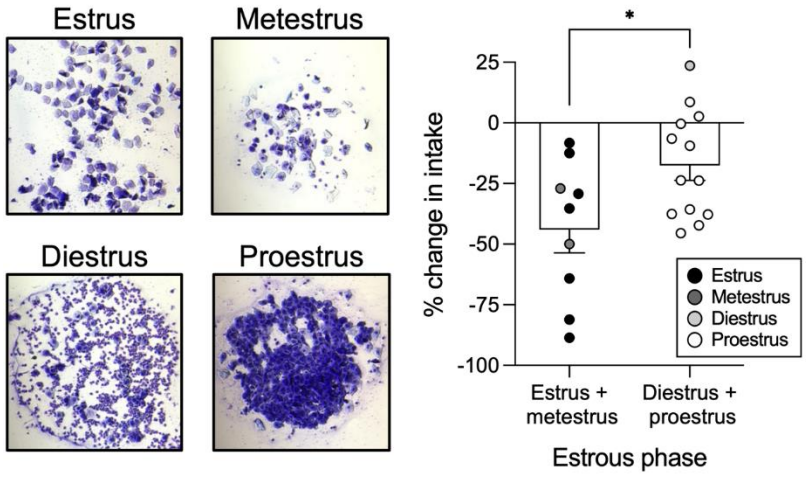

Figure 10. Tolcapone effects during phases of the estrous cycle. Representative images of stained estrous swab images and the proportion of rats in each phase when treated with tolcapone. Abscissae: estrous phase on day of tolcapone pretreatment. Ordinate: percentage decrease in ethanol intake after administration of tolcapone for individual rats in estrus and metestrus (left bar, black and dark grey circles) or in diestrus and proestrus (right bar, light grey and white circles). Bar represents mean and error represents S.E.M. * is $p < 0.05$ for a t-test.

Acknowledgements

Chapter 2, in part, contains reprints of materials as it appears in Doyle, M. R., Dirik, S., Martinez, A. R., Hughes, T. E., Iyer, M. R., Sneddon, E. A., Seo, H., Cohen, S. M., & De Guglielmo, G. (2024). Catechol-O-Methyltransferase inhibition and alcohol use disorder: Evaluating the efficacy of tolcapone in ethanol-dependent rats. Neuropharmacology, 242, 109770. The thesis author was an author of this paper.

Chapter 3 DISCUSSION

The present study aimed to investigate the relationship between the gut microbiome, metabolome, and alcohol addiction, focusing on differences between sexes, addiction severity, and treatment response. The results demonstrate significant variations in addiction-like behaviors and microbiome composition between males and females, as well as between high-addiction and low-addiction rats. Moreover, specific microbial and metabolomic profiles associated with treatment response were identified, highlighting the potential for personalized medicine approaches in the treatment of Alcohol Use Disorder (AUD).

Sex Differences in Addiction-Like Behaviors and Microbiome Composition

The findings reveal notable sex differences in addiction-like behaviors, with females exhibiting higher ethanol intake compared to males. These differences may be attributed to hormonal variations, as previous studies have shown that estradiol administration increases drug self-administration (Hu et al., 2004). Microbial analysis also revealed sex-specific differences, with females having higher abundances of Proteobacteria and Cyanobacteria compared to males. While previous animal studies have reported microbial sex differences, the specific phyla identified vary across studies (Kim et al., 2020; Yurkovetskiy et al., 2013), emphasizing the need for further research to elucidate the underlying mechanisms and potential confounding factors.

The observed sex differences in addiction-like behaviors and microbiome composition are consistent with the growing body of literature highlighting the importance of considering sex as a biological variable in addiction research (Becker and Koob, 2016; Becker et al., 2017). Sex hormones, particularly estrogen, have been shown to modulate the reward system and influence drug-seeking behavior (Becker and Hu, 2008; Carroll and Anker, 2010). Moreover, the gut

microbiome has been implicated in the bidirectional communication between the gut and the brain, known as the gut-brain axis (Cryan et al., 2019; Martin et al., 2018). Sex differences in the gut microbiome may contribute to the differential susceptibility to addiction and treatment response observed between males and females (Kiraly et al., 2016; Temko et al., 2017).

Predictive Value of the Microbiome in Addiction Severity and Treatment Response

Interestingly, the study found that the microbiome may have predictive value for addiction severity in females, with lower Tenericutes abundance pre-dependence in high-addiction rats. This finding suggests that specific microbial profiles could be used to identify individuals at higher risk for developing severe AUD, allowing for early intervention and personalized prevention strategies. Additionally, higher levels of Verrucomicrobia, Fusobacteria, and SR1 were observed to correlate with better treatment outcomes, indicating that the microbiome may also play a role in treatment response. While previous studies have not found significant microbial differences associated with naltrexone response (Gicquelais et al., 2020), the results highlight the importance of considering addiction severity when investigating treatment efficacy.

The predictive value of the microbiome in addiction severity and treatment response is a novel finding that warrants further investigation. The gut microbiome has been implicated in various aspects of brain function and behavior, including mood, cognition, and addiction (Cryan et al., 2019; Meckel and Kiraly, 2019). Preclinical studies have shown that manipulating the gut microbiome can influence drug-seeking behavior and alter the reinforcing properties of drugs (Kiraly et al., 2016; Ning et al., 2017). Moreover, the gut microbiome has been proposed as a potential biomarker for psychiatric disorders, including addiction (Kiraly et al., 2016; Temko et

al., 2017). The findings suggest that specific microbial profiles may serve as predictive markers for AUD severity and treatment response, particularly in females. Future studies should aim to validate these findings in larger, more diverse samples and explore the mechanisms underlying the observed associations.

Metabolomic Differences Between Sexes and Addiction Levels

Metabolomic analysis revealed clear differences between sexes and more subtle variations between addiction levels. Bile acids and lipids were identified as the most prominent distinguishing metabolites, with Ursocholic Acid, Phosphatidylcholine (PC), Taurocholic Acid, Glycodeoxycholic Acid, and Linoleic Acid being consistently featured in both analyses. These findings are consistent with previous studies that have implicated bile acids in alcohol use and related comorbidities (Rachakonda et al., 2014; Zhang et al., 2022). However, conflicting results regarding the direction of change in specific bile acids (Brandl et al., 2018; Xie et al., 2013) underscore the need for further research to clarify their role in AUD.

The observed metabolomic differences between sexes and addiction levels provide valuable insights into the underlying mechanisms of AUD. Bile acids have been shown to play a critical role in the regulation of lipid and glucose metabolism, as well as in the modulation of inflammation and the gut microbiome (de Aguiar Vallim et al., 2013; Wahlstrom et al., 2016). Alterations in bile acid metabolism have been linked to various metabolic disorders, including non-alcoholic fatty liver disease (NAFLD) and obesity (Arab et al., 2017; Ferslew et al., 2015), which are common comorbidities of AUD (Traversy and Chaput, 2015). Moreover, bile acids have been implicated in the regulation of brain function and behavior through the gut-brain axis (Mertens et al., 2017; Xie et al., 2015). The identification of specific bile acids and lipids as key

metabolites associated with AUD highlights the importance of further investigating their role in the pathophysiology and treatment of this disorder.

Implications of the COMT Study and the Role of Linoleic Acid in AUD

The COMT study provided further evidence for the involvement of the metabolome in addiction, identifying linoleic acid as a key metabolite associated with addiction severity. differences in linoleic acid levels were hypothesized to influence COMT activity through modulation of S-adenosylmethionine (SAM) concentration (Pita and Delgado, 2000). SAM is a crucial methyl donor for various methylation reactions, including those catalyzed by COMT (Zhu, 2002). COMT is an enzyme that plays a key role in the degradation of catecholamines, such as dopamine, which are involved in the reward and reinforcement pathways associated with addiction (Apud et al., 2007; Gasparini et al., 1997). Alterations in SAM levels due to differences in linoleic acid could potentially influence COMT activity, leading to changes in catecholamine metabolism and, consequently, alcohol consumption, ultimately affecting catecholamine metabolism and alcohol consumption.

COMT inhibitors have been implicated as novel treatments for AUD due to their ability to improve cognitive function. Although tolcapone, an FDA-approved COMT inhibitor, has been used in randomized, double-blind, placebo-controlled studies in people (Coker et al., 2020b; Schacht et al., 2022b) and in rodent models of ethanol drinking (McCane et al., 2014; McCane et al., 2018b), its ability to decrease operant ethanol self-administration specifically in dependent subjects had not been evaluated. In this study, tolcapone was found to decrease ethanol drinking in rats made dependent using the CIE model, but not in nondependent rats or rats drinking saccharin. These findings suggest tolcapone, and perhaps other COMT inhibitors, may be effective treatments for people with AUD.

Tolcapone decreased ethanol self-administration in rats made dependent using the CIE model but did not affect ethanol intake in non-dependent rats or response for saccharin in a different group of rats. There was also a nonsignificant trend towards tolcapone reducing alcohol drinking in male, non-dependent rats. These findings are consistent with prior reports that, in a cued access protocol, tolcapone was ineffective at reducing ethanol intake in non-dependent Wistar rats, but was effective at decreasing ethanol intake in selectively bred, alcohol-preferring P rats (McCane et al., 2018a; McCane et al., 2014). Tolcapone can also decrease cue seeking in the absence of an ethanol reinforcer delivery (McCane et al., 2018a). Notably, the same dose of tolcapone can decrease ethanol drinking in two, distinct models that capture different aspects of AUD in different laboratories, especially since there have been pushes to evaluate potentially therapeutic compounds under conditions that model different human conditions (Becker and Lopez, 2016; Lynch, 2018; Vendruscolo and Roberts, 2014). Using models of alcohol dependence, such as the CIE model, is a key factor in evaluating whether pharmacotherapies may be effective in the human population.

The mechanism through which tolcapone can decrease drinking in dependent rats is still unclear. Others have reported that tolcapone may be effective at suppressing excessive reward-seeking due to altered dopaminergic transmission in the prefrontal cortex (McCane et al., 2014). Given that CIE alters dopaminergic activity in the prefrontal cortex and COMT inhibitors are used as cognitive enhancers (Apud et al., 2007; Bhakta et al., 2017; Gasparini et al., 1997), it is possible that regulating dopaminergic activity in the prefrontal cortex is responsible for the decrease in alcohol drinking.

Another potential mechanism could involve the interaction between tolcapone, and stress-related neurotransmitter systems implicated in alcohol dependence and withdrawal

symptoms, such as the corticotropin-releasing factor (CRF) (Koob, 2008). Recent evidence suggests an interaction between the COMT and CRF systems in the context of withdrawal from other substances of abuse, such as methamphetamine (Garcia-Carmona et al., 2018). In recent work, García-Carmona, and colleagues (2018) demonstrated that methamphetamine withdrawal induces activation of CRF neurons in the brain stress system, which occurs in parallel with increased activity of cardiac sympathetic pathways, including COMT. Given the role of CRF in alcohol withdrawal and dependence (de Guglielmo et al., 2019; Funk and Koob, 2007; Funk et al., 2006; Gilpin and Koob, 2008; Koob and Volkow, 2010; Roberto et al., 2010), a similar interaction between COMT and CRF systems could be an explanation for the selective effects of tolcapone observed in dependent rats. However, in the present study, withdrawal symptoms such as anxiety or handling-induced convulsions were not directly assessed. Future studies should investigate the effects of tolcapone on these withdrawal measures and explore the potential interactions between tolcapone, dopamine signaling, and stress-related neurotransmitter systems, particularly CRF, to provide further insight into the mechanisms underlying its selective action in dependent animals.

In this study, tolcapone was effective in both male and female subjects at similar doses. This is somewhat surprising given prior work with tolcapone in rats (McCane et al., 2018b) as well as in a mouse model of COMT gene disruption (Tammimaki et al., 2008). In those studies, decreasing COMT activity pharmacologically or increasing COMT activity genetically altered drinking in male subjects without affecting drinking in female subjects. It has been long known that COMT activity changes as a function of estrous cycle (Parvez et al., 1978), so differences in these studies could be related to estrous cycle. In fact, COMT activity in the rat whole brain and hypothalamus specifically is highest during estrus, compared to the other phases (Parvez et al.,

1978), when estrogen levels are lowest (Smith et al., 1975). Additionally, estrogens decrease COMT activity (Cohn and Axelrod, 1971). This is consistent with the findings that tolcapone administered during estrus and metestrus, when COMT activity is highest and estrogens are lowest, could increase the likelihood of a beneficial response to tolcapone compared to proestrus and diestrus, when estrogen is increasing. It is important to note that tolcapone was also generally effective in the non-estrus phases (Goldman et al., 2005), and these studies have a low number of subjects, particularly in metestrus and diestrus. However, given these findings and that there are estrogen response elements in the promotor region of the COMT gene through which estrogen can inhibit the formation of COMT (Jiang et al., 2003; Xie et al., 1999), the use of COMT inhibitors as a treatment for AUD in women/female subjects and during different phases of the menstrual/estrous cycle needs to be further explored to understand the interaction between circulating estrogens and COMT inhibitors.

There is a well-characterized polymorphism in the COMT gene that substantially increases COMT activity in humans, which suggests COMT inhibitors may be more effective in a subset of individuals that are homozygous for the val allele at the rs4680 single nucleotide polymorphism (Bhakta et al., 2017; Boettiger et al., 2007; Coker et al., 2020b; Comasco et al., 2015; Schacht et al., 2022b). These individuals tend to be more impulsive and drink more, which is consistent with the genetic mouse model that increases COMT levels (Tammimaki et al., 2008). Importantly, these individuals are also more responsive to tolcapone (Schacht et al., 2022b), suggesting a personalized medicine approach may be particularly useful when considering COMT inhibitors as a treatment for AUD or other psychiatric disorders.

Notable side effects of tolcapone in acute treatment were not observed, which is an encouraging finding. Moreover, tolcapone did not affect water or saccharin consumption, nor did

it affect ethanol consumption in non-dependent rats. The fact that tolcapone did not affect water or saccharin consumption indicates that the drug is not causing a general suppression of consummatory behavior or locomotor activity, which are common concerns with pharmacological treatments for substance use disorders. Moreover, it was previously shown that tolcapone can increase locomotor activity at the doses tested (Mihaylova et al., 2019). Instead, the selective reduction of ethanol consumption in dependent rats suggests that tolcapone may be specifically targeting the neurobiological mechanisms underlying AUD.

An additional consideration in evaluating tolcapone as a treatment for AUD is the possibility that it might alter alcohol metabolism, which could influence its ability to reduce alcohol self-administration. Although alcohol metabolism was not measured, and there is no direct evidence in the literature suggesting that tolcapone could affect alcohol metabolism, it seems unlikely based on the available data. Behaviorally, tolcapone did not alter alcohol drinking in nondependent rats, which would be expected if it had a significant impact on ethanol metabolism. Furthermore, a comparison of the metabolism pathways for both tolcapone and alcohol reveals that they are both partially metabolized by UDP-glucuronosyltransferases. However, only ~15% of tolcapone is metabolized via this pathway (Jorga et al., 1999), and less than 0.1% of ethanol metabolism occurs through this pathway (Wurst et al., 2015), suggesting that tolcapone is unlikely to directly alter ethanol metabolism. No other overlapping metabolism pathways were identified that might interact.

It is important to note that the lack of side effects observed may be due to the acute treatment paradigm. In clinical settings, tolcapone is often administered chronically, and it is during this long-term administration that concerns about liver dysfunction have arisen arisen (Olanow and Panel, 2000). Moving forward, novel COMT inhibitors with better side effect

profiles would be better candidates for a novel pharmacotherapy for AUD. Although tolcapone is FDA-approved to treat Parkinson's disorder, there are concerns about liver dysfunction that limit its use arisen (Olanow and Panel, 2000). Entacapone and opicapone are two other FDA-approved COMT inhibitors that are also suboptimal due to their poor bioavailability and low efficacy, or undesirable side effects such as dyskinesia and difficulty sleeping, respectively (Fabbri et al., 2018; Keranen et al., 1994). Future studies must evaluate novel COMT inhibitors with better side effect profiles to determine whether COMT inhibition is effective at treating AUD without problematic side effects. These next-generation COMT inhibitors should also have reduced toxicity and metabolic liability, while being blood-brain-barrier penetrant with improved efficacy and safety profiles. The present study combined with prior work in humans (Coker et al., 2020b; Schacht et al., 2022b) and rodents (Coker et al., 2020b; McCane et al., 2014; McCane et al., 2018b; Schacht et al., 2022b) suggests COMT inhibitors show promise for AUD treatment, but future studies should evaluate novel COMT inhibitors, particularly as a part of personalized medicine.

Limitations and Future Directions

While the study provided valuable insights into the role of the gut microbiome and metabolome in AUD, it is not without limitations. While allowing for greater control over environmental factors, animal models may not fully capture the complexity of human AUD. Additionally, the sample size and limited number of timepoints investigated may have influenced the observed results. Future research should aim to replicate these findings in larger, more diverse samples and incorporate longitudinal designs to understand better the temporal dynamics of microbial and metabolomic changes in AUD. Moreover, the mechanisms underlying the observed associations between the microbiome, metabolome, and addiction remain to be

elucidated. Future studies should investigate the functional implications of specific microbial and metabolomic profiles, as well as their interactions with host genetics and environmental factors. The identification of key microbial species or metabolites that confer resistance or vulnerability to AUD could lead to the development of novel therapeutic strategies, such as probiotics or metabolite-based interventions.

Acknowledgements

Chapter 3, in part, contains reprints of materials as it appears in Doyle, M. R., Dirik, S., Martinez, A. R., Hughes, T. E., Iyer, M. R., Sneddon, E. A., Seo, H., Cohen, S. M., & De Guglielmo, G. (2024). Catechol-O-Methyltransferase inhibition and alcohol use disorder: Evaluating the efficacy of tolcapone in ethanol-dependent rats. Neuropharmacology, 242, 109770. The thesis author was an author of this paper.

CONCLUSION

The study demonstrated the significant role of the gut microbiome and metabolome in alcohol addiction, highlighting sex differences, predictive value for addiction severity and treatment response, and the importance of personalized treatment approaches. The identification of specific microbial and metabolomic profiles associated with AUD vulnerability and treatment efficacy opens new avenues for the development of targeted prevention and intervention strategies. Considering the unique microbial and metabolomic makeup of each individual, creates a more personalized approach to AUD treatment, ultimately improving outcomes for those struggling with this debilitating disorder.

The COMT study provides further evidence for the involvement of the metabolome in addiction, identifying linoleic acid as a key metabolite associated with addiction severity and implicating COMT inhibitors as potential novel treatments for AUD. The selective efficacy of tolcapone in reducing ethanol consumption in dependent rats, particularly in females during high estradiol phases, highlights the importance of considering individual factors such as sex, hormonal status, and genetic polymorphisms in the development of personalized treatment strategies.

Future research should focus on validating these findings in larger, more diverse samples, exploring the mechanisms underlying the observed associations, and evaluating novel COMT inhibitors with improved safety and efficacy profiles. Integrating behavioral, microbiome, metabolomic, and pharmacological data gives a more comprehensive understanding of the complex nature of AUD and develop targeted, personalized interventions to address this devastating disorder.

Acknowledgements

Conclusion, in part, contains material as it appears in Doyle, M. R., Dirik, S., Martinez, A. R., Hughes, T. E., Iyer, M. R., Sneddon, E. A., Seo, H., Cohen, S. M., & De Guglielmo, G. (2024). Catechol-O-Methyltransferase inhibition and alcohol use disorder: Evaluating the efficacy of tolcapone in ethanol-dependent rats. Neuropharmacology, 242, 109770. The thesis author was an author of this paper.

REFERENCES

Apud JA, Mattay V, Chen J, Kolachana BS, Callicott JH, Rasetti R, Alce G, Iudicello JE, Akbar N, Egan MF, Goldberg TE and Weinberger DR (2007) Tolcapone improves cognition and cortical information processing in normal human subjects. *Neuropsychopharmacology* **32**:1011-1020.

Arab JP, Karpen SJ, Dawson PA, Arrese M and Trauner M (2017) Bile acids and nonalcoholic fatty liver disease: Molecular insights and therapeutic perspectives. *Hepatology* **65**:350-362.

Aron AT, Gentry EC, McPhail KL, Nothias LF, Nothias-Esposito M, Bouslimani A, Petras D, Gauglitz JM, Sikora N, Vargas F, van der Hooft JJJ, Ernst M, Kang KB, Aceves CM, Caraballo-Rodriguez AM, Koester I, Weldon KC, Bertrand S, Roullier C, Sun K, Tehan RM, Boya PC, Christian MH, Gutierrez M, Ulloa AM, Tejeda Mora JA, Mojica-Flores R, Lakey-Beitia J, Vasquez-Chaves V, Zhang Y, Calderon AI, Tayler N, Keyzers RA, Tugizimana F, Ndlovu N, Aksenov AA, Jarmusch AK, Schmid R, Truman AW, Bandeira N, Wang M and Dorrestein PC (2020) Reproducible molecular networking of untargeted mass spectrometry data using GNPS. *Nat Protoc* **15**:1954-1991.

Association AP (1994) Diagnostic and Statistical Manual of Mental Disorders, (Association AP ed).

Bahji A, Bach P, Danilewitz M, Crockford D, Devoe DJ, El-Guebaly N and Saitz R (2022) Pharmacotherapies for Adults With Alcohol Use Disorders: A Systematic Review and Network Meta-analysis. *J Addict Med* **16**:630-638.

Becker HC and Lopez MF (2016) An Animal Model of Alcohol Dependence to Screen Medications for Treating Alcoholism. *Int Rev Neurobiol* **126**:157-177.

Becker JB and Hu M (2008) Sex differences in drug abuse. *Front Neuroendocrinol* **29**:36-47.

Becker JB and Koob GF (2016) Sex Differences in Animal Models: Focus on Addiction. *Pharmacol Rev* **68**:242-263.

Becker JB, McClellan ML and Reed BG (2017) Sex differences, gender and addiction. *J Neurosci Res* **95**:136-147.

Bercik P, Collins SM and Verdu EF (2012) Microbes and the gut-brain axis. *Neurogastroenterol Motil* **24**:405-413.

Bhakta SG, Light GA, Talledo JA, Balvaneda B, Hughes E, Alvarez A, Rana BK, Young JW and Swerdlow NR (2017) Tolcapone-Enhanced Neurocognition in Healthy Adults: Neural Basis and Predictors. *Int J Neuropsychopharmacol* **20**:979-987.

Boettiger CA, Mitchell JM, Tavares VC, Robertson M, Joslyn G, D'Esposito M and Fields HL (2007) Immediate reward bias in humans: fronto-parietal networks and a role for the catechol-O-methyltransferase 158(Val/Val) genotype. *J Neurosci* **27**:14383-14391.

Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ, Brown CT, Callahan BJ, Caraballo-Rodriguez AM, Chase J, Cope EK, Da Silva R, Diener C, Dorrestein PC, Douglas GM, Durall DM, Duvallet C, Edwardson CF, Ernst M, Estaki M, Fouquier J, Gauglitz JM, Gibbons SM, Gibson DL, Gonzalez A, Gorlick K, Guo J, Hillmann B, Holmes S, Holste H, Huttenhower C, Huttley GA, Janssen S, Jarmusch AK, Jiang L, Kaehler BD, Kang KB, Keefe CR, Keim P, Kelley ST, Knights D, Koester I, Kosciolek T, Kreps J, Langille MGI, Lee J, Ley R, Liu YX, Loftfield E, Lozupone C, Maher M, Marotz C, Martin BD, McDonald D, McIver LJ, Melnik AV, Metcalf JL, Morgan SC, Morton JT, Naimey AT, Navas-Molina JA, Nothias LF, Orchanian SB, Pearson T, Peoples SL, Petras D, Preuss ML, Pruesse E, Rasmussen LB, Rivers A, Robeson MS, 2nd, Rosenthal P, Segata N, Shaffer M, Shiffer A, Sinha R, Song SJ, Spear JR, Swafford AD, Thompson LR, Torres PJ, Trinh P, Tripathi A, Turnbaugh PJ, Ul-Hasan S, van der Hooft JJJ, Vargas F, Vazquez-Baeza Y, Vogtmann E, von Hippel M, Walters W, Wan Y, Wang M, Warren J, Weber KC, Williamson CHD, Willis AD, Xu ZZ, Zaneveld JR, Zhang Y, Zhu Q, Knight R and Caporaso JG (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* **37**:852-857.

Brandl K, Hartmann P, Jih LJ, Pizzo DP, Argemi J, Ventura-Cots M, Coulter S, Liddle C, Ling L, Rossi SJ, DePaoli AM, Loomba R, Mehal WZ, Fouts DE, Lucey MR, Bosques-Padilla F, Mathurin P, Louvet A, Garcia-Tsao G, Verna EC, Abraldes JG, Brown RS, Jr., Vargas V, Altamirano J, Caballeria J, Shawcross D, Starkel P, Ho SB, Bataller R and Schnabl B (2018) Dysregulation of serum bile acids and FGF19 in alcoholic hepatitis. *J Hepatol* **69**:396-405.

Breit S, Kupferberg A, Rogler G and Hasler G (2018) Vagus Nerve as Modulator of the Brain-Gut Axis in Psychiatric and Inflammatory Disorders. *Front Psychiatry* **9**:44.

Carrette LL, de Guglielmo G, Kallupi M, Maturin L, Brennan M, Boomhower B, Conlisk D, Sedighim S, Tieu L, Fannon MJ, Velarde N, Kononoff J, Kimbrough A, Simpson S, Smith LC, Shankar K, Ramirez F, Jr., Chitre AS, Lin B, Polesskaya O, Solberg Woods LC, Palmer AA and George O (2021) The cocaine and oxycodone biobanks, two repositories from genetically diverse and behaviorally characterized rats for the study of addiction. *eNeuro*.

Carroll ME and Anker JJ (2010) Sex differences and ovarian hormones in animal models of drug dependence. *Horm Behav* **58**:44-56.

Chitre AS, Polesskaya O, Munro D, Cheng R, Mohammadi P, Holl K, Gao J, Bimschleger H, Martinez AG, George AM, Gileta AF, Han W, Horvath A, Hughson A, Ishiwari K, King CP, Lamparelli A, Versaggi CL, Martin CD, St Pierre CL, Tripi JA, Richards JB, Wang T, Chen H, Flagel SB, Meyer P, Robinson TE, Solberg Woods LC and Palmer AA (2023) An exponential increase in QTL detection with an increased sample size. *Genetics* **224**.

Cohn CK and Axelrod J (1971) The effect of estradiol on catechol-O-methyltransferase activity in rat liver. *Life Sci I* **10**:1351-1354.

Coker AR, Weinstein DN, Vega TA, Miller CS, Kayser AS and Mitchell JM (2020a) The catechol-O-methyltransferase inhibitor tolcapone modulates alcohol consumption and impulsive choice in alcohol use disorder. *Psychopharmacology (Berl)* **237**:3139-3148.

Coker AR, Weinstein DN, Vega TA, Miller CS, Kayser AS and Mitchell JM (2020b) The catechol-O-methyltransferase inhibitor tolcapone modulates alcohol consumption and impulsive choice in alcohol use disorder. *Psychopharmacology* **237**:3139-3148.

Comasco E, Hellgren C, Olivier J, Skalkidou A and Sundström Poromaa I (2015) Supraphysiological hormonal status, anxiety disorders, and COMT Val/Val genotype are associated with reduced sensorimotor gating in women. *Psychoneuroendocrinology* **60**:217-223.

Cryan JF, O'Riordan KJ, Cowan CSM, Sandhu KV, Bastiaanssen TFS, Boehme M, Codagnone MG, Cussotto S, Fulling C, Golubeva AV, Guzzetta KE, Jaggar M, Long-Smith CM, Lyte JM, Martin JA, Molinero-Perez A, Moloney G, Morelli E, Morillas E, O'Connor R, Cruz-Pereira JS, Peterson VL, Rea K, Ritz NL, Sherwin E, Spichak S, Teichman EM, van de Wouw M, Ventura-Silva AP, Wallace-Fitzsimons SE, Hyland N, Clarke G and Dinan TG (2019) The Microbiota-Gut-Brain Axis. *Physiol Rev* **99**:1877-2013.

de Aguiar Vallim TQ, Tarling EJ and Edwards PA (2013) Pleiotropic roles of bile acids in metabolism. *Cell Metab* **17**:657-669.

de Guglielmo G, Carrette LL, Kallupi M, Brennan M, Boomhower B, Maturin L, Conlisk D, Sedighim S, Tieu L, Fannon MJ, Martinez AR, Velarde N, Othman D, Sichel B, Ramborger J, Lau J, Kononoff J, Kimbrough A, Simpson S, Smith LC, Shankar K, Bonnet-Zahedi S, Sneddon EA, Avelar A, Crook C, Chun L, Vang A, Milan KK, Schweitzer P, Lin B, Peng B, Chitre A, Polleskaya O, Woods LCS, Palmer AA and George O (2023a) Large-scale characterization of cocaine addiction-like behaviors reveals that escalation of intake, aversion-resistant responding,

and breaking-points are highly correlated measures of the same construct. *bioRxiv*:2021.2007.2022.453410.

de Guglielmo G, Kallupi M, Pomrenze MB, Crawford E, Simpson S, Schweitzer P, Koob GF, Messing RO and George O (2019) Inactivation of a CRF-dependent amygdalofugal pathway reverses addiction-like behaviors in alcohol-dependent rats. *Nat Commun* **10**:1238.

de Guglielmo G, Simpson S, Kimbrough A, Conlisk D, Baker R, Cantor M, Kallupi M and George O (2023b) Voluntary and forced exposure to ethanol vapor produces similar escalation of alcohol drinking but differential recruitment of brain regions related to stress, habit, and reward in male rats. *Neuropharmacology* **222**:109309.

Douglas GM, Maffei VJ, Zaneveld JR, Yurgel SN, Brown JR, Taylor CM, Huttenhower C and Langille MGI (2020) PICRUSt2 for prediction of metagenome functions. *Nat Biotechnol* **38**:685-688.

Fabbri M, Ferreira JJ, Lees A, Stocchi F, Poewe W, Tolosa E and Rascol O (2018) Opicapone for the treatment of Parkinson's disease: A review of a new licensed medicine. *Mov Disord* **33**:1528-1539.

Ferslew BC, Xie G, Johnston CK, Su M, Stewart PW, Jia W, Brouwer KL and Barritt ASt (2015) Altered Bile Acid Metabolome in Patients with Nonalcoholic Steatohepatitis. *Dig Dis Sci* **60**:3318-3328.

Funk CK and Koob GF (2007) A CRF(2) agonist administered into the central nucleus of the amygdala decreases ethanol self-administration in ethanol-dependent rats. *Brain Res* **1155**:172-178.

Funk D, Li Z and Le AD (2006) Effects of environmental and pharmacological stressors on c-fos and corticotropin-releasing factor mRNA in rat brain: Relationship to the reinstatement of alcohol seeking. *Neuroscience* **138**:235-243.

Garcia-Carmona JA, Georgiou P, Zanos P, Bailey A and Laorden ML (2018) Methamphetamine withdrawal induces activation of CRF neurons in the brain stress system in parallel with an increased activity of cardiac sympathetic pathways. *Naunyn Schmiedebergs Arch Pharmacol* **391**:423-434.

Gasparini M, Fabrizio E, Bonifati V and Meco G (1997) Cognitive improvement during Tolcapone treatment in Parkinson's disease. *J Neural Transm (Vienna)* **104**:887-894.

Gicquelais RE, Bohnert ASB, Thomas L and Foxman B (2020) Opioid agonist and antagonist use and the gut microbiota: associations among people in addiction treatment. *Sci Rep* **10**:19471.

Gilpin NW and Koob GF (2008) Neurobiology of alcohol dependence: focus on motivational mechanisms. *Alcohol Res Health* **31**:185-195.

Goldman D, Oroszi G, O'Malley S and Anton R (2005) COMBINE genetics study: the pharmacogenetics of alcoholism treatment response: genes and mechanisms. *J Stud Alcohol Suppl*:56-64; discussion 33.

Gonzalez A, Navas-Molina JA, Kosciolek T, McDonald D, Vazquez-Baeza Y, Ackermann G, DeReus J, Janssen S, Swafford AD, Orchanian SB, Sanders JG, Shorenstein J, Holste H, Petrus S, Robbins-Pianka A, Brislawn CJ, Wang M, Rideout JR, Bolyen E, Dillon M, Caporaso JG, Dorrestein PC and Knight R (2018) Qiita: rapid, web-enabled microbiome meta-analysis. *Nat Methods* **15**:796-798.

Grant BF, Saha TD, Ruan WJ, Goldstein RB, Chou SP, Jung J, Zhang H, Smith SM, Pickering RP, Huang B and Hasin DS (2016) Epidemiology of DSM-5 Drug Use Disorder: Results From the National Epidemiologic Survey on Alcohol and Related Conditions-III. *JAMA Psychiatry* **73**:39-47.

Hopf FW and Lesscher HM (2014) Rodent models for compulsive alcohol intake. *Alcohol* **48**:253-264.

Hu M, Crombag HS, Robinson TE and Becker JB (2004) Biological basis of sex differences in the propensity to self-administer cocaine. *Neuropsychopharmacology* **29**:81-85.

Jandhyala SM, Talukdar R, Subramanyam C, Vuyyuru H, Sasikala M and Nageshwar Reddy D (2015) Role of the normal gut microbiota. *World J Gastroenterol* **21**:8787-8803.

Jiang H, Xie T, Ramsden DB and Ho SL (2003) Human catechol-O-methyltransferase down-regulation by estradiol. *Neuropharmacology* **45**:1011-1018.

Jorga K, Fotteler B, Heizmann P and Gasser R (1999) Metabolism and excretion of tolcapone, a novel inhibitor of catechol-O-methyltransferase. *Br J Clin Pharmacol* **48**:513-520.

Kallupi M, Carrette LLG, Kononoff J, Solberg Woods LC, Palmer AA, Schweitzer P, George O and de Guglielmo G (2020) Nociceptin attenuates the escalation of oxycodone self-

administration by normalizing CeA-GABA transmission in highly addicted rats. *Proc Natl Acad Sci U S A* **117**:2140-2148.

Keranen T, Gordin A, Karlsson M, Korpela K, Pentikainen PJ, Rita H, Schultz E, Seppala L and Wikberg T (1994) Inhibition of soluble catechol-O-methyltransferase and single-dose pharmacokinetics after oral and intravenous administration of entacapone. *Eur J Clin Pharmacol* **46**:151-157.

Kim YS, Unno T, Kim BY and Park MS (2020) Sex Differences in Gut Microbiota. *World J Mens Health* **38**:48-60.

Kiraly DD, Walker DM, Calipari ES, Labonte B, Issler O, Pena CJ, Ribeiro EA, Russo SJ and Nestler EJ (2016) Alterations of the Host Microbiome Affect Behavioral Responses to Cocaine. *Sci Rep* **6**:35455.

Koob GF (2008) A role for brain stress systems in addiction. *Neuron* **59**:11-34.

Koob GF (2014) Neurocircuitry of alcohol addiction: synthesis from animal models. *Handb Clin Neurol* **125**:33-54.

Koob GF (2024) Alcohol Use Disorder Treatment: Problems and Solutions. *Annu Rev Pharmacol Toxicol* **64**:255-275.

Koob GF and Volkow ND (2010) Neurocircuitry of addiction. *Neuropsychopharmacology* **35**:217-238.

Leclercq S, Le Roy T, Furgiuele S, Coste V, Bindels LB, Leyrolle Q, Neyrinck AM, Quoilin C, Amadieu C, Petit G, Dricot L, Tagliatti V, Cani PD, Verbeke K, Colet JM, Starkel P, de Timary P and Delzenne NM (2020) Gut Microbiota-Induced Changes in beta-Hydroxybutyrate Metabolism Are Linked to Altered Sociability and Depression in Alcohol Use Disorder. *Cell Rep* **33**:108238.

Lynch WJ (2018) Modeling the development of drug addiction in male and female animals. *Pharmacol Biochem Behav* **164**:50-61.

Martin CR, Osadchiy V, Kalani A and Mayer EA (2018) The Brain-Gut-Microbiome Axis. *Cell Mol Gastroenterol Hepatol* **6**:133-148.

McCane AM, Ahn S, Rubchinsky LL, Janetsian-Fritz SS, Linsenbardt DN, Czachowski CL and Lapish CC (2018a) COMT Inhibition Alters Cue-Evoked Oscillatory Dynamics during Alcohol Drinking in the Rat. *eNeuro* **5**.

McCane AM, Czachowski CL and Lapish CC (2014) Tolcapone suppresses ethanol intake in alcohol-preferring rats performing a novel cued access protocol. *Alcohol Clin Exp Res* **38**:2468-2478.

McCane AM, DeLory MJ, Timm MM, Janetsian-Fritz SS, Lapish CC and Czachowski CL (2018b) Differential COMT expression and behavioral effects of COMT inhibition in male and female Wistar and alcohol preferring rats. *Alcohol* **67**:15-22.

Meckel KR and Kiraly DD (2019) A potential role for the gut microbiome in substance use disorders. *Psychopharmacology (Berl)* **236**:1513-1530.

Mertens KL, Kalsbeek A, Soeters MR and Eggink HM (2017) Bile Acid Signaling Pathways from the Enterohepatic Circulation to the Central Nervous System. *Front Neurosci* **11**:617.

Mihaylova A, Zlatanova H, Doncheva N, Delev D and Kostadinov I (2019) Catechol-o-methyltransferase inhibitor tolcapone improves learning and memory in naive but not in haloperidol challenged rats. *Iran J Basic Med Sci* **22**:695-702.

Ning T, Gong X, Xie L and Ma B (2017) Gut Microbiota Analysis in Rats with Methamphetamine-Induced Conditioned Place Preference. *Front Microbiol* **8**:1620.

Olanow CW and Panel atTA (2000) Tolcapone and Hepatotoxic Effects. *Archives of Neurology* **57**:263-267.

Parvez S, Ismahan G, Raza-Bukhari A and Youdim MB (1978) Activity of catechol-o-methyltransferase in brain regions and adrenal gland during the oestrus cycle. *J Neural Transm* **42**:305-312.

Pita ML and Delgado MJ (2000) Folate administration increases n-3 polyunsaturated fatty acids in rat plasma and tissue lipids. *Thromb Haemost* **84**:420-423.

Rachakonda V, Gabbert C, Raina A, Bell LN, Cooper S, Malik S and Behari J (2014) Serum metabolomic profiling in acute alcoholic hepatitis identifies multiple dysregulated pathways. *PLoS One* **9**:e113860.

Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W and Smyth GK (2015) limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* **43**:e47.

Roberto M, Cruz MT, Gilpin NW, Sabino V, Schweitzer P, Bajo M, Cottone P, Madamba SG, Stouffer DG, Zorrilla EP, Koob GF, Siggins GR and Parsons LH (2010) Corticotropin releasing factor-induced amygdala gamma-aminobutyric Acid release plays a key role in alcohol dependence. *Biol Psychiatry* **67**:831-839.

Schacht JP, Yeongbin I, Hoffman M, Voronin KE, Book SW and Anton RF (2022a) Effects of pharmacological and genetic regulation of COMT activity in alcohol use disorder: a randomized, placebo-controlled trial of tolcapone. *Neuropsychopharmacology* **47**:1953-1960.

Schacht JP, Yeongbin Im, Hoffman M, Voronin KE, Book SW and Anton RF (2022b) Effects of pharmacological and genetic regulation of COMT activity in alcohol use disorder: a randomized, placebo-controlled trial of tolcapone. *Neuropsychopharmacology* **47**:1953-1960.

Schlosburg JE, Whitfield TW, Jr., Park PE, Crawford EF, George O, Vendruscolo LF and Koob GF (2013) Long-term antagonism of kappa opioid receptors prevents escalation of and increased motivation for heroin intake. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **33**:19384-19392.

Sedighim S, Carrette LLG, Venniro M, Shaham Y, de Guglielmo G and George O (2021) Individual differences in addiction-like behaviors and choice between cocaine versus food in Heterogeneous Stock rats. *Psychopharmacology* **238**:3423-3433.

Simpson S, McLellan R, Wellmeyer E, Matalon F and George O (2022) Drugs and Bugs: The Gut-Brain Axis and Substance Use Disorders. *J Neuroimmune Pharmacol* **17**:33-61.

Smith MS, Freeman ME and Neill JD (1975) The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. *Endocrinology* **96**:219-226.

Solberg Woods LC and Palmer AA (2019) Using Heterogeneous Stocks for Fine-Mapping Genetically Complex Traits. *Methods Mol Biol* **2018**:233-247.

Spanagel R (2017) Animal models of addiction. *Dialogues Clin Neurosci* **19**:247-258.

Tammimaki A, Forsberg MM, Karayiorgou M, Gogos JA and Mannisto PT (2008) Increase in free choice oral ethanol self-administration in catechol-o-methyltransferase gene-disrupted male mice. *Basic Clin Pharmacol Toxicol* **103**:297-304.

Temko JE, Bouhlal S, Farokhnia M, Lee MR, Cryan JF and Leggio L (2017) The Microbiota, the Gut and the Brain in Eating and Alcohol Use Disorders: A 'Menage a Trois'? *Alcohol Alcohol* **52**:403-413.

Thistlethwaite LR, Li X, Burrage LC, Riehle K, Hacia JG, Braverman N, Wangler MF, Miller MJ, Elsea SH and Milosavljevic A (2022) Clinical diagnosis of metabolic disorders using untargeted metabolomic profiling and disease-specific networks learned from profiling data. *Sci Rep* **12**:6556.

Traversy G and Chaput JP (2015) Alcohol Consumption and Obesity: An Update. *Curr Obes Rep* **4**:122-130.

Vendruscolo LF, Barbier E, Schlosburg JE, Misra KK, Whitfield TW, Jr., Logrip ML, Rivier C, Repunte-Canonigo V, Zorrilla EP, Sanna PP, Heilig M and Koob GF (2012) Corticosteroid-dependent plasticity mediates compulsive alcohol drinking in rats. *J Neurosci* **32**:7563-7571.

Vendruscolo LF and Roberts AJ (2014) Operant alcohol self-administration in dependent rats: focus on the vapor model. *Alcohol* **48**:277-286.

Wahlstrom A, Sayin SI, Marschall HU and Backhed F (2016) Intestinal Crosstalk between Bile Acids and Microbiota and Its Impact on Host Metabolism. *Cell Metab* **24**:41-50.

Walf AA and Frye CA (2007) The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat Protoc* **2**:322-328.

Wang X, Li L, Bian C, Bai M, Yu H, Gao H, Zhao J, Zhang C and Zhao R (2022) Alterations and correlations of gut microbiota, fecal, and serum metabolome characteristics in a rat model of alcohol use disorder. *Front Microbiol* **13**:1068825.

White AM, Castle IP, Powell PA, Hingson RW and Koob GF (2022) Alcohol-Related Deaths During the COVID-19 Pandemic. *JAMA* **327**:1704-1706.

Wurst FM, Thon N, Yegles M, Schruck A, Preuss UW and Weinmann W (2015) Ethanol metabolites: their role in the assessment of alcohol intake. *Alcohol Clin Exp Res* **39**:2060-2072.

Xia J, Psychogios N, Young N and Wishart DS (2009) MetaboAnalyst: a web server for metabolomic data analysis and interpretation. *Nucleic Acids Res* **37**:W652-660.

Xie G, Wang Y, Wang X, Zhao A, Chen T, Ni Y, Wong L, Zhang H, Zhang J, Liu C, Liu P and Jia W (2015) Profiling of serum bile acids in a healthy Chinese population using UPLC-MS/MS. *J Proteome Res* **14**:850-859.

Xie G, Zhong W, Li H, Li Q, Qiu Y, Zheng X, Chen H, Zhao X, Zhang S, Zhou Z, Zeisel SH and Jia W (2013) Alteration of bile acid metabolism in the rat induced by chronic ethanol consumption. *FASEB J* **27**:3583-3593.

Xie T, Ho SL and Ramsden D (1999) Characterization and implications of estrogenic down-regulation of human catechol-O-methyltransferase gene transcription. *Mol Pharmacol* **56**:31-38.

Yurkovetskiy L, Burrows M, Khan AA, Graham L, Volchkov P, Becker L, Antonopoulos D, Umesaki Y and Chervonsky AV (2013) Gender bias in autoimmunity is influenced by microbiota. *Immunity* **39**:400-412.

Zhang YL, Li ZJ, Gou HZ, Song XJ and Zhang L (2022) The gut microbiota-bile acid axis: A potential therapeutic target for liver fibrosis. *Front Cell Infect Microbiol* **12**:945368.

Zhu BT (2002) Catechol-O-Methyltransferase (COMT)-mediated methylation metabolism of endogenous bioactive catechols and modulation by endobiotics and xenobiotics: importance in pathophysiology and pathogenesis. *Curr Drug Metab* **3**:321-349.