EFFECTS OF ALCOHOL AND FECAL MICROBIOTA TRANSPLANTATION ON CORTICOTROPIN-RELEASING FACTOR

by

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Abstract

The human gut microbiome is composed of trillions of microorganisms that represent a diverse population of microbes in healthy individuals (Ghaisas et al., 2016). Microbial representation in the gut plays an important role in general health and illness. The gut has a special relationship with the brain via the gut-brain axis (GBA), allowing bi-directional communication between the gut and brain. Thus, disruption in the gut can clearly lead to interruptions in the brain and vice versa. For example, alcohol use is known to directly influence the brain through activation of the hypothalamic pituitary adrenal axis leading to the release corticotropin-releasing factor (CRF). However, alcohol consumption can also directly, and indirectly, impact the gut via the gut-brain axis. Due to this, when alcohol consumption persists, CRF becomes dysregulated. Fecal microbiota transplanatation (FMT) has been shown to be successful in rebalancing the gut microbiome, and ultimaltely replenishing the GBA. In an effort to investigate the mechanism that drives the relationship between these factors, the present study examined the effects of alcohol consumption and FMT on CRF expression in the paraventricular nucleus of the hypothalamus and central nucleus of the amygdala. Adult male, Sprague-Dawley rats were exposed to alcohol during a drinking-in-the-dark paradigm. After three rounds of DID, omeprazole was administered to reduce gastric acid secretion and to promote a successful FMT. FMT was administered via oral gavage using fecal samples obtained from either self donations or those from healthy donor rats. Following FMT, the animals were exposed to a two-bottle choice paradigm with water and a 10% ethanol solution for 14 days to measure alcohol craving. Additionally, an elevated plus maze was used to measure anxious behavior in all animals. Brain tissue was collected, sliced, and stained using immunohistochemistal techniques. CRF was quantified using densotimetry. A factorial ANOVA revealed no significant effects of alcohol and FMT on CRF stain density in the PVN of the hypothalamus or the CeA. An unexpected

significant cohort effect was observed. Though results did not yield significant results, it underscores the necessity of continuing to investigate the complicated mechanisms involved in alcohol consumption, FMT, and CRF.

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Chapter 1. Introduction

Gut Microbiome

The human microbiome is composed of millions of microorganisms living on and in the human body, and it contributes to a multitude of bodily functions. The human microbiome consists of different ecosystems, such as the skin microbiota; however, in comparison to other sites on the body, the microbiota in the gut appears to be the most diverse among healthy individuals (Human Microbiome Project Consortium, 2012). The gut microbiota is estimated to consist of 100 trillion microbes (Ghaisas et al., 2016). The abundant community of microorganisms living in the human gastrointestinal tract is suggested to encode about 150 times more unique genes than the human genome (Qin et al., 2010). Gut microbes greatly outnumber the human cells in the gut and play a significant role in the development, physiology, and overall health of the human host (Qin et al., 2010). Due to individual differences in birth, environment, and diet, the gut displays variation in microbe composition between individual hosts (Quigley, 2017).

Despite the differences in bacterial species between individual hosts, a healthy adult gut microbiota will generally host the same class of microorganisms. More than 1,000 species of bacteria, most of which belong to the phyla, *Bacteroidetes* and *Firmicutes* will inhabit a healthy adult gut microbiota. The different functions occupied by the microorganisms in the human gastrointestinal tract hold a pivotal role in the general health of the host. A healthy human gut microbiota contributes to metabolic functions by aiding in the digestion of fermented insoluble polysaccharides and fibers, thus retrieving nutrients the host otherwise would be unable to receive (Berry, 2016; Johnson et al., 2016; Schreiner et al., 2015). Additionally, the gut microbiota and immune system share a bi-directional relationship where the microbiota can influence immune homeostasis and in turn, the immune system regulates the community

structure and function of the microbiota (Kamada & Nunez, 2015; Mittal et al., 2017). By educating the immune system to differentiate bacterial species that promote anti-inflammatory activity, the gut microbiota can regulate the development of important immune cells necessary for the body's defense to pathogens (Schreiner et al., 2015). Therefore, if specific bacterial species are necessary to facilitate immune homeostasis, the immune system has the capability to impact the composition of the microbiome. Furthermore, specific microorganisms (i.e., *Candida)* also contribute to the synthesis of essential neurotransmitters (i.e., serotonin) in the gut that have the potential to travel to the central nervous system (Heintz-Buschart & Wilmes, 2018). With the ability to travel from the gut to the brain, neurotransmitters can either positively or negatively affect the brain and subsequently alter mood and behavior (Mittal et al., 2017).

The Gut-Brain-Axis

The human nervous system is divided into two major divisions, the central nervous system (CNS) and the peripheral nervous system (PNS). Collectively, the nervous system functions to collect, integrate, process, interpret, and respond appropriately to sensory information from the body and external environment. However, the gut is unique in that it has its own intrinsic nervous system called the enteric nervous system (ENS). The ENS was exclusively known for its role in both small and large intestinal function (Furness et al., 2014), but more recently, research has shown a pivotal connection between the ENS and CNS that allows the gut and brain to communicate (Lerner et al., 2017; Mittal et al., 2017). The pathway of communication between the gut and brain has been termed the gut-brain axis (GBA) and has revealed the bidirectional influence they are capable of imposing on one another. Discovery of the GBA was imperative because it links cognitive and emotional areas in the brain with gastrointestinal functions.

Consequently, the discovery of a bidirectional system between the gut and brain led to the investigation of the mechanism through which the gut could exert influence on the CNS (Carabotti et al., 2015). The GBA may utilize two different pathways for communication between the brain and gut: (1) using the autonomic nervous system (ANS) and the vagus nerve to communicate via the spine or (2) through the bidirectional pathway where the exchange of information to and from the gut occurs between the ENS and the ANS and vagus nerve (Wang & Wang, 2016). Consequently, with an ease of communication within the GBA, the gut microbiota has the potential to affect cognitive, behavioral, and physiological function due to its influence on the GBA (Wang & Wang, 2016). For example, if the gut is experiencing a lack of a *Candida* population to produce serotonin, serotonin neurotransmission will decrease, and less serotonin will travel from the GBA to the brain. A reduced level of serotonin in the central nervous system will lead to negative changes in mood, sleep, and behavior.

The bacterial composition of the microbiota is vital in maintaining metabolic balance in the body. Specific microorganisms in the gut are associated with the synthesis of specific corresponding neurotransmitters, thus allowing the gut microbiota to manage pathophysiological effects often displayed as a result of the GBA. For example, γ-aminobutyric acid (GABA) is produced by the bacteria *Lactobacillus brevis* and *Bifidobacterium dentium* in the gut (Kim & Shin, 2018). Despite GABA's notorious inhibitory role in the CNS, the neurotransmitter has an excitatory function in the ENS, specifically through the $GABA_A$ receptor (Seifi et al., 2014). GABAergic signaling in the gut is known for its role in moving food from the stomach to the ileum, but GABA neurotransmission also influences other body systems. Through the activation of $GABA_A$ receptors, research has found that GABA may encourage necessary immune responses against antigens, such as T cells (Mittal et al., 2017; Tian et al., 1999). As a

neurotransmitter with a variety of purposes, GABAergic signaling is also implicated in the prevention and onset of anxiety disorders. Research suggests that low GABA activity in the central nervous system, among other things, may result in anxiety (Nuss, 2015). $GABA_A$ receptors in the amygdala have allosteric sites that allow other molecules to either activate or deactivate the effects of the receptor. GABA synthesized in the gut can project its effects on the brain and possibly contribute to the development of anxiety (Cryan et al., 2005). Therefore, if GABA synthesis in the gut is impacted, it will likely influence the brain, and may ultimately create serious implications for the development of anxiety.

In addition, 5-hydroxytryptamine (5-HT), or serotonin, is also produced in both the ENS and CNS. When produced in the CNS, 5-HT is synthesized in serotonergic neurons and can affect mood and sleep; however, when 5-HT is created in the gut, it is produced by enterochromaffin cells, mucosal mast cells, and myenteric neurons (Banskota et al., 2018). Ninety-five percent of total 5-HT in the body can be found in the gut, while the remaining 5% can be found in the brain (Yano et al., 2016). Gut-derived 5-HT has the capability to bind to 14 different 5-HT receptor subtypes found on the immune cells (Baganz & Blakely, 2013), enteric neurons in the gut (Mawe & Hoffman, 2013), and enterocytes (Hoffman et al., 2012). When released in the gut, 5-HT is involved in immune responses to inflammation and pathogens (Baganz & Blakely, 2013), gastric secretion in preparation for the digestion of food (O'Mahony et al., 2015), and enteric motility (Yano et al., 2016). However, due to 5-HT traveling to the brain via the GBA, serotonergic malfunction in the gastrointestinal system can lead to complications in the brain such as unregulated mood, impaired sleep, and behavioral issues (Berger et al., 2009; Delgado et al., 1990), but it can also cause chaos in the gut. With 5-HT receptors located on immune cells and being involved in immune responses, anything that

creates serotonergic dysfunction in the gut can lead to intestinal inflammatory diseases such as Crohn's disease, celiac disease, and ulcerative colitis (Cloez-Tayarani & Changeux, 2007; Shaw et al., 2010).

Influence of an Unbalanced Microbiota

Therefore, a well-balanced gut microbiota is vital for maintaining functional neurotransmission of various signals that are essential in regulating immune responses and implicated in the pathophysiology of various diseases. An imbalanced microbiota extends its effects beyond the gut to the brain, the immune system, and the endocrine system. Due to the multi-system influence of the gut microbiota, it is vital to understand what can and will cause an imbalance of microbes in the gut, otherwise known as gut dysbiosis. Numerous factors can contribute to gut dysbiosis, such as significant diet alterations, stress, and alcohol consumption. Research has shown that, depending on microbial representation in an individual's gut, diet can either be beneficial or detrimental to gut health. For example, a high-fat diet will significantly decrease the number of bacteria associated with healthy metabolism, such as *Akkermansia muciniphila* and *Lactobacillus* (Singh et al., 2017).

In addition, psychological, environmental, and physiological stressors may also affect gut dysbiosis by disrupting the microbiota homeostasis and signaling to the body's stress response system to mediate the dysbiosis. Ultimately, these stressors will disrupt the lining of epithelial cells in the gastrointestinal wall, and encourage systemic inflammation by releasing molecules from the gut into blood circulation, thus altering the composition of the microbiota (Karl et al., 2018). For example, alcohol consumption is considered a physiological stressor to the body and may elicit inflammation to the gut by altering the permeability of the intestinal mucosal barrier and making it likely that important components of bacteria, such as their metabolites, will escape

the gut (Engen et al., 2015). Unfortunately, dysbiosis has implications for many negative consequences, such as mental health disorders (Temko et al., 2017). As a result, dysbiosis will not only affect the gastrointestinal system but invite a host of possible diseases. Therefore, understanding the potential factors responsible for gut dysbiosis is vital to identify the illnesses that can result from such inflammation. With easy accessibility and prevalence of consumption, alcohol is an important culprit in gut dysbiosis that must be examined.

Alcohol

According to the 2017 report from the National Survey on Drug Use and Health, 56% of people 18 and older in the United States reported consuming alcohol in the last month (SAMHSA, 2017). Alcohol can be found in different forms, such as beer, wine, and liquor, with varying ethanol percentages across the different forms. A standard drink contains 0.6 ounces of pure alcohol and can generally be found in 12 ounces of beer, 5 ounces of wine, and 1.5 ounces of 80 proof distilled spirits (Center for Disease Control and Prevention, 2018). Alcohol is an available, accessible, and frequently used drug with dose-dependent toxic effects (McIntosh & Chick, 2004).

Dose, duration, and pattern of use are important factors for defining alcohol consumption. When alcohol is consumed in low dosages, it can produce a blood alcohol concentration (BAC) of up to .05% that may result in short term anxiolytic effects. However, as alcohol consumption increases, BAC levels will also increase, causing symptoms to become progressively worse and increase the risk for impaired judgement and speech, vomiting, and, at worse, death (South Australia Health, 2019). Moreover, alcohol is a central nervous system depressant and may likely produce impairments in balance, cognitive reasoning, and motor incoordination. Increases in dose and duration of alcohol consumption may lead to a series of health illnesses, such as

alcoholic liver disease, compromised immune function, and gastrointestinal inflammation (Bishehsari et al., 2017; Purohit et al., 2008; Szabo & Mandrekar, 2009). With a capacity to influence multiple systems in the body, the pattern of use in which people consume alcohol can affect gastrointestinal permeability, resulting in a leaky gut.

After alcohol is absorbed in the gastrointestinal tract, it is metabolized by the liver. The breakdown of alcohol metabolites differs slightly based on the level of alcohol consumption. Within normal social drinking limits, often described as an average of two drinks, liver cells have no difficulty breaking down the contents of alcohol (Bishehsari et al., 2017). Typically, when consumed in this manner, alcohol is eliminated by way of the aldehyde dehydrogenase pathway. Alcohol is broken down by alcohol dehydrogenase, an enzyme, and turns into a toxic substance, acetaldehyde. Then, aldehyde dehydrogenase metabolizes acetaldehyde into acetate, which is then excreted via the kidneys as waste (Edenberg, 2007). However, when alcohol is ingested frequently and in large amounts, it activates the microsomal ethanol-oxidizing system (MEOS) to help metabolize alcohol (Lieber, 1999). MEOS breaks down alcohol similarly to the aldehyde dehydrogenases pathway except that it uses the help of an enzyme, cytochrome P450 2E1, to turn alcohol into acetaldehyde (Wallner & Olsen, 2008). The activation of this system may lead to intestinal issues because the MEOS releases oxygen free radicals, which can elicit cell damage due to similar intestinal enzymes involved in oxidative metabolism of alcohol (Cederbaum, 2012). Therefore, the breakdown of alcohol can also occur in the intestines, compromising intestinal permeability and creating susceptibility to gut dysbiosis.

Alcohol in Psychology

Alcohol consumption and the effects thereof have been extensively explored in psychology with a large portion of studies utilizing animal models. Scientists have investigated

alcohol consumption through a variety of ways, such as forced consumption models, operant conditioning, and voluntary consumption models, among others. As a forced consumption model, vapor chambers utilize vaporized ethanol intermittently released into an airtight sealed chamber where the animal will inhale the content being released. Despite its use for achieving extremely high levels of alcohol intake, the level of consumption is based on the experimenter, which lacks validity for any human drinking situation (Kang et al., 2004; O'Dell et al., 2004). In addition, operant conditioning seeks to examine cue-induced drug-seeking behaviors (Spanagel, 2017). This approach allows researchers to assess drug-seeking habits in drug addiction; however, it fails to capture the social drinking phenomenon displayed in human behavior.

The previous models are ideal for assessing extremely high levels of alcohol consumption, but they lack accurate representation of human drinking patterns typically observed in social settings. The best known voluntary consumption paradigm for modeling social drinking in humans is the drinking-in-the-dark (DID) paradigm originally described by Rhodes and colleagues (2005). In this model, water bottles are replaced with 20% ethanol solutions for 2-4 hours beginning at 3 hours into the advent of the dark phase of the light/dark cycle. The DID paradigm forces the increased consumption of alcohol beyond the BAC levels that rats will normally drink. The paradigm takes advantage of the fact that rats are nocturnal and display frequent periods of high energy during the night, and thus are willing to consume more alcohol (Thiele & Narvarro, 2015). The DID paradigm is unique from other models because it possesses high validity for typical human drinking situations. Animals are given the choice to drink or avoid the ethanol solution, while in most other models, alcohol is administered by an experimenter, which may cause more stress to the animal and serve as a confounding variable in the experiment.

Similarly, social drinking in humans is typically voluntary; humans can decide what they will drink, how much they will consume, and how frequently they will participate in alcohol consumption. Human drinking in typical social settings can elicit high enough levels of blood alcohol concentration to affect the GBA via activation of the HPA axis and leaking of microbes from the gut. Therefore, when gut disruptions negatively impact the GBA, there are serious implications for alcohol's effects on the brain and mental health disorders. As a result, the current study examined voluntary, limited access of alcohol consumption because it is most closely representative of human social drinking and will likely induce disruptive activity in the GBA, as opposed to the free access consumption of alcohol (two-bottle choice). Alternatively, in the free access consumption of alcohol model, animals are more likely to not consume enough alcohol to create gut dysbiosis.

The Stress Response System and Corticotropin-Releasing Factor (CRF)

Alcohol consumption will compromise the microbiome by placing significant stress on the gut. The dysbiotic state of the gut results in increased permeability of the intestinal walls, often coined "leaky gut syndrome." Increased permeability may allow inflammatory molecules to flow out of the gut and, in extreme cases, infiltrate systemic circulation (Clapp et al., 2017). In addition, bacteria metabolites may be found floating throughout the blood and interacting with other systems of the body. Due to comprised permeability, an increased presence of foreign molecules impacts the gut and reinforces the weakened intestinal walls. This provides compromised access through the blood brain barrier (BBB), allowing molecules to invade the brain. Foreign molecules traveling systemically cause an inflammatory response, alerting the immune system, and subsequently releasing cytokines and neurotransmitters from the gut into the blood and ultimately across the BBB into the brain (Clapp et al., 2017).

Inflammation in the gut will activate the Hypothalamic Pituitary Adrenal (HPA) axis, which is responsible for the body's stress response. The HPA axis is part of the limbic system and is activated by environmental stress and pro-inflammatory cytokines (Carabotti et al., 2015). For example, the inflammation induced by the regular consumption of alcohol will prompt the release of pro-inflammatory cytokines, creating more inflammation, which activates the HPA axis. When the HPA axis is alerted, corticotropin-releasing factor (CRF) from the hypothalamus will be released and initiate the delivery of adrenocorticotropic hormone (ACTH) from the pituitary gland to the adrenal gland. ACTH will prompt the adrenal glands on the kidney to release cortisol, a major stress hormone that affects the entire human body. If the stress remains, cortisol will continue to be released and commence a positive feedback or hyper-reactivity of the HPA axis (Muscatello et al., 2014). As a result, the inflammation in the gut will persist and the HPA axis and immune system will become overactive in an attempt to correct the inflammation. Therefore, the overactive HPA axis and comprised immune health will leave the human body susceptible to illnesses.

Immune Responses and CRF

As an important component of the HPA axis, CRF is essential in the immune system's attempt to mediate inflammation. CRF is a 41 amino acid peptide released from the hypothalamus during the activation of the body's stress response system (Risbrough & Stein, 2006). When released, CRF targets three major areas: the anterior pituitary gland, immune cells, and the gut. CRF also signals to specific cells in the immune system to release anti-inflammatory cytokines, such as interleukin-4 (IL-4), to regulate and mediate the inflammatory response elicited by pro-inflammatory cytokines (Zhang & An, 2007).

Cytokines are proteins released by cells specific to the immune system and signal to either pro-inflammatory or anti-inflammatory molecules (Zhang & An, 2009). Cytokines, such as interleukin-6 (IL-6), released during inflammatory responses are proinflammatory, therefore increasing inflammatory reactions from the immune system. The ratio of anti- to pro-inflammatory cytokines may determine whether the cytokines will help resolve a disease or contribute to its development. Elevated levels of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and tumor necrosis factor (TNF-α) seem to be negatively linked with the risk of developing diseases such as inflammatory bowel disease (IBS), depression, and autism spectrum disorder (Ashwood et al., 2011; Foster & McVey Neufeld, 2013; Maes et al., 2002; Potvin et al., 2008;). For example, a study conducted by Gao observed the relationship between the anxiety-depression status and cytokines in a small diarrhea-predominant IBS (IBS-D) sample and suggested that IL-1β levels increase while anti-inflammatory cytokine levels, such as IL-10, decrease in patients with anxiety-depression IBS-D (Gao, 2013).

Moreover, research regarding depression and pro-inflammatory markers seems to suggest that increased pro-inflammatory cytokines are found circulating in the blood in patients with idiopathic major depression in comparison to those without depression (Sluzewska et al., 1996). Elevated levels of pro-inflammatory cytokines have also been suggested in autism spectrum disorder (ASD), specifically in ASD patients who express exacerbated symptoms (Ashwood et al., 2011). Due to CRF initiating the release of cytokines, it is important to consider the consequences of a hyperactive HPA axis and the release of inflammatory markers, such as mental health illnesses and diseases. For instance, research has shown that CRF is relevant in alcohol dependence in that CRF signaling in the amygdala helps create negative emotionality-induced drinking (Heilig & Koob, 2007). With a high comorbidity rate with alcohol use

disorders, depression and anxiety are also indicated to be influenced by the CRF system. Moreover, CRF has been shown to signal to brain areas other than the hypothalamus and amygdala, such as the locus coeruleus, and pontine nuclei, which all have expressed high CRF neurotransmission in postmortem major depression and suicide cases (Binder & Nemeroff, 2010). The study of CRF is vital as it assumes a multitude of roles in mental health illnesses and diseases that may provide insight into effective treatment or prevention measures.

Fecal Microbiota Transplantation (FMT)

Fecal microbiota transplantation (FMT) is the use of healthy human feces for transplantation to an individual with gut dysbiosis for microbiota regulation (Kim & Shin, 2018). FMT can be administered via multiple routes, such as oral, nasogastric, rectal, and more (Sarin et al., 2019). FMT has demonstrated the capability to restore the composition of microbes and functionality of the gut microbiome (Khoruts &Sadowsky, 2016). FMT has a history of successful treatment outcomes in *Clostridium difficile (C.diff) infection* and inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis in humans (Wilson et al., 2019). *C.diff* is a pathogen in the gut that is suppressed when the microbiota expresses diverse representation of microbes; however, when the microbiota is not diverse, *C.diff* will take over the gut and release enterotoxins that will create inflammation. *C.diff* cases using FMT as a treatment have reported a cure rate of 92% across 30 *C.diff* cases (Quraishi et al., 2017). FMT is quickly on the rise as regulations for its use are being formulated and approved in many countries (Tsai et al., 2019). Research also shows FMT as an effective treatment for illnesses, such as IBS, providing a remission rate of 36-89% after FMT treatment (Evrensel & Ceylan, 2016).

More importantly, FMT helps restore the gut microbiota by regulating the bacterial community. FMT treatment is significant because it completely changes the gut microbiota into

that of the healthy donor. FMT recipients experience a significant increase in microbiota diversity, which aids in ultimately reducing inflammation in the gut by making the HPA axis become less hyperactive. It is possible that when FMT recipients receive a new gut, they will no longer experience inflammation and a comprised HPA axis and immune system. Essentially, FMT re-establishes the gut microbiota, stabilizes the HPA axis and immune system, and influences the GBA. Due to the bidirectional relationship in the GBA, the newly established gut holds promise for FMT improving mental health and diseases as well.

Current Study Overview

The present study examined alcohol conditions (alcohol or water) and FMT treatment (donor or self) on CRF levels in the paraventricular nucleus of the hypothalamus (PVN) and central nucleus of the amygdala (CeA). Based on previous literature, it is known that alcohol contributes significantly to gut dysbiosis by the increased permeability of the intestinal walls that result in a leaky gut. After leaving the gut, molecules flow into systemic circulation and elicit a cascade of changes, resulting in signaling to immune cells in the gut due to the presence of foreign molecules in the blood. Thus, the HPA axis activation acts as an attempt to counteract the inflammation in the gut by releasing various chemicals, such as CRF, that initiate anti-inflammatory responses. However, due to persistent alcohol consumption, the HPA axis becomes hyperactive and continues to release CRF. The continuous release of CRF signals the release of pro-inflammatory cytokines in the immune system, subsequently reinforcing gut dysbiosis. As a result, successful FMT treatment rates provide evidence for FMT as a resolution to re-establishing the gut by replenishing the gut microbiota, thus regulating the functionality of the HPA axis by decreasing inflammation.

Consequently, the current study observed the effects of alcohol conditions and FMT treatment on CRF levels in the PVN of the hypothalamus and CeA. The levels for the alcohol variable were as follows: alcohol-exposed group (experimental) and a non-alcohol-exposed group (control). In addition, the levels for the FMT variable were as follows: FMT from healthy donor (experimental) and FMT from self (control).

The study focused on CRF levels in both the PVN of the hypothalamus and CeA due to the increased presence of CRF signaling in these regions and the implications of alcohol use and its comorbidities within these brain regions. The present study proposed the following hypotheses: (1) It was hypothesized there would be a main effect of alcohol on CRF levels, such that the animals in the alcohol-exposed conditions would exhibit higher levels of CRF expression in the PVN of the hypothalamus and CeA than the animals who were not exposed to alcohol. (2) Also, it was predicted there would be a main effect of FMT on CRF, such that among animals in the alcohol-exposed condition, those who received FMT from a donor would express lower levels of CRF expression in the PVN of the hypothalamus and CeA than animals who received FMT from self. (3) Finally, it was hypothesized there would be an interaction between alcohol and FMT on CRF levels in the subregions within the hypothalamus and amygdala such that CRF expression would be higher in the alcohol-exposed animals who received FMT from self than the alcohol exposed animals who received FMT from self.

Chapter 2. Methods

Subjects

Fifty-four adult, male, Sprague-Dawley rats were bred in-house from rats originally obtained from Charles River Laboratories. The animals were housed in standard rodent cages in a temperature- and humidity-controlled vivarium. Lights in the room were controlled by a timer to establish a light/dark cycle with lights on for 12 hours. Animals were allowed free access to standard rodent chow and water except where noted. Cages were changed according to federal regulations of best practice (approximately weekly). Six of the 54 animals were used as fecal donors and group housed in large plastic tubs, three rats per tub, while the experimental animals were individually housed. The 48 experimental animals were semi-randomly assigned to one of the following conditions: (1) ethanol-exposed who received FMT from healthy donor, (2) ethanol-exposed who received FMT of their own fecal matter, (3) water only with FMT from healthy donor, and (4) water only who received FMT from their own fecal matter. One animal was removed from the study due to fatal internal injuries during the oral gavage procedure. Due to experimental limitations (i.e., time and laboratory equipment), subjects were randomly assigned to one of two cohorts for experimental tasks, which was not expected to influence results.

Measures

Initial Ethanol Exposure. The animals underwent a repeated 4-day drinking-in-the-dark (DID) paradigm modified from Rhodes and colleagues (2005). The well-established paradigm is a limited access model of consumption that utilizes the fact that rodents are most active nocturnally in order to artificially increase levels of voluntary consumption observed in rodents (Rhodes et al., 2005). Animals allowed free access to ethanol do not typically achieve more than

a .08 blood ethanol concentration (BEC) (Thiele & Navarro, 2014) and, as such, models like DID are required to model the human social drinking phenomenon. Therefore, beginning 3 hours into the dark cycle, all animals in the ethanol condition had their water bottles replaced with a new bottle containing 20% ethanol while under red lights. On days 1-3, animals received access to the ethanol for 2 hours. However, during the fourth day of the ethanol treatment, the animals were allowed 4 hours of alcohol exposure. This additional time is crucial for the escalation of alcohol consumption. After the ethanol exposure time periods, all bottles were collected and replaced with their original bottle of water. Following the 4-day DID paradigm, the animals experienced a 3-day abstinence period where they had no access to ethanol. The DID paradigm occurred in three repetitions with a longer abstinence period occurring during trial 2 due to experimental complications (repetition 1 = 4 days on, 3 days off; repetition 2 = 4 days on, 5 days off; repetition 3 = 4 days on, 3 days off). The animals in the water only condition did not undergo the DID paradigm, but they received a fresh bottle of water every time the ethanol-exposed animals received ethanol. The total amount of liquid consumed per animal was recorded, and for ethanol drinking it was converted to grams per kilograms (g/kg).

Fecal Microbiota Transplantation. Half of the animals in the ethanol-exposed condition received an FMT using a donor sample and the other half received an FMT using their own fecal samples. All FMTs were performed the day after completion of the final omeprazole administration. In preparation for fecal donations, all animals were given fresh cages approximately 6 hours before donations occurred and fecal matter was collected from the new containers and used fresh. The FMT stool suspension was prepared by mixing of 2 g (within 0.05 g) of assigned fecal matter with 10 mL of non-preserved, 0.9% saline solution. The mixture was filtered through a gauze pad to help eliminate larger particles. Each rat received 5 ml of their

designated FMT suspension (donor or self) through oral gavage. To properly administer the FMT, the animal's head was immobilized by restraining the animal's shoulders, head, and neck. The stool suspension was administered using a 16-gauge, 4-inch-long metal cannula inserted into the mouth down the esophagus and into the stomach. All animals were assessed for medical complications at 30 minutes, 1 hour, and then 3-4 hours post oral gavage.

Omeprazole Administration. In order to increase the likelihood of bacterial survival following the FMT, gastric acid secretions were reduced in the stomach by administering 50 mg/kg over-the-counter omeprazole suspended in 50% Ensure solution to all experimental animals. The omeprazole administration occurred once a day for 3 consecutive days during the final trial's abstinence period.

Ethanol Craving. A two-bottle choice voluntary consumption paradigm was utilized to measure ethanol craving because higher ethanol consumption is indicative of higher levels of ethanol craving. All animals in the experimental conditions were given free access to two bottles for 14 days: one bottle containing water and the other bottle containing a 10% v/v ethanol solution. The bottles were weighed every 24 hours to determine the amount of liquid consumed from each bottle. Grams per kilograms of ethanol consumed by each animal was then calculated.

Behavorial Testing. Three days after the completion of ethanol craving testing, all animals were tested for anxiety-related behaviors using the Elevated-Plus-Maze (EPM). This maze has four long arms: two open arms and two enclosed arms that intersect in a center square. If an animal spends more time in one type, as opposed to the other, this is indicative of its anxiety levels. When an animal spends more time in the enclosed arms, as opposed to the open arms, it is considered anxious.

Tissue Collection and Preparation

All animals were sacrificed in accordance with the IACUC-approved protocol using sodium pentobarbital (150 mg/kg in Fatal Plus solution) injection as an anesthetic overdose. Then, the animals received transcardial perfusion using 0.1 M phosphate buffered saline (PBS) followed by a 4% paraformaldehyde. The brains were extracted and post-fixed in 4% paraformaldehyde for 24 hours then transferred to 0.1 M PBS for storage, both at 4°C. The brain tissue was sliced into 40 µm coronal sections in a 1:8 series using the Leica VT1000 S vibratome machine. The start and stop points for tissue collection were determined using the second edition of the Rat Brain in Stereotaxic Coordinates atlas (Paxinos and Watson, 1986). Collection started at the striatum (bregma 2.20 mm) and finished at the end of the posterior end of the dorsal hippocampus (bregma -6.04 mm), yielding approximately three quarters of the entire brain being collected. The sliced brain tissue was stored in cryoprotectant at -20 °C until staining.

Immunohistochemistry (IHC) and Quantification

To examine CRF levels in the paraventricular nucleus of the hypothalamus (PVN) and central nucleus of amgydala (CeA), the brain tissue was rinsed in tris-buffered saline (TBS) three times for 5 minutes to remove any traces of cryoprotectant, followed for 30 minutes by incubation in 0.6% of 30% hydrogen peroxide (H_2O_2) to block endogenous peroxidases. The tissue underwent a second round of wash in TBS three times for 5 minutes each. Then, the tissue was incubated in blocking solution (3% normal horse serum, 0.1 % triton, and TBS) for 30 minutes, and before being transferred to blocking solution plus primary rabbit anti-CRF antibody (1:200; Peninsula Laboratories) at 4 °C for 4 days.

Following 4 days of incubation of anti-bodies, tissue underwent three, 10-minute washes in the blocking solution. Then, the tissue was incubated in biotinylated goat anti-rabbit secondary

antibodies (1.5% normal goat serum, goat anti-rabbit {1:200} and TBS) for 1 hour. The tissue was rinsed in TBS again and placed in avidin-biotin complex solution (ABC) kit for 1 hour. After the third round of TBS washes, the tissue was stained using 3,3'-diaminobenzidine (DAB) with cobalt chloride, nickle ammonium sulfate, and 30% H_2O_2. The tissue was rinsed for the final time in TBS, then stored in TBS at 4 °C. The tissue was mounted onto glass slides and coverslipped using cytoseal. The experimenter was made blind to treatment condition by covering the animal ID with tape to prevent experimenter bias. The slides were stored until quantification.

CRF expression in the subregions of the hypothalamus and amygdala was examined at 40 or 100 times magnification respectively using an Olympus BX43 microscope with the Olympus U-TV1XC camera. Q-capture camera software was utilized to obtain images of the target regions and Image J software allowed for densitometric analysis of CRF staining. See Figure 1 for complete procedural timeline.

Denistometry Criterion

Images of the PVN and CeA were captured for both the left and right side of the brain using parameters stated above. Using Image J, a threshold technique was applied to differentiate between foreground and background pixels. After thresholding, the correct anatomical subregion of interest was outlined from the broader hypothalamus and amygdala photos. The area and density measurements were collected. The area calculation referred to the size of the zone outlined on each image. Therefore, a non-significant difference in area indicates that the size of the zone selected remained consistent per image. In addition, the density represents the difference between true staining and background. The delineated area on the left and right side of each subregion was averaged for each animal. See Figure 2 for picture array displaying how the PVN and CeA zones were selected and outlined for densitometric calculations.

Statistical Analyses

The current study used a 2 x 2 x 2 factorial ANOVA to examine the effects of alcohol treatment (alcohol or water), FMT treatment (donor or self), and cohort (first or second) on CRF levels in the PVN of the hypothalamus and CeA. Statistical analyses were conducted to examine main effects and interactions among the aforementioned variables within the PVN and CeA for both area and density.

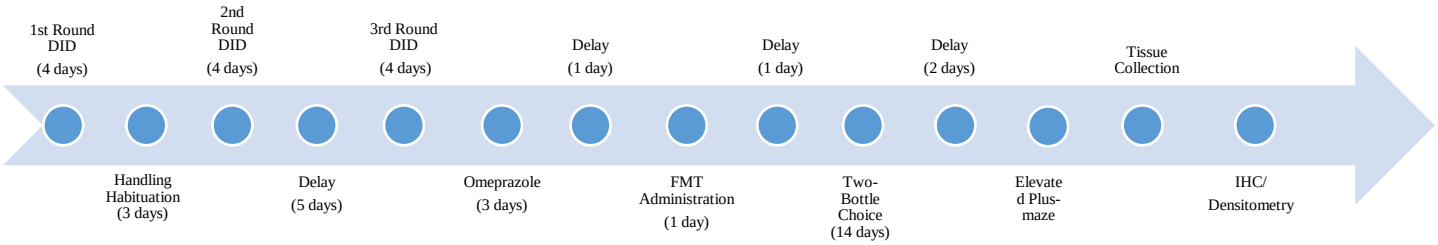

Figure 1. This figure displays the procedure timeline of the present study.

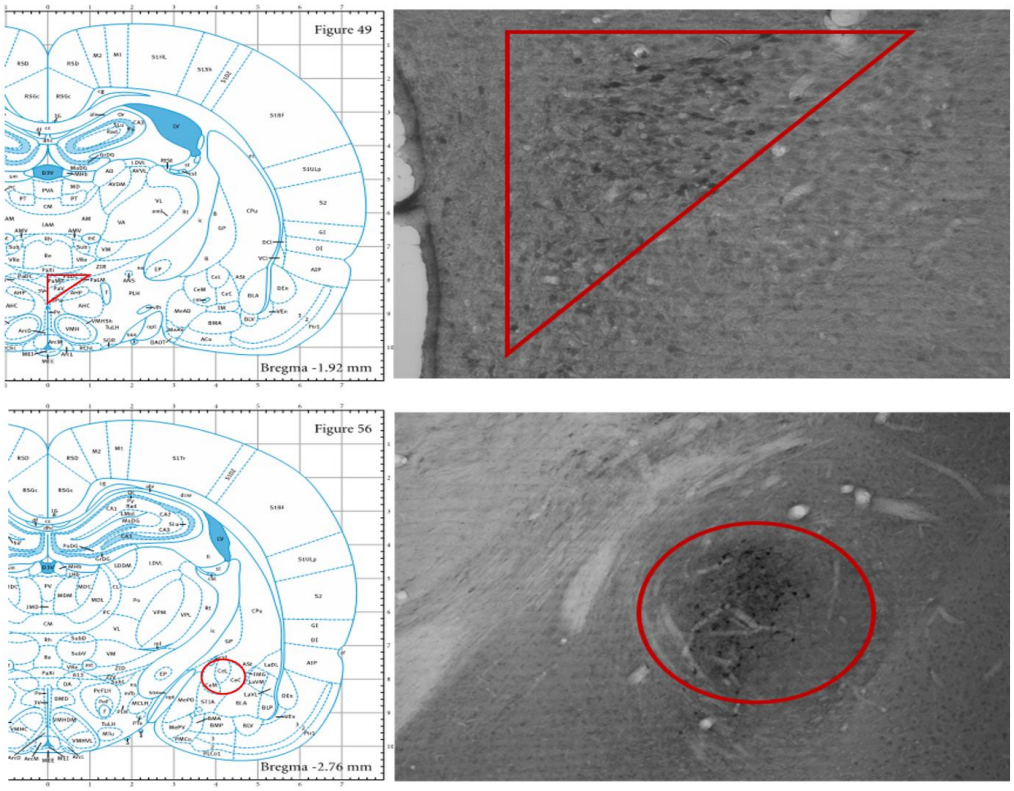

Figure 2. Left: The images on the left are of the PVN of the hypothalamus (top) and CeA (bottom) from the Paxinos and Watson atlas. The images on the right reveal staining in the PVN of the hypothalamus (top) and CeA (bottom) from the current study. It also illustrates the manner in which the targeted areas were outlined.

Chapter 3. Results

General Subject Data

Behavorial data was collected from 48 adult, male Sprague Dawley rats. Prior to IHC techniques, one subject was removed from the study due to gavage complications. Additionally, four more subjects were excluded from the staining process due to the inability to achieve complete and consistent tissue slices. These brain samples were determined to be unusable due to their shredded composition, likely resulting from brain extraction or tissue slicing. As a result, a total of 43 brains were utilized to conduct IHC techniques and densitometry. Despite the removal of subjects in the study, the number of animals in cohort 1 (N = 21) and cohort 2 (N = 22) remained generally balanced.

The remaining 43 subjects ranged in age from PND 263 to 338 (approximately 8-11 months; M = 292.44, SD = 21.14) and ranged in body weight from 630 to 1,008 grams (M = 850.23, SD = 92.71) at the beginning of the study. A 2 x 2 x 2 factorial analysis revealed no significant differences in body weight across variables. However, a similar analysis conducted on the age variable revealed a significant difference in age among cohorts, (F (1, 42) = 102.80, p = .00, η_p^2 = .75). The age difference between cohorts is a result of the first cohort needing to complete all testing prior to cohort 2 starting testing, yielding a 35.58 day age difference. Both cohorts had subjects well within the adult range for Sprague Dawley rats; therefore, the differences in age should have little to no impact on the current study (Sengupta, 2013). See Table 1 for mean and standard deviations for age, body weight, and ethanol consumed during the DID paradigm separated by cohort.

Table 1. Mean & SEM for Age, Weight, & Ethanol.

Cohort	***Age***		***Body Weight***		***Ethanol Consumed***	
	M	*SEM*	*M*	*SEM*	*M*	*SEM*
1	274.24	2.45	840.57	16.78	.27	.06
2	309.82	2.40	859.45	22.74	.54	.11
Total	292.44	3.22	850.23	14.14	.41	.07

Analysis of the Paraventricular Nucleus of Hypothalamus

An alcohol consumption (alcohol or water) by FMT (donor or self) by cohort (first and second) factorial ANOVA was performed to examine consistency of size of area selected in the PVN. As expected, this analysis failed to reveal any significant main effects of alcohol treatment (F (1, 27) = 1.05, p = .32, η_p^2 = .04), FMT treatment (F (1, 27) = .11, p = .75, η_p^2 = .00), or cohort (F (1, 27) = .01, p = .91, η_p^2 = .00). Further, there were no significant interactions amongst the variables on PVN area. Refer to Table 2 for statistical values regarding PVN stain area.

In addition, a separate factorial analysis was conducted to analyze darkness of pixels over background, or stain density, of CRF staining in the PVN using the same variables. There were no significant main effects of alcohol treatment (F (1, 27) = .03, p = .86, η_p^2 = .00), FMT treatment (F (1, 27) = 1.28, p = .27, η_p^2 = .05), or cohort (F (1, 27) = .34, p = .57, η_p^2 = .01). There were no interactions of CRF stain density in across the variables. Please refer to Table 2 for statistical values concerning the interactions and Figure 3 contains graphical representation of the above results.

Table 2. Statistical Values for PVN Interactions.

PVN Area		***F***	***p***	η_p^2
Interactions				
	Alcohol x Cohort	.00	.96	.00
	FMT x Cohort	.17	.68	.01
	Alcohol x FMT	.07	.80	.00
	Alcohol x FMT x Cohort	1.41	.25	.05
CRF Density in PVN				
Interactions				
	Alcohol x Cohort	.05	.83	.00
	FMT x Cohort	.08	.78	.00
	FMT x Alcohol	.02	.90	.00
	Alcohol x FMT x Cohort	.02	.89	.00

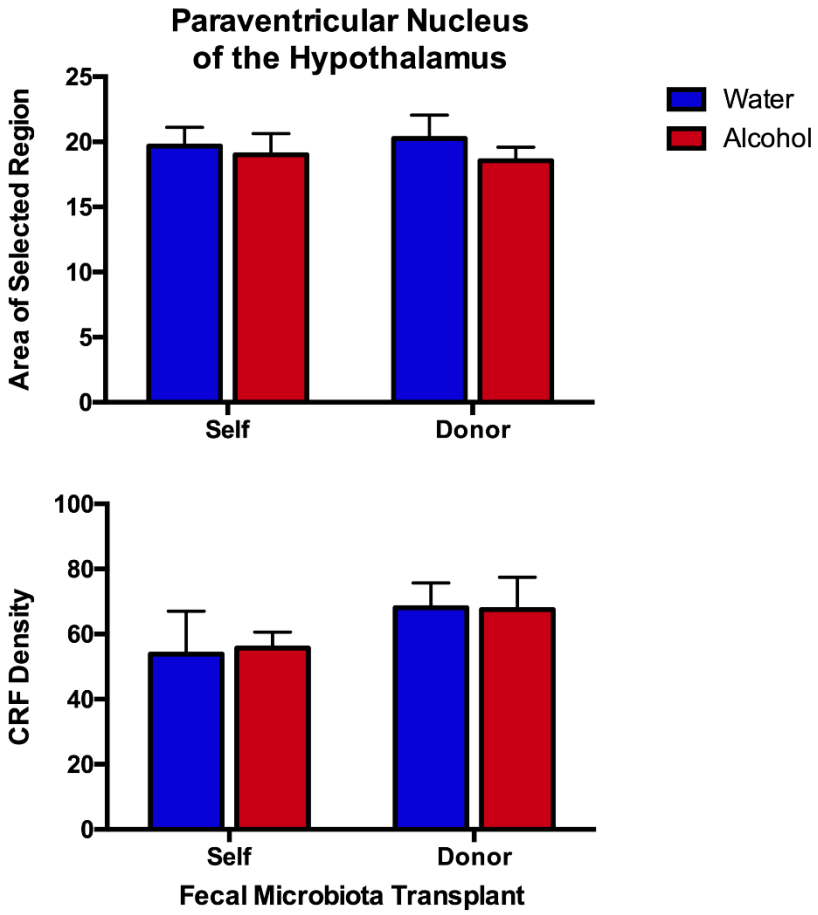

Figure 3. Top image represents the selected area while the bottom represents CRF density in the PVN. Error bars represent the standard error of mean.

Analysis of Central Nucleus of the Amygdala

As expected, a 2 x 2 x 2 factorial ANOVA failed to reveal significant main effects of alcohol treatment (F (1, 28) = 1.15, p = .29, η_p^2 = .04), FMT treatment (F (1, 28) = .47, p = .50, η_p^2 = .02), or cohort (F (1, 28) = 3.10, p = .09, η_p^2 = .10) on CeA selected area. There was no significant main effect of alcohol treatment (F (1, 28) = .41, p = .53, η_p^2 = .02) or FMT treatment (F (1, 28) = .29, p = .60, η_p^2 = .01). Please refer to Table 3 for statistical values of the associated interactions and Figure 4 for graphs representing the area selected and CRF stain density within the CeA. There was, however, a main effect of cohort (F (1, 28) = 6.58, p = .02, η_p^2 = .19) on CRF stain density in CeA, such that cohort 2 (M = 123.75, SEM = 8.71) expressed higher levels of CRF staining than cohort 1 (M = 98.82, SD = 6.41; see figure 5 for cohort effects). There were no significant interactions for area selected or CRF stain density in the CeA observed. However, the interaction between alcohol and FMT did show a moderate effect size, which may indicate this relationship should be probed further (F (1, 27) = 2.35, p = .14, η_p^2 = .08; see table 3). Please refer to Table 4 for all means and standard errors for all dependent variables across all independent variables.

Table 3. Statistical Values for CeA Interactions.

CeA Area		*F*	*p*	η_p^2
Interactions				
	Alcohol x Cohort	.02	.88	.00
	FMT x Cohort	.05	.83	.00
	FMT x Alcohol	.26	.61	.01
	Alcohol x FMT x Cohort	1.53	.23	.05
CRF Density in CeA				
Interactions				
	Alcohol x Cohort	1.19	.18	.06
	FMT x Cohort	.73	.40	.03
	FMT x Alcohol	2.35	.14	.08
	Alcohol x FMT x Cohort	1.92	.18	.06

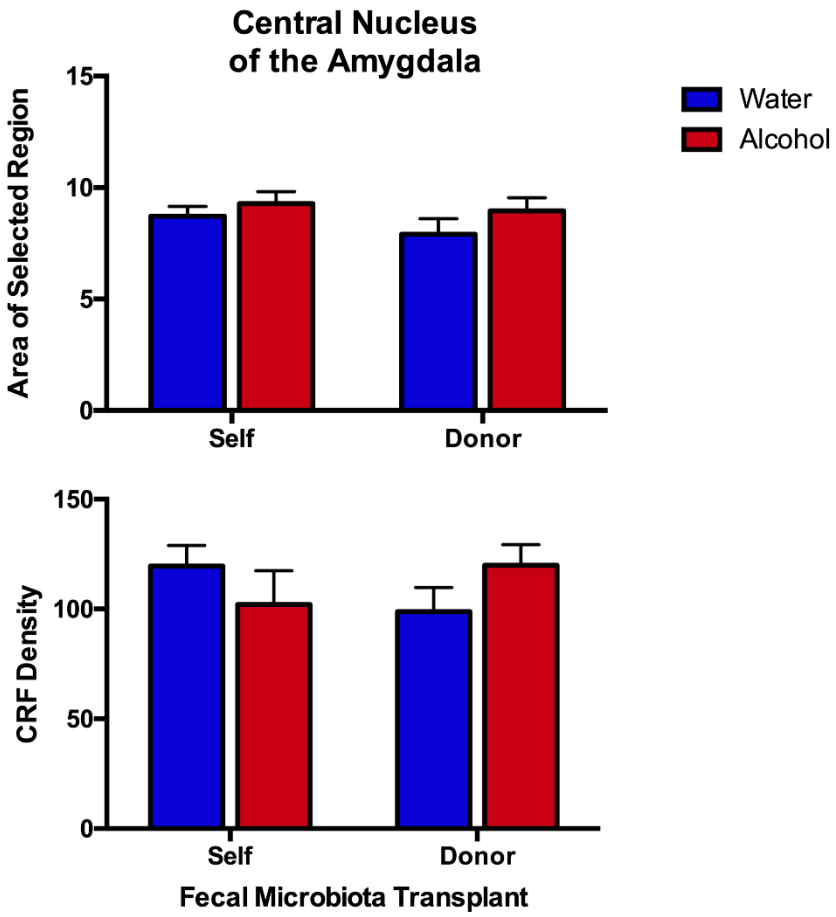

Figure 4. Top image displays the area selected while the bottom image represents the CRF stain density in the CeA. Error bars represent the standard error of mean.

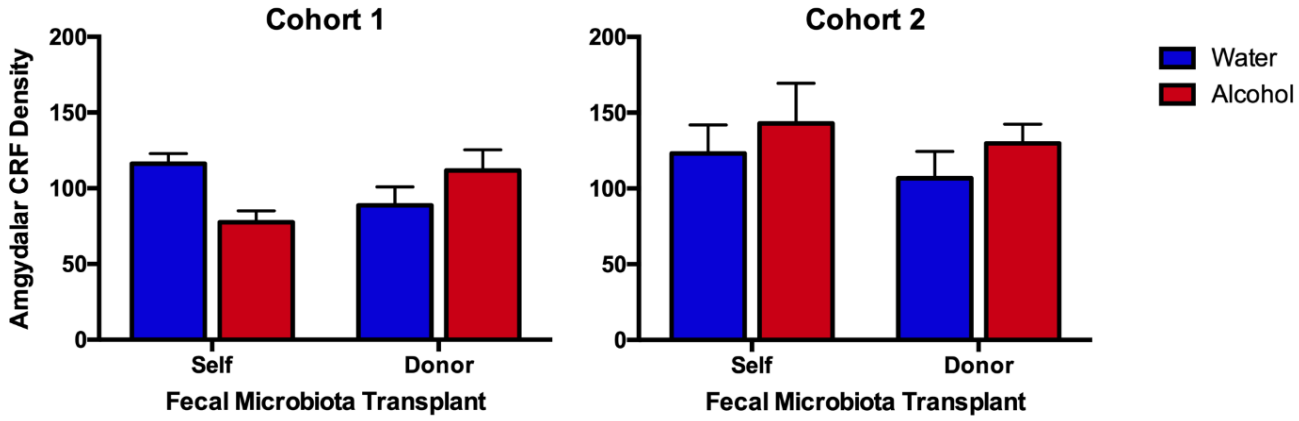

Figure 5. The left image displays the CeA stain density for cohort 1. The right image represents the CeA stain density for cohort 2. Error bars represent the standard error of mean.

Table 4. Mean & SEM for PVN & CeA.

	Alcohol Treatment				**FMT Treatment**				**Cohort**			
	Water		Alcohol		FMT Self		FMT Donor		1		2	
	M	*SEM*	*M*	SEM	*M*	SEM	*M*	*SEM*	*M*	*SEM*	*M*	*SEM*
PVN Area	20.02	.77	18.73	.87	19.34	1.06	19.37	.69	19.06	1.04	19.67	.522
PVN Density	63.27	6.98	62.84	6.39	54.76	6.74	67.77	6.18	58.91	7.38	66.42	5.61
CeA Area	8.29	.42	9.90	.40	9.00	.34	8.49	.45	9.23	.36	8.14	.44
CeA Density	108.57	7.55	112.40	8.48	110.80	9.02	110.42	7.38	98.82	6.41	123.75	8.71

Chapter 4. Discussion

The current study investigated the effects of alcohol and FMT treatment on the gut-brain axis. It specifically aimed to examine the relationship between alcohol, FMT treatment as a gastrointestinal restorative effort, and their ability to alter corticotropin releasing factor in the paraventricular nucleus of the hypothalamus and central nucleus of the amygdala. The hypothalmus is a component of the stress response system and is known to be involved in feeding behaviors. However, the amygdala has implications for anxiety and other emotions.

The current study was unable to confirm well-established theories in the literature, which suggest the methodology and procedures utilized were not ideal and require amending for future studies. However, the current study is important for a variety of reasons. To our knowledge, this study is the first to incorporate alcohol and FMT treatment to measure CRF levels in the PVN of the hypothalamus and CeA. Despite the findings of the study, its novelty lends insight into the importance of examining the interconnected effects of alcohol consumption, gastrointestinal system function, and brain function to each other. Furthermore, assessing restorative measures for the gut-brain axis, such as FMT, may help develop treatment options for those experiencing gastrointestinal illnesses, mental illnesses, or alcohol-induced illnesses.

It was anticipated that animals in the alcohol-exposed condition would express higher levels of CRF in the PVN of the hypothalamus and CeA than the animals in the non-alcohol-exposed condition. Despite the results of the current study, extensive literature supports the influence of alcohol on the CRF system in various brain regions. For example, a study by Sims et al. (2013) examined CRF in alcohol-exposed animals and found intermittent access to alcohol dysregulated CRF function. The researchers also found that blocking CRF signaling with the use of an antagonist ultimately reduced inflammation and alcohol consumption. A similar study

observed that injecting the CRF antagonist into CeA also decreased binge drinking (Lowery-Gionta et al., 2012). In addition, other studies have investigated the functionality of CRF receptors in the hypothalamic brain regions during alcohol use and found that alcohol consumption modifies CRF expression (Haass-Koffler et al., 2016; Quadros et al., 2016). These studies suggest there lies a complex relationship between CRF systems and stressors to the body, such as alcohol.

Although the current study revealed there was no significant effect of alcohol on CRF in either the PVN and CeA, expression of CRF was present. It is possible the animals did not consume enough alcohol to alter CRF expression. The current study performed three DID rounds where the animals were exposed to alcohol for 4 days per round. During the final day of the first round, animals consumed 1.87 g/kg on average during the session. By round three, the value escalated to 2.92 g/kg, showing that each round increased overall drinking levels, and that these levels are equivalent to human social drinking levels. Nevertheless, although the alcohol level achieved was acute, I found no evidence of upregulated CRF expression (Quadros et al., 2016). However, other studies have found that even higher levels of alcohol consumption can be achieved with a drinking-in-the-dark model consisting of more rounds (up to six; Thiele & Navarro, 2014). Therefore, the number of DID rounds may be critical for escalating the levels of alcohol drinking to levels that will impact CRF expression. Another discrepancy between the current study and literature is that the DID model in the current study had an abstinence period of 3 days, whereas other studies used DID models with abstinence periods 24 hours or shorter (Lowery-Gionta et al., 2012). Existing studies suggest an acute abstinence period may increase plasma levels of hormones such as corticosterone and ACTH, which are associated with CRF activity in the PVN of the hypothalamus (Rivier, et al., 1990).

Despite the suggested modifications to the DID model used in the present study, it is important to note the strength and validity of the current model as well. This voluntary, binge-like model of alcohol administration is an established and accepted tool for producing pharmacologically relevant blood ethanol concentrations in rodents (Fritz & Boehm, 2016). One benefit of the DID model is that it eliminates alcohol dependence characteristics, such as anxiety-like behavior. Alternatively, forced consumption models, such as oral gavage, tend to produce higher blood ethanol concentrations, but elicit stress in the animals (McBride & Li, 1998). For this reason, oral gavage was not selected as the main route of alcohol administration, so as to avoid introducing confounding variables.

Other forced consumption models, such as vapor chambers, involve alcohol exposure via inhalation. Vapor chambers are helpful because they allow the researcher precise control over alcohol intoxication levels, which can be utilized to model alcohol dependence, as opposed to social drinking levels. In addition, alcohol administration via inhalation has been shown to elicit alterations to the gut via the brain-gut axis (Peterson et al., 2017). Due to the bidirectional relationship between the gut and the brain, the gut microbiota is indirectly impacted. Though DID is a valid model, it is most representative of social intake. However, it may be beneficial to investigate different alcohol consumption models, such as dependence drinking models, to observe changes in CRF levels.

Additionally, it was expected that animals in the alcohol-exposed condition who received an FMT from a donor would express lower levels of CRF in the regions than the alcohol-exposed animals who received an FMT using their own fecal sample. FMT from healthy donors has been shown to serve as a restorative technique in reestablishing a dysbiotic gut microbiota (Wilson et al., 2019). The transfer of microbes from the donor to the recipient is vital in that the donor

microbes should be comparatively richer than that of the recipient for a successful transfer (Ericsson et al., 2017). The initial study prepared for this by randomly selecting animals as donors from the same litters as the experimental animals. By choosing donor animals from the same litters, it ensured the donor and experimental animals would share similar gut microbiomes when initially beginning the experiment. After the experimental animals consumed alcohol, it was assumed their microbes would change and no longer resemble the donors. Therefore, they would need the richer sample from the donor for a successful transplant. However, it is possible the animals selected as healthy donors were not much different than experimental animals, therefore, resulting in an unsuccessful FMT. Ongoing research is investigating microbial consituients. It is likely alcohol consumption levels were not high enough to elicit expected gut dysbiosis. Thus, there was no damage to be repaired by FMT. A colloborative follow-up study between the Hayes and Caughron labs is examining intestinal samples collected prior to FMT to verify the presence of gut dysbiosis.

Additionally, previous literature suggests the present study may have benefitted from repeated FMT procedures over the span of several weeks. A study using the DID model repeated FMT procedures three times per week for 3-5 weeks (Xu et al., 2018). The researchers observed a steady reestablishment of the gut microbes and a decrease in alcohol-induced anxiety-like behaviors during withdrawal in animals. This may suggest that the single performance of FMT was not enough to completely replace the microbes in the alcohol-exposed animals. However, the current study administered omeprazole three times prior to FMTs to reduce gastric acid secretions and increase the likelihood of the survival of bacteria in the FMT. Also, it may be beneficial to explore other treatment options for a dysbiotic gut, such as probiotics. Probiotics are live microorganisms that are typically found in the human gut microbiota and can be used to

replace the microbes that escaped from the leaky gut. Probiotics have the ability to act as preventive treatment, or can be used to help rebalance dysbiosis by replenishing microbes in the gut (Gagliardi et al., 2018). Research shows that probiotics possess a host of benefits, such as suppressing inflammatory cytokines by limiting their production (D'Mello et al., 2015; Desbonnet et al., 2008), intact intestinal barrier (Clapp et al., 2017), and reducing depression and anxiety (Liu et al., 2015). Despite the many benefits of probiotics, it is unclear whether it may have the same effect as FMT on the gut, as it only increases the presence of certain species of bacteria found in the gut (Clapp et al., 2017). Applying FMT as a treatment method for gut dysbiosis is an extreme treatment option when compared to the use of probiotics, as it is possibly more disruptive to the gut. Therefore, future studies should compare the possible effects of probiotic treatment to FMT in alcohol-related dysbiosis to examine which treatment option would best resolve gut dysbiosis.

The results of the study suggest that alterations to CRF expression may be time sensitive following FMT. After the FMT procedure, there was a two-week period where the animals continued to consume alcohol via the two-bottle choice procedure prior to collecting their brains. Available research suggests that any attempt to restore the gut microbiome via FMT may have been reversed by the continued alcohol administration leading to no effects on CRF. Research shows that as few as one round of DID can alter CRF levels which remain affected through an acute abstinence (Lowery-Gionta et al., 2012; Rivier et al., 1990; Zorrilla et al., 2001). It is likely time may play an important role in CRF expression, as well as give further insight into the CRF mechanisms. Future studies should investigate time sensitivity of CRF expression by comparing CRF levels in brain samples collected soon after FMT and those collected following re-exposure

to alcohol post FMT procedure. As a result, this will allow researchers to better understand any time constraints that may also influence CRF expression.

Limited research investigating the relationship between FMT and CRF exists, however that does not negate the connection between the two. The study conducted by Xiao and colleagues (2018) examined the impact of chronic alcohol drinking, withdrawal, and FMT on gut microbiome composition, as well as the expression of genes implicated in alcohol addiction. Their study revealed that chronic alcohol altered the composition of the gut microbiome and that FMT influenced the gene expression of CRF receptor 1. Another study observed that when rats with irritable bowel syndrome were given an FMT, they expressed decreased levels of CRF receptor 1 (Ma et al., 2019). Both studies depict the indirect influence FMT can have on CRF expression via restoring the gut microbiome. Notably, the current study examined CRF expression, which binds to CRF receptor 1. Therefore, the results of the previous studies suggest that CRF expression in the present study may have been altered since its receptor expression can be modified by alcohol consumption and FMT. Evidently, it is critical to produce more research observing the relationship between FMT and CRF levels, as it may help define the relationship between the two.

An unexpected result from the current study was a cohort effect on stain density in the CeA. Specifically, the alcohol-exposed animals that received an FMT using their own fecal sample had drastically increased CRF levels in cohort 2 compared to cohort 1. An inevitable limitation was the inability to conduct the experiment with all the animals at once. It would be impossible to avoid conducting the experiment in cohorts because there was not sufficient lab equipment and time to accomplish this. In fact, a few cohort differences were observed. For example, cohort 2 was older than cohort 1 at the beginning of the study. Previous research

explains that age could play a role in the amount of alcohol consumed, though it is unclear whether older animals drink more or less alcohol as the results vary across studies (Abel & York, 1979; Schramm-Sapyta et al., 2010). However, this was unlikely to produce a cohort effect in the current study as the cohort differences in age did not place the animals in differing developmental stages (Sengupta, 2013). Sprague Dawley rats have an average lifespan of 2.5-3 years and enter into adulthood at about PND 90, thus all animals in the study were within the adult range (McCutcheon & Marinelli, 2009; Sengupta, 2013).

Importantly, cohort 2 consumed higher levels of alcohol than cohort 1 across all 14 days of the two bottle choice measurements and repetitions of DID. The increased intake of alcohol in cohort 2 may drive the cohort effect of CRF expression in the CeA, as high levels of CRF are often associated with higher levels of alcohol drinking. However, a follow-up analysis failed to reveal a correlation between alcohol consumption and CRF expression in the CeA, r (34) = .11, p = .54. An additional rationale for the cohort effects, the cohorts differed on the time of day the two-bottle choice measurements were taken due to changes in researcher availability. Researcher availability to collect data necessitated a shift in light/dark cycle timing for cohort 2. Though both cohorts had a consistent DID paradigm where they had access to alcohol beginning 3 hours into the dark cycle, there was a shift in the time of day the measurements were taken for the two-bottle choice measurements by the experimenter. Furthermore, cohort 1 had bottles put on at 11 a.m. and cohort 2 had bottles put on at 4 p.m. everyday. Given the animals were allowed more than two weeks to adjust to the change in light cycles, it is not expected that this would produce any changes among the cohorts. Moreover, research shows that rats can socially transfer pain to rats they are familiar with due to empathic responses (Li et al., 2018). Empathy allows the sharing of emotional states in both humans and animals. The animals received the oral gavage

procedure in the same romm where they consumed alcohol, due to a lack of additional available rooms. Therefore, it is possible the acute stress cohort 1 experienced during the oral gavage procedure and other behavioral testing may have elicited an empathic response in cohort 2, likely increasing the levels of alcohol consumed by cohort 2, as well as their CRF levels.

A statistically insignificant interaction between alcohol and FMT was observed on CRF stain density in the CeA. However, the effect size was moderate, indicating that both alcohol and FMT account for a significant amount of variance in CRF stain density in the CeA. Despite the insignificant interaction between alcohol and FMT on CRF, an increase in sample size within each condition may increase the likelihood of observing statistical significance. Future studies should conduct a power analysis to determine the sample size necessary to observe statistical significance.

The current literature supports alcohol's effects on inflammation as a conduit to negatively influence the gut microbiome by activating the body's stress response system, the HPA axis. The activation of the HPA axis can result from alcohol-induced inflammation (Carabotti et al., 2015). When released, CRF has the ability to signal to immune cells that it will either further promote or slow down inflammation (Zhang & An, 2009). This strongly suggests that CRF may play a vital role in mediating inflammation in the gut-brain axis. In order to investigate this relationship further, fecal samples were collected throughout the experiment and will be analyzed for pro- and anti-inflammatory cytokines during ongoing research between the Hayes and Caughron labs. This may provide insight into a mechanism that further explains this relationship.

The results of the current study strongly suggest the necessity of further investigations of the interplay between alcohol and FMT on the gut-brain axis. Despite the results, this novel

study provided a theoretical foundation for the relationship between alcohol, FMT, and CRF. Existing literature is able to connect one component to the another, but has failed to determine how the three may influence each other. Understanding the connection between alcohol, FMT, and CRF may provide valuable insight into the functioning of the gut-brain axis. Additionally, it is important that mechanisms aiding in the function of this complex relationship, such as inflammation, are examined as it will likely contribute towards the development of a treatment for inflammatory illnesses. Since humans experience gut and mental health disorders, understanding these mechanisms becomes beneficial as it may help translate animal research findings to human mental health and gastrointestinal diseases.

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