Voluntary binge-patterned alcohol drinking and sex-specific influences on monoamine-related neurochemical signatures in the mouse gut and brain

Ella E. Bauer\textsuperscript{1} | Allyse Shoeman\textsuperscript{1} | Trevor J. Buhr\textsuperscript{1} | Karrie M. Daniels\textsuperscript{2} | Mark Lyte\textsuperscript{2} | Peter J. Clark\textsuperscript{1}

\textsuperscript{1}Department of Food Science and Human Nutrition, Iowa State University, Ames, IA, USA
\textsuperscript{2}Department of Veterinary Microbiology and Preventative Medicine, Iowa State University, Ames, IA, USA

Correspondence
Ella Bauer, Iowa State University, 220 MacKay Hall, 2302 Osborn Dr., Ames, IA 50011, USA.
Email: elbauer@iastate.edu

Abstract

\textbf{Background:} Altered monoamine (i.e., serotonin, dopamine, and norepinephrine) activity following episodes of alcohol abuse plays key roles not only in the motivation to ingest ethanol, but also physiological dysfunction related to its misuse. Although monoamine activity is essential for physiological processes that require coordinated communication across the gut–brain axis (GBA), relatively little is known about how alcohol misuse may affect monoamine levels across the GBA. Therefore, we evaluated monoamine activity across the mouse gut and brain following episodes of binge-patterned ethanol drinking.

\textbf{Methods:} Monoamine and select metabolite neurochemical concentrations were analyzed by ultra-high-performance liquid chromatography in gut and brain regions of female and male C57BL/6J mice following “Drinking in the Dark” (DID), a binge-patterned ethanol ingestion paradigm.

\textbf{Results:} First, we found that alcohol access had an overall small effect on gut monoamine-related neurochemical concentrations, primarily influencing dopamine activity. Second, neurochemical patterns between the small intestine and the striatum were correlated, adding to recent evidence of modulatory activity between these areas. Third, although alcohol access robustly influenced activity in brain areas in the mesolimbic dopamine system, binge exposure also influenced monoaminergic activity in the hypothalamic region. Finally, sex differences were observed in the concentrations of neurochemicals within the gut, which was particularly pronounced in the small intestine.

\textbf{Conclusion:} Together, these data provide insights into the influence of alcohol abuse and biological sex on monoamine-related neurochemical changes across the GBA, which could have important implications for GBA function and dysfunction.

\textbf{Keywords:}
alcohol abuse, gut-brain axis, neurochemical changes, sex differences
INTRODUCTION

Alcohol abuse has been linked to the development of mood disorders, neurodegenerative diseases, and digestive issues (Bettioli et al., 2015; Fratiglioni et al., 1993; Kamal et al., 2020; Kathryn McHugh & Weiss, 2019; Shield et al., 2013). Given the high prevalence of alcohol abuse worldwide (World Health Organization, 2018), there is a need for effective treatments to curb the severity of its consequences. However, much remains unknown about the body's response to regular episodes of alcohol misuse. The bidirectional communication network between the gut organs and the brain, known as the gut-brain axis (GBA), is a particularly interesting candidate to investigate the physiological responses to alcohol misuse. Indeed, alcohol ingestion can modify neural, neuroendocrine, metabolic, and immune pathways involved in GBA communication (Barve et al., 2017; Bode, 1997; Bravo et al., 2011; Forsyth et al., 2017; Gorky & Schwaber, 2016; Jerlhag, 2019). For example, changes in patterns of nerve activity within the gut can signal through the vagus nerve to influence cognitive modalities including reward, motivation, and mood processes that may be relevant to alcohol abuse (Foster et al., 2017; Han et al., 2018; Kaelberer et al., 2018; de Lartigue, 2014). Furthermore, evidence suggests the gut may be an origin of neurobiological disorders that have also been linked to alcohol abuse, including major depressive disorder and Parkinson's disease (Foster et al., 2017; Gorky & Schwaber, 2016; Kelly et al., 2016; Zheng et al., 2016). However, the possible mechanisms behind how high-volume ethanol ingestion modifies the GBA to increase the risk of gastrointestinal (GI) disorders, neurobiological diseases, or motivation to abuse alcohol remain poorly understood.

Monoamine neurochemicals like serotonin (5HT), dopamine (DA), and norepinephrine (NE) play key roles in the GBA, including mediation of GI function, energy homeostasis, and neural communication (Mittal et al., 2017). Alcohol ingestion can alter the activity of monoamines in the gut, which play vital roles in GI motility, gastric secretions, immunity, nutrient absorption, and systemic energy homeostasis (Namkung et al., 2015; Rubi & Maechler, 2010; Tank & Wong, 2015). Ethanol and primary metabolite acetaldehyde can alter the activity of enzymes (i.e., alcohol and aldehyde dehydrogenase) involved in the breakdown of serotonin, norepinephrine, and dopamine, which could influence the activity of these neurochemicals in the gut (Huang, 2010; Rosenfeld, 1960). In addition to interfering with metabolism, alcohol can disturb entire populations of neurochemical producing and utilizing GI microbes, thereby altering monoamine levels in host intestinal tissues (Leclercq et al., 2014; Szabo & Saha, 2015). The gut produces nearly 50% of the body's serotonin (Bellono et al., 2017; Eisenhofer et al., 1997); thus, altered monoamine activity following episodes of alcohol abuse could not only have an impact on intestinal function and communication across the entire GBA but also contribute to GI dysfunction. For example, alcohol ingestion can exacerbate symptoms of GI disorders characterized by abnormal monoaminergic activity (e.g., Crohn's disease, ulcerative colitis, and irritable bowel syndrome) (Mittal et al., 2017). Moreover, disturbances to the balance of monoamines in the gut can influence cognitive processes that require monoaminergic signaling (Borghammer, 2018; Breit et al., 2018; Davey et al., 2013; McVey Neufeld et al., 2019; Suarez et al., 2018). Evidence also suggests intestinal nerves may be initial target of selective serotonin reuptake inhibitors (i.e., SSRIs) capacity to mitigate rodent depression-like behavior mediated by abnormal monoaminergic activity in the brain (Desbonnet et al., 2015; Liang et al., 2015; McVey Neufeld et al., 2019). Therefore, alcohol-induced changes to gut monoamine concentrations may modify communication across the entire GBA network, potentially increasing the risk for the development of neurobiological disorders (Bonaz et al., 2017; Kelly et al., 2016; Lionnet et al., 2018). However, despite the critical roles of monoamines in GBA function and dysfunction, monoaminergic changes in the gut remain uncharacterized following alcohol abuse.

Ingesting high volumes of alcohol can also potently alter the activity of monoamines within several brain pathways including reward, motor, the hypothalamic-pituitary-adrenal axis (HPA), and limbic system. In fact, the altered activity of monoamine neurotransmitters in these circuits is linked to the motivation to misuse alcohol, risk for the development of neurobiological disease, and alcohol-related cognitive dysfunction (Koob & Volkow, 2016). Research into the impact of alcohol abuse on brain monoamines has predominately focused on dopaminergic activity in reward circuit structures, such as the ventral tegmental area and the nucleus accumbens (Koob, 2014; Lovinger & Alvarez, 2017; Weiss et al., 1996). Monoamine activity in other brain regions that comprise parts of the limbic, motor, reward, and stress circuits remains less characterized, despite probable contributions to the motivation to consume alcohol or cognitive dysfunction related to alcohol ingestion (Blaine & Sinha, 2017). Moreover, brain monoaminergic-related neurochemical levels in rodent models following voluntary binge-patterned ethanol ingestion also remains less characterized (Bell et al., 2014, 2016; Ferguson et al., 2019; Iancu et al., 2018). The voluntary ingestion of ethanol at high volumes is an important consideration for understanding the neurobiological underpinnings of the motivation to misuse alcohol. This is a departure from past research, which commonly utilized rodent models of forced ethanol exposure (e.g., gavage, vapor chambers, and i.p. injection) to investigate brain monoaminergic responses to binge-like alcohol drinking (Brown et al., 2000; Gouveia & Hurst, 2013; Stuart & Robinson, 2015). Therefore, even considering the decades of research that has gone into investigating the influence of alcohol abuse on brain monoaminergic activity, much remains to be understood.

The purpose of this study was to evaluate the effects of binge-patterned ethanol drinking on changes in mouse brain and gut monoaminergic activity, using the “Drinking in the Dark” (DID) paradigm. During the DID procedure, C57BL/6J mice voluntarily consume ethanol to levels exceeding the standard of binge drinking set by the National Institute of Alcohol Abuse and Alcoholism, defined as a BAC of 80 mg% within a 2-hour period (Crabbe et al., 2011; Rhodes et al., 2005). A BAC above this level is sufficient to induce intoxication-like behavior in mice, including ataxia and anxiety (Thiele & Navarro,
Animal Care and Use Committee. Procedures were approved by the Iowa State University Institutional except for the DID procedure (see section 2.2 for details). One con ad libitum the DID paradigm (as described in Rhodes et al., 2005). Mice had daily drinks ethanol to levels exceeding 0.8 mg/ml (0.08 BAC) using -incorporated because evidence suggests that this strain voluntar... hypertalamus, cerebellum, brainstem, and remaining cerebral cortex. These regions of the brain comprise limbic, reward, motor, and HPA pathways that contribute to alcohol misuse and related dysfunction (Uhl et al., 2019). Finally, relationships between monoamine neurochemical signatures across regions of the gut and brain were considered, as this information could provide insight into the possible factors contributing to alcohol-altered communication within the GBA. The results of this study provide detailed information regarding the relationship between voluntary binge-patterned ethanol ingestion and monoaminergic activity in the GBA. These data could have implications for understanding the motivation to misuse alcohol, but also the increased risk of alcohol abuse-related pathological conditions. Moreover, these data also provide insight into sex differences in gut and brain neurochemicals.

MATERIALS AND METHODS

Animals

Upon arrival, 24 6-week-old male and female C57BL/6J mice (Jackson Laboratory) were housed individually in standard laboratory cages with a reverse 12-h light/dark cycle (dark at 6 a.m.) and kept at constant room temperature (21 ± 2°C). C57BL/6J mice were incorporated because evidence suggests that this strain voluntarily drinks ethanol to levels exceeding 0.8 mg/ml (0.08 BAC) using the DID paradigm (as described in Rhodes et al., 2005). Mice had ad libitum access to food and water during the entire experiment, except for the DID procedure (see section 2.2 for details). One control male mouse was prematurely removed due to malocclusion. All procedures were approved by the Iowa State University Institutional Animal Care and Use Committee.

Drinking in the dark

Mice began the DID procedure 1 week after arrival. During DID, mice had 2-hour access to graduated sipper tubes containing water (i.e., water group; 6 female, 5 male) or 20% v/v ethanol in water (20% ethanol group; 6 female, 6 male) for 3 consecutive days of acclimation and 4-hour access ±0.25 hours on the fourth day. Four-hour access to sipper tubes has previously shown to yield the highest BEC, which we hypothesized would increase the likelihood of observing neurochemical changes. The DID procedure was initiated 3 hours after the start of the dark cycle, as this coincides with peak consummatory behavior in mice. The amount of fluid ingested was recorded from each sipper tube every 30 minutes to measure drinking patterns over the course of the DID periods. Daily total ethanol consumed was calculated as grams of ethanol per kilogram body weight to control for differences in body mass across mice.

Blood ethanol concentration via gas chromatography

Trunk blood was collected immediately following 4-hour DID access on day 4 for all mice and stored at −80°C. Whole blood was thawed on ice, aliquoted with the ethanol solvent 1,4-dioxane, sonicated, vortexed, and centrifuged (10,000 x g). The supernatant was purified via filtered centrifugation (10,000 x g). Duplicates of purified samples were detected via a gas chromatography–flame ionization detector (GC-FID; Keck Metabolomics Research Laboratory, Iowa State University). To reduce potential ethanol evaporation, samples were kept on ice and recapped quickly throughout the protocol. Ethanol was not detected in the blood of mice that had access to water.

Tissue preparation and UHPLC analysis

Immediately following DID on the fourth day, mice were decapitated, and brain and gut samples were rapidly collected on a chilled platform simultaneously by separate experimenters. First, a flat edge razor was used to discard the olfactory bulbs and make coronal slice through the brain at approximately 1.97 mm from bregma to collect the area containing the prefrontal cortex. Second, the circle of Willis blood vessels surrounding the exterior of the hypothalamus were discarded via fine point forceps before collecting the hypothalamus (approximate coordinates 0.13 mm, −2.69 anteroposterior from bregma). Third, a coronal slice was taken approximately −0.83 mm from bregma to isolate the striatum. Within this brain slice, the cortex superior and lateral to the corpus callosum was removed from the striatum containing section. Next, the cerebellum was carefully excised. After a mid-sagittal cut, a small flat edge spatula was used to carefully unfold the surrounding cortex and collect the hippocampus. The posterior brainstem area was isolated and collected (see approximate locations −2.69 mm, −8.15 mm from bregma), leaving just the remaining cortical area. For the intestines, an incision was made immediately after the pyloric sphincter and before the ileocecal valve. The duodenum included a 2 cm segment of the proximal end of the small intestine. The jejunum included a 2 cm segment at approximately 15 cm or the middle of the small intestine. The ileum...
included a 2 cm segment at the distal end of the small intestine. Intestinal segments between duodenum and jejunum as well as between jejunum and ileum were discarded to remove any ambiguity in discerning the small intestine regions collected. Next, an incision was made on the distal end of the large intestine, the cecum was excised, and entire contents of cecum were collected. A segment of the caudate lobe was excised from the liver. Brain and intestinal regions, liver, and cecal contents were immediately placed into 0.2 M HClO₄ acid and stored at −80°C until UHPLC processing.

Brain, intestinal, liver, and cecal content samples were homogenized using the Omni Bead Ruptor Elite (Omni International) and centrifuged (3000 x g) at 4°C for 30 minutes. After centrifugation, the supernatant from brain samples was aliquoted and stored at −80°C until UHPLC quantification. The remaining gut tissue and cecal samples were further purified and centrifuged (3000 x g) using a 0.45-μm filter, aliquoted, and stored at −80°C until UHPLC quantification.

Monoamine quantification was performed in separate batches of brain and gut subregions (to accommodate the large number of samples) using ultra-high-performance liquid chromatography with electrochemical detection (UHPLC-ECD) on a Dionex UltiMate 3000 UHPLC System (Thermo Fisher Scientific). Prior to injection, samples were held in the UHPLC autosampler at 4°C. Neurochemical separation was achieved using Thermo Scientific MD-TM mobile phase at a flow rate of 0.6 ml/min and a Hypersil BDS C18 column (Thermo Scientific). Electrochemical detection was achieved with a 6041RS glassy carbon electrode set to 400 mV.

Neurochemical standards (DOPAC, HVA, DA, 5-HT 5-HIAA, NE, DHMA, SAL, and the internal standard ISO-A) were purchased from Sigma-Aldrich.

Statistics

Two-way ANOVA was used to compare the mean volume (ml/kg) consumed between 20% ethanol group (water vs 20% ethanol) and sex (males vs. females) for each day of DID. Additionally, Pearson’s correlation coefficients were also used to investigate relationships between BAC and total ethanol consumed on day 4 of DID (see section 2.1).

Concentrations of monoamines and metabolites are expressed as mean μg/g tissue weight ±SEM. Neurotransmitter-to-metabolite ratios (DOPAC/DA, HVA/DA, SAL/DA, DHMA/NE, 5-HIAA/5-HT) were reported as the mean ±SEM for each subregion, as a marker of neurotransmitter turnover. The neurotransmitter ratio DA/L-DOPA mean ±SEM was also reported as a measure of dopamine synthesis. Data points that exceeded ±2 standard deviations from the mean were removed from the analyses, which can be observed in the degrees of freedom. Mean neurochemical values were compared using 2-way ANOVAs with ethanol treatment (water vs. ethanol) and sex (male vs. female) as between-subject factors. Post hoc analyses were performed using Tukey’s HSD following either a significant main effect of ethanol treatment and sex, or interaction between ethanol treatment and sex (see section 3.2 and 3.3). Pearson’s correlation was used to follow up on a significant effect of ethanol access to investigate a potentially interesting relationship between BAC and the striatal dopamine metabolites DOPAC and HVA.

Principal component analysis (PCA) was used to identify potentially meaningful trends in the neurochemical data by reducing the large number of variables (regional neurochemical measurements) and incorporating these data into unique, linear combinations called principal components (PCs). PCA is advantageous for visualizing trends in data that has high dimensionality and exhibit collinearity, as is the case for this extensive data set that includes multiple regional neurochemical measurements. The top 2 PCs that explained the most neurochemical variation were used in subsequent analyses (see sections 3.2.4 and 3.4). First, PCA was used to investigate alcohol access and sex-specific differences in neurochemical signatures within the entire gut, as well as regions of the small intestine, and large intestine. ANOVA was used to follow up on significant group differences among these regional signatures (see section 3.2.4). Second, PCA was performed separately for each brain and gut region to create PCs which represent unique neurochemical signatures for each region collected (see section 3.4). The relationship between the gut and brain PCs as well as the individual neurochemical relationships that followed (Tables 1 and 2) were evaluated using Pearson’s correlation with conservative p < 0.01 significance level.

RESULTS

Drinking behavior and blood ethanol concentration

No differences in ethanol ingestion (corrected for body mass) or BAC were observed between male and female mice (see Figure 1A,B). Male mice with water access consumed less fluid compared with the other groups on day 2 of DID (Figure 1A). On day 3 of DID, mice with ethanol access consumed mildly more fluid compared to mice with water access. On day 4 of DID, there was no difference in drinking behavior between any of the groups. Mean ethanol consumption on day 4 of DID was 9.47 ± 0.41 g/kg and 7.82 ± 0.69 g/kg for female and male mice, respectively. Blood ethanol concentration (BEC) was correlated with ethanol consumed (g/kg) on the fourth day of DID (r = 0.6172, p = 0.0431) (Figure 1B).

Gut neurochemical quantification

Small intestine subregion

In the duodenum, mouse sex and access to alcohol interacted to influence DA/L-DOPA ratio, F(1, 22) = 6.0604, p = 0.0241 (Figure 2A,B). Post hoc analyses revealed that male mice with ethanol access had higher DA/L-DOPA ratios compared to female mice with ethanol access (p = 0.0498). No other neurochemical differences reached statistical significance as defined by p ≤ 0.05 in this subregion.

In the jejunum, access to ethanol increased levels of dopamine, F(1, 22) = 8.1942, p = 0.0100 (Figure 2C,D), without influencing any
other neurochemicals. Moreover, sex affected the expression of NE- and DA-related neurochemicals. Female mice had a higher ratio of DA/L-DOPA, $F(1, 22) = 13.5625, p = 0.0016$, due to a decrease in L-DOPA concentration, $F(1, 22) = 5.8114, p = 0.0262$, with no change in DA concentration. Moreover, compared to male mice, female mice had higher ratios of DHMA/NE, $F(1, 22) = 11.4244, p = 0.0031$, which was due to higher levels of DHMA, $F(1, 22) = 6.9292, p = 0.0164$, and lower levels of NE, $F(1, 22) = 6.7268, p = 0.0178$.

Access to ethanol did not play a role in determining neurochemical concentrations in the ileum as no significant differences as defined by $p \leq 0.05$ were observed. However, the ileum showed sex differences in the concentrations of DA- and 5-HT-related neurochemicals (Figure 2E,F). Similar to the jejunum, the ileum in female mice had higher ratios of DA/L-DOPA, $F(1, 22) = 6.7214, p = 0.0179$, compared to males, which was due to lower levels of L-DOPA, $F(1, 22) = 11.9103, p = 0.0027$, without a change in DA. Additionally, female mice had lower concentrations of 5-HIAA, $F(1, 22) = 7.2323, p = 0.0145$, compared to males.

Large intestine subregions

In the cecum, both access to ethanol and sex influenced neurochemical measures (Figure 3A,B). Moreover, access to ethanol reduced the ratio of DOPAC/DA, $F(1, 22) = 6.5860, p = 0.0189$. L-DOPA was detected in the cecum; however, measurement was not achieved due to obstruction from a nearby peak.

In the cecal contents, both access to ethanol and sex influenced neurochemical measures (Figure 3C,D). Moreover, similar to the cecum, ethanol access lowered ratio of DOPAC/DA, $F(1, 22) = 11.2104, p = 0.0036$, in the cecal contents compared to mice with water access. Dopamine concentrations were higher in female mice, $F(1, 22) = 4.8760, p = 0.0404$, compared to male mice. Additionally, female mice had higher concentrations of 5-HT, $F(1, 22) = 6.5550, p = 0.0191$, compared to male mice.

In the proximal colon, both access to ethanol and sex influenced neurochemical measures (Figure 3E,F). Ethanol access decreased the ratio of DOPAC/DA, $F(1, 22) = 7.3694, p = 0.0137$, as well as levels of HVA, $F(1, 22) = 5.2201, p = 0.034$, compared to mice with water access. Both access to ethanol, $F(1, 22) = 8.5225, p = 0.0088$, and sex, $F(1, 22) = 7.5067, p = 0.0130$, altered the ratios of DHMA/NE. Post hoc analyses revealed female mice with water access have higher DHMA/NE ratio compared to male mice with ethanol access ($p = 0.0031$).

In the distal colon, both access to ethanol and sex influenced neurochemical measures (Figure 3G,H). Moreover, ethanol access to ethanol increased ratios of DOPAC/DA, $F(1, 22)=9.5333, p = 0.006$, which was due to increased levels of DOPAC, $F(1, 22) = 7.1936, p = 0.0147$, without a change in DA. Additionally, both access to ethanol, $F(1, 22) = 5.8997, p = 0.0252$, and sex, $F(1, 22) = 5.3108, p = 0.0326$, increased concentrations of NE. Post hoc analysis of this interaction reveals that the male mice with access to ethanol have higher concentrations of NE compared to female mice with water access ($p < 0.0012$), female mice with ethanol access ($p < 0.0015$), and male mice with water access ($p < 0.0193$).

Liver

In the liver, both access to ethanol and sex influenced 5-HT (Figure 4A,B). Access to ethanol increased concentrations of 5-HT, $F(1, 22) = 9.5337, p = 0.0061$, compared to mice with water access. Additionally, female mice had higher levels of 5-HT, $F(1, 22) = 6.9438, p = 0.0163$, compared to male mice. Post hoc analyses revealed that female mice with ethanol access had higher levels of...
5-HT compared to male mice with water access ($p < 0.0043$), female mice with water access ($p < 0.005$), and male mice with ethanol access ($p < 0.0103$).

### Neurochemical signatures in the gut

A relatively large data set was acquired for this experiment containing monoamine-related neurochemical concentrations across gut regions, while taking into consideration experimental factors such as access to ethanol and sex. From this data set, different neurochemical concentration changes related to alcohol, sex, and the interaction between these factors were observed (Figures 2–4). Therefore, principal component analysis (PCA) was utilized in order to summarize potentially meaningful trends in this large data set in a manner that can be more easily visualized. Principal components (PCs) for each mouse represented a combination of each neurochemical measure (i.e., the neurochemical signature) in the gut. ANOVAs considering

![Figure 2](image-url)  
**Figure 2** Neurochemical concentrations and markers of neurotransmitter turnover in the small intestine, including the duodenum (A) markers of neurotransmitter turnover and (B) μg/g of neurochemical, the jejunum (C) markers of neurotransmitter turnover and (D) μg/g of neurochemical, and the ileum (E) markers of neurotransmitter turnover and (F) μg/g of neurochemical, +/- SEM. The * represents significant main effect of alcohol; *= $p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Significant main effects of sex are represented as ♂ for males or ♀ for females, each of which indicates which sex had higher values. Letters that are distinct (“a” and “b”) represent a statistically significant post hoc analysis.
mouse sex and ethanol access were only completed for 2 PCs that contained the most variation in the data (i.e., PC1 and PC2). Across the entire gut, PC1 (representing 15.5% of the variation in the data) indicated there is a statistically significant difference in overall neurochemical signatures in the gut between male and female mice, \(F(1, 20) = 8.5591, p = 0.0094\), but no significant effect of alcohol (Figure 5A,D,G). To investigate whether sex-specific trends existed more prominently in a particular region of the intestines, a separate
PCA was performed just on neurochemicals in the small intestine (duodenum, jejunum, ileum) and again for the large intestine (cecum, proximal, and distal colon). ANOVA of small intestine PC1 (representing 19.782% of the variation in the data) indicates that neurochemical signatures in the small intestine are different between males and females, $F(1, 21) = 17.8329, p = 0.0005$, but alcohol access had no effect (Figure 5B,E,H). Alternatively, an ANOVA of the large intestine PC1 (representing 27.8% of the variation in the data) indicated that there was not a difference in the neurochemical signatures in the large intestine between male and female mice; however, a statistically significant main effect of alcohol treatment was observed, $F(1, 22) = 5.1399, p = 0.0342$ (Figure 5C,F,I). There were no statistically significant interactions in either the gut, small intestine, or large intestine ANOVAs. Taken together, these data may suggest that rodent’s sex may influence monoamine neurochemicals in the small intestine to a greater degree than the large intestine, whereas alcohol may influence markers for monoamine activity in the large intestine to a greater degree than the small intestine.

Brain neurochemicals

Striatum subregion

Ethanol access had a robust impact on DA-related neurochemicals in the striatum of both male and female mice (Figure 6A,B). Mice with access to ethanol had increased markers of DA turnover, as indicated by greater ratios of HVA/DA, $F(1, 22) = 15.1284, p = 0.001$, and DOPAC/DA, $F(1, 22) = 5.8086, p = 0.0262$. However, ethanol access decreased SAL/DA ratio, $F(1, 22) = 6.4289, p = 0.0202$. The DA metabolite HVA also increased in concentration following ethanol access, $F(1, 22) = 9.8227, p = 0.0055$. Evidence suggests a dose-dependent DA response to ethanol ingestion; therefore, Pearson’s correlation was used to follow up on the effects from ethanol on dopamine metabolites DOPAC and HVA. Moreover, BAC was positively correlated with the sum of DOPAC and HVA [$r = 0.7803, p = 0.0028$] (Figure 6C).

Additionally, the concentrations of DA-related neurochemicals were also influenced by the interaction between sex and access to ethanol during DID. A statistically significant interaction was observed between sex and ethanol access for the DA metabolite SAL, $F(1, 22) = 9.1741, p = 0.0069$. Post hoc analysis revealed that female mice with water access had higher levels of SAL than female mice with ethanol access ($p = 0.0094$), males with ethanol access ($p = 0.0098$), or males with water access ($p = 0.0099$). Additionally, there was a significant interaction between sex and ethanol access for L-DOPA, $F(1, 22) = 7.232, p = 0.0145$. Post hoc analyses revealed that female mice with ethanol access had lower levels of L-DOPA compared to female mice with water access ($p = 0.0178$).

Mouse sex and ethanol access interacted to influence the concentrations of other monoamines and metabolites, aside from DA-related neurochemicals. A statistically significant sex by ethanol access interaction was observed for 5-HIAA, $F(1, 22) = 8.7141, p = 0.0082$. Post hoc analysis revealed that female mice with water access maintained overall greater concentrations of 5-HIAA than males with water access ($p = 0.0066$) and female mice with ethanol access ($p = 0.0343$), with males with ethanol access falling between these groups failing to reach statistical significance ($p = 0.0672$). Moreover, sex and ethanol access interacted to impact striatal NE content, $F(1, 22) = 4.8627, p = 0.0400$; however, post hoc analysis failed to reveal statistically significant differences.

Hypothalamus subregion

The hypothalamus had the most changes to monoaminergic neurochemical concentrations as a result of ethanol access compared to other brain areas (Figure 7A,B). Ethanol access had an impact on 5-HT and NE activity in the hypothalamus, independent of sex. Access to ethanol increased concentrations 5-HT, $F(1, 22) = 6.2517, p = 0.0217$, and the metabolite 5-HIAA, $F(1, 22) = 10.2327, p = 0.0047$.

Access to ethanol influenced the activity of NE-related neurochemicals in the hypothalamus. Moreover, ethanol access
increased levels of NE, \( F(1, 22) = 6.464, p = 0.0199 \), and DHMA, \( F(1, 22) = 9.2762, p = 0.0067 \). A statistically significant interaction between sex and ethanol access was observed in the ratio of DHMA/NE, \( F(1, 22) = 4.5172, p = 0.0479 \); however, between-group differences failed to reach significance in post hoc analyses.

Finally, ethanol access also increased DA-related neurochemical concentrations and production in the hypothalamus. Regarding individual neurochemicals, ethanol access increased L-DOPA, \( F(1, 22) = 7.0866, p = 0.0154 \), DOPAC, \( F(1, 22) = 5.8809, p = 0.0254 \), and HVA, \( F(1, 22) = 7.6798, p = 0.0122 \), independent of sex. Dopamine metabolite ratios in mice with ethanol access failed to reach statistical significance. However, there was a statistically significant interaction between sex and ethanol access in the ratio of DOPAC/DA, \( F(1, 22) = 4.389, p = 0.0498 \), but post hoc analysis failed to reveal statistically significant group differences.

**Brainstem, hippocampus, cerebellum, prefrontal cortex, cerebral cortex subregions**

Compared to the striatum and hypothalamus, relatively fewer statistically significant changes were observed for monoaminergic-related
neurochemicals in areas containing the brainstem, cerebellum, and hippocampus. For the brainstem, access to ethanol increased markers of dopamine turnover in the brainstem as indicated by greater ratio of HVA/DA, $F(1, 22) = 17.3976, p = 0.0005$ (Figure 8A,B). Additionally, female mice have significantly higher levels of 5-HIAA, $F(1, 22) = 6.0657, p = 0.0235$; however, there is no significant main effect of alcohol on 5-HIAA levels.
effect for ethanol access in the brainstem. For the hippocampus, ethanol access significantly lowered concentrations of DOPAC, $F(1, 22) = 4.4887$, $p = 0.0475$ (Figure 8E,F). Additionally, ethanol access lowered hippocampal DHMA/NE ratio, $F(1, 22) = 11.5929$, $p = 0.0030$. In the cerebellum (Figure 8C,D), access to ethanol increased the ratio of DOPAC/DA, $F(1, 22) = 7.7080$, $p = 0.0120$, and SAL/DA, $F(1, 22) = 4.3923$, $p = 0.0407$, consistent with heightened markers of DA turnover in this region. Finally, the prefrontal cortex and cortex areas had no statistically significant neurochemical differences from either ethanol access or sex (figure not shown).

Gut–brain neurochemical axis

While several lines of evidence suggest the gut and the brain communicate and influence species behavior, the role of monoamines in GBA communication remains less understood. Therefore, the next objective was to investigate potential relationships between neurochemical signatures within gut and the brain, which could provide some insight into communication across the GBA. Because this study collected a large data set summarizing monoamine-related neurochemicals across several gut and brain regions, as well as...
experimental conditions, the data were reduced using PCA in an effort to capture potentially meaningful trends in a manner that can be more easily visualized. First, PCA was performed to reduce neurochemical signatures in each gut and brain region to individual principal components (PCs). Next, Pearson correlation was used to evaluate relationships between gut and brain PCs that contained the most variation in the data (i.e., PC1 and PC2). Two correlations were found below a conservative significance level of $p < 0.01$, between the jejunum PC2 (representing 28% of the neurochemical variation) and the striatum PC1 (representing 60.7% of the neurochemical variation), and the jejunum PC1 (representing 35.6% of the neurochemical variation) and the cerebellum PC2 (representing 15.5% of the neurochemical variation) (Table 1). This finding was particularly interesting because past work has indicated a role for the cerebellum in small intestinal motility (Manchanda et al., 1972; Zhu & Wang, 2008), and recent work suggests neural networks originating in the small intestine may contribute to the modulation of monoamine activity in the striatum (Han et al., 2018).

To follow up on the aforementioned relationship between the jejunum with both the cerebellum and striatum, Pearson’s correlation was then used to evaluate whether factors like access to ethanol, or sex, drives this relationship. PCA correlations suggest that the relationship between the striatum and jejunum is driven by the mice with water access (Table 1). Table 2 presents data showing notable neurochemical correlations that were observed between the striatum and jejunum.

## DISCUSSION

Alcohol binge drinking is a prevalent form of substance abuse that can have severe consequences on the body. GI dysfunction has been recently implicated in neurobiological diseases that are also linked with regular alcohol abuse; however, little is currently known about how alcohol abuse influences neurochemicals across the GBA in manners that may be related to the manifestation of disease (Bruce-Keller et al., 2018; Foster & McVey Neufeld, 2013; Lionnet et al., 2018). The influence of voluntary binge-patterned alcohol ingestion on monoamine neurochemical activity across the GBA remains underexplored, despite the known roles of monoamines in both gut and brain function (Mittal et al., 2017). The results of this study provided several key findings related to the influence of ethanol ingestion and sex on monoamine-related neurochemical expressions in mouse gut and brain structures. First, ethanol had a relatively mild effect on gut monoamine-related neurochemical concentrations, primarily influencing intestinal DA activity. Second, some evidence suggests that associations may exist between neurochemical signatures in the small intestine and the striatum, a major component of the brain reward circuit. This finding is consistent with recent work implicating the activity of neural networks in small intestinal with the release of dopamine in the striatum (Han et al., 2018). Third, the DID paradigm yielded some parallels to previous work using forced exposure to high volumes of ethanol, which primarily included augmented dopaminergic activity in striatal and brainstem areas. However, relatively few NE- and 5HT-related changes were observed across brain, outside of the hypothalamus. Finally, several sex-related differences in monoamine-related neurochemicals were observed across gut structures, which could have importance for understanding broader differences in gut function and GI disease between males and females. Together, these data provide insight into the influence of binge-patterned ethanol ingestion and sex on monoamine-related neurochemicals in the GBA.

To the best of our knowledge, this is the first study to investigate the impact of high-volume acute ethanol ingestion on gut monoamine-related neurochemical composition in a rodent model. One of the most surprising outcomes of this study was that alcohol ingestion only had a relatively mild influence on neurochemical concentrations across the gut (see Figures 2–4), despite mice exceeding NIAAA standards for pharmacologically significant BECs (see Figure 1) (Cranke et al., 2011; Gilpin & Koob, 2008). This outcome was unanticipated, given evidence suggesting that ethanol can inhibit the activity of enzymes (i.e., alcohol and aldehyde dehydrogenase) involved in the breakdown of all 3 monoamines measured in this study, which could influence the levels of these neurochemicals in the gut (Eisenhofer et al., 2004; Some et al., 2000; Zimak et al., 2006). Yet still, neurochemical changes, primarily DA-related, were detected in the gut following alcohol ingestion. A reduction in the ratio of DOPAC to DA concentration was observed across large intestine regions of mice that had access to alcohol during DID (see Figure 3), which may be indicative of an ethanol-related interference with DA turnover (Deitrich et al., 2006). In the small intestine, mice with access to alcohol during DID also displayed a mild increase of jejunal DA (see Figure 2). This finding could be similarly related to alcohol-induced interference with DA metabolism, thereby resulting in greater DA levels, as DA metabolite concentrations in this region were low, failing to reach the detectable limits of UHPLC. While the source of these dopamine-related changes from alcohol ingestion remains unknown, DA is in the GI tract, including multiple bacteria

| Principal Component | Correlation | All groups (n = 23) | Water (n = 11) | 20% Ethanol (n = 12) |
|---------------------|-------------|---------------------|--------------|---------------------|
|                     | r | p     | r | p     | r | p     |
| Striatum PC1        | 0.5289 | 0.0095 | 0.8273 | 0.0017 | -0.328 | 0.2979 |
| Jejunum PC2         | -0.5468 | 0.0069 | -0.5323 | 0.0919 | -0.5698 | 0.0531 |
| Cerebellum PC2      | 0.5328 | 0.0017 | 0.8273 | 0.0017 | -0.328 | 0.2979 |
| Jejunum PC1         | -0.5468 | 0.0069 | -0.5323 | 0.0919 | -0.5698 | 0.0531 |
A relationship was observed between neurochemical signatures in the jejunum and striatum of mice with water, but not ethanol, access (see Table 1). Taken in the context of other recent findings, this relationship may provide new insight into the monoaminergic influences on GBA communication. Indeed, the optogenetic stimulation of neural networks located in the small intestine has been shown to potentiate the release of dopamine in the striatum (Han et al., 2018), a critical component of the brain’s natural reward circuit. In the current study, the absence of relationship between small intestine and striatum neurochemicals of mice with alcohol access could provide novel evidence that ethanol ingestion might disrupt synchronous patterns of neural activity across the GBA. Monoamines in the intestine have been shown to mediate GI sensory information toafferent sensory nerve fibers (Bellono et al., 2017; Kaelberer et al., 2018; Martin et al., 2018). Therefore, it is possible that changes in luminal contents either directly from ethanol or indirectly (e.g., inflammation, bacterial changes, and bacterial by-products) affect the sensory signals being relayed to the striatum. However, ethanol has a potent influence directly on brain neurochemicals, which makes it difficult to disentangle the influence of intestinal modulators. Nonetheless, a potential alcohol-induced desynchronization of the coordinated communication between the gut and the brain could underlie the increased risk of disease, for example, in Parkinson’s disease and depression (Foster & McVey Neufeld, 2013; Gorky & Schwaber, 2016; Klingelhofer & Reichmann, 2015). Our data, taken together with previous work, provide new evidence suggesting synchronized neurochemical activity may exist between the small intestine and the brain’s natural reward circuit. The potential role for monoamines in coordinated communication between the gut and brain, as well as how ethanol ingestion may alter the GBA, warrants a more detailed investigation in future studies.

Many traditional models of binge drinking, such as oral ethanol gavage, intragastric administration, intraperitoneal injection, or water restriction to motivate drinking behavior, have provided foundational knowledge of brain neurochemistry following high-volume ethanol ingestion. While there is considerable variability...
in outcomes from these models with regard to levels of 5HT and NE (Das et al., 2016; De Witte, 1996; Gongwer et al., 1989; Milio & Hadfield, 1992; Murphy et al., 1988; Nurmi et al., 1994; Pohorecky & Jaffe, 1975), the preponderance of evidence suggests a role for the mesolimbic dopaminergic system (i.e., ventral tegmental area and nucleus accumbens) in the acute reinforcing effects of alcohol (Koob, 2014). The current DID paradigm parallels previous findings that elevated dopaminergic activity in brain reward structures following binge ethanol administration (Barbaccia et al., 1982; Dar & Woolies, 1984; Fadda et al., 1989; Honkanen et al., 1994; Imperato & Di Chiara, 1986; Murphy et al., 1988; Nurmi et al., 1994), while also observing fewer and less robust brain-wide monoaminergic responses outside of the hypothalamus (see Figures 6–8). Indeed, access to ethanol increased concentrations of DOPAC and HVA in the striatum area (see Figure 6), suggesting ethanol augments activity in the DA reward pathway. Consistent with other studies, the DA metabolite SAL was also present in the brain regions that contain prevalent DA pathways, including areas like the striatum and brainstem. In these regions, elevated SAL activity has received some attention for its possible role in linking the motivation to ingest high volumes of alcohol and neurodegeneration (Kurnik-Lucka et al., 2018; Peana et al., 2016). Given that SAL is a product of DA and acetaldehyde, it could be hypothesized that an increase in the ratio of SAL to DA might also be observed in the brainstem following a period of alcohol access. Contrary to expectations, the ratio of SAL to DA was actually lower in the striatum and unchanged in the brainstem following ethanol access (see Figures 6 and 8). However, the conditions that lead to the augmented SAL production remain poorly understood and may be more than just a consequence of alcohol abuse, also involving exogenous or endogenous sources of dysregulation within catecholamine enzymatic pathways (Kurnik-Lucka et al., 2018). Recent in vivo work found that SAL production was most stimulated following the intraperitoneal administration of high volumes of ethanol and L-DOPA in combination with a cocktail of inhibitors of enzymes involved in the breakdown catecholamines (Boston, 2016). Taken together, the current data underscore the importance of using rodent models of voluntary binge-patterned ethanol drinking (e.g., DID) to gain a more complete understanding of the acute effects that alcohol abuse has on brain function.

While not initially the primary focus of this study, several interesting sex-specific neurochemical responses were observed across the gut (see Figure 5). In fact, data collected from PCA suggested the sex differences in neurochemical concentrations may be pronounced in the small intestine (see Figure 5B). This finding adds to a body of growing literature suggesting the small intestine many contain a more robust sexually dimorphic neurochemical response than the large intestine. For instance, differential gene expression in the small and large intestine of mice found the majority of sexually dimorphic genes in the small intestine compared to the large intestine, and many of these genes were involved in regulating neurotransmission (Steegenga et al., 2014). Consistent with this observation, monoamine synthesis enzymes (i.e., L-amino acid decarboxylase) were found in higher concentrations in the small intestine, but not the colon, of male compared to female mice (López-Contreras et al., 2008). Thus, this could help explain differences in monoamine concentrations or turnover, like those observed in the current study (see Figure 2). Additionally, several studies have reported sex differences in the microbiome (de la Cuesta-Zuluaga et al., 2019; Hormone-dependent et al., 2013; Kim et al., 2020; Vemuri et al., 2019), which could be relevant because some microbial species synthesize 5-HT and DA in the gut (Galland, 2014). Sex differences in monoamine-synthesizing enzymes and the microbiome could account from at least some of the sex differences (less L-DOPA in the female jejunum and ileum, less 5-HIAA in the ileum, greater levels of NE turnover in the jejunum, and more DA and 5HT in the large intestine cecal contents) observed in the current study (see Figures 2 and 3). Taken together, these data may provide some insight for understanding sex-biased GI function or dysfunction. For instance, sex differences found in GI monoaminergic systems may contribute to the variation in treatment efficacy between males and females with irritable bowel syndrome (Viramontes et al., 2001). However, monoaminergic sex differences in the intestinal tract have not been well characterized, and consequently, the potential functions currently remain less clear should be followed up with more detail in future work.

Furthermore, sex differences in liver 5HT concentrations following ethanol ingestion in the current study could have relevance for understanding susceptibility to liver damage. Indeed, the liver is the body’s main source of alcohol metabolism and can become acutely damaged following ingestion of ethanol. In the current study, female mice with access to ethanol displayed higher concentrations of liver 5HT than male mice (see Figure 4). Female sex-specific hormones, such as estradiol, reduce the activity of 5HT reuptake transporters and degradation enzymes (Benmansour et al., 2012; Koldzic-Zivanovic et al., 2004). Therefore, it is possible that alcohol-stimulated 5HT activity in the liver will be less likely to clear in females than males, thereby leading to greater tissue concentrations of 5HT in the female liver (see Figure 4). This potential outcome is particularly noteworthy because evidence indicates that livers of women who drink alcohol excessively may be more prone to damage than males. In rodent models, females are more likely to display alcohol-induced liver steatosis following a single episode of high-volume alcohol exposure, a process dependent on serotonin receptor activity (Pang et al., 2020; Wagnerberger et al., 2013). Taken together, sex-related hormones may be driving the ethanol-induced increase in 5HT concentrations in a manner that contributes to female susceptibility to alcohol-related liver injury. Future studies could investigate this topic as it could have importance for the etiology of liver disease in women that struggle with alcohol use disorder.

The present study added several new perspectives furthering our understanding of influence of alcohol binge-like drinking and sex on GBA physiology. Alcohol’s influence on monoamine concentrations in the gut and brain could play key roles in altering communication across the GBA in manners that may contribute to the development, or severity, of neurobiological and intestinal diseases. Moreover, potential sexual dimorphism of monoaminergic
activity within the gut could have key implications for reported differences in gut functions between males and females, as well as sex-specific treatments for intestinal disorders known to involve abnormal monoamine activity. Together, these findings lay the foundation for understanding the role of monoamines in GI function, GBA communication, and provide insight into targeted interventions (based on diet, lifestyle, sex, etc.) for diseases associated with alcohol misuse and altered GBA communication. The functional implications of GBA monoaminergic-related neurochemical differences due to alcohol or mouse sex should be topics for future research.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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