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**Prenatal alcohol exposure reduces 5-HT concentration in mouse intestinal muscle and mucosa.**

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## Abstract

1  
2 The influence of prenatal alcohol exposure on the serotonergic system in the brain has been well  
3 studied, however its influence on the serotonergic system in the gastrointestinal system remains  
4 unknown. The objective of the study was to use a mouse model of prenatal alcohol exposure to  
5 investigate the effects on serotonin and its metabolites and precursors in colonic tissue. This study  
6 used treatment of mouse breeding harems with 5% ethanol with saccharin via drinking water  
7 throughout pregnancy and compared the results with a saccharin control group. Tryptophan,  
8 serotonin (5-HT) and 5- hydroxyindoleacetic acid (5-HIAA) concentrations were measured in the  
9 longitudinal muscle myenteric plexus (LMMP) and mucosa of intestinal tissue by high-performance  
10 liquid chromatography (HPLC). Decreased 5-HT concentrations in mucosa and LMMP (females only)  
11 were observed in prenatally exposed mice compared to controls. Increases in mucosal and LMMP  
12 tryptophan concentration were only observed in prenatally exposed female mice. In conclusion,  
13 prenatal alcohol exposure causes a decrease in conversion of tryptophan to 5-HT in both muscle and  
14 mucosa although the effect is more pronounced in females. The observed sex difference may be  
15 related to changes associated with the estrous cycle.  
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41 Keywords: prenatal alcohol exposure, gastrointestinal, serotonin, tryptophan, colon, myenteric  
42 plexus  
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44 Abbreviations:  
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46 5-HT – serotonin  
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48 5-HIAA- 5- hydroxyindoleacetic acid  
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50 HPLC- high-performance liquid chromatograph  
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52 LMMP – longitudinal muscle myenteric plexus  
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## 1. Introduction

In 1973 Jones et al. described the characteristics of Fetal Alcohol Syndrome (Jones, 2011). It is now recognized that prenatal alcohol exposure causes a wide range of neurobehavioral deficits, encompassed by the term fetal alcohol spectrum disorders (Hoyme et al., 2016). Fetal alcohol syndrome is considered the most severe form of these disorders and is characterised by growth retardation, such as low birth weight, lack of weight gain over time, disproportional low weight to height and a characteristic pattern of facial anomalies, such as short palpebral fissures, thin vermilion border and flattened philtrum. There are also specific neurological signs such as impaired fine motor skills, neurosensory hearing loss and cognitive impairment (Kodituwakku, 2010; Mukherjee et al., 2006; Willford et al., 2004).

The effect of prenatal alcohol exposure on the serotonergic system in the brain has been studied specifically. Tajuddin et al. described a decrease in serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) in the cerebrocortex of the offspring of ethanol-fed rats (Tajuddin and Druse, 1988). It has also been demonstrated that there is a remarkable deficit of 5-HT<sub>1</sub> receptors in both motor and somatosensory cortex of rats prenatally exposed to alcohol (Clausing et al., 1996) and that serotonin uptake is decreased in the motor cortex (Tajuddin and Druse, 1988). The serotonergic system of the gastrointestinal tract is based on analogous mechanisms to the serotonergic system of the brain (De Ponti, 2004) however the effects of prenatal alcohol exposure have not been studied.

Clinical evidence suggests that prenatal alcohol exposure results in long-lasting effects on the gastrointestinal tract. In a Finnish cross-sectional study the morbidity of gastrointestinal system among individuals with FASD was as high as 26% (Autti-Ramo et al., 2006). Werts et al. reported constipation being a common problem among children prenatally exposed to alcohol (Werts et al., 2014) while Kvigne et al. emphasized the high prevalence of feeding problems and diarrhoea (Kvigne et al., 2009). There is also evidence that individuals with fetal alcohol spectrum disorders experience gastrointestinal motility disorders presenting as chronic pseudoobstruction syndrome (Uc et al., 1997). However limited studies have explored the mechanisms behind these functional changes in gastrointestinal motility.

5-HT is a key signaling molecular within the colon, where it is located within the enterochromaffin (EC) cells present within the mucosal epithelium and neurons within the myenteric plexus. Numerous studies have shown that intestinal serotonin is a key pro-kinetic molecule that drives motility (Bertrand and Bertrand, 2010; Gershon, 2004; Mawe and Hoffman, 2013). The aim of

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this study was to use a mouse model of prenatal alcohol exposure to investigate the effects on 5-HT and its metabolites and precursors in gut tissue.

## 2. Material and Methods

### 2.1 Animal Husbandry

All procedures were licensed under the UK Animals (Scientific Procedures) Act 1986 and EU directive 2010/63/EU and complied with the ARRIVE guidelines. They were approved by the University of Brighton animal welfare and ethics review board. C57BL/6J mice were maintained at  $19.0 \pm 1$  °C, 55 % humidity and fed on either a breeding diet (RM3 (E) 801002 chow, Special Diet Services) (breeding harems) or a maintenance diet (RM1 (E) 801002 chow, Special Diet Services) (offspring) *ad libitum*. The mice were maintained on a 12-hour light/ dark schedule, lights on 0700h (60 Lux at cage level).

### 2.2 Prenatal Alcohol exposure (PAE)

Breeding harems were established with one male to 3-4 females. Following an adaptation of the maternal ethanol consumption model described by Kleiber et al. (Kleiber et al., 2011), the harems received fluid *ad libitum* under a two-bottle choice. The alcohol exposure groups had 24 hour access to both a bottle of 5% ethanol sweetened with 0.066% saccharin solution and a bottle of tap-water. In order to eliminate the influence of saccharine on the experiment results, the control group had 24 hour access to a bottle of 0.066% saccharin solution and a bottle of tap-water. The volume of liquid consumed from each of the two bottles in each cage was recorded daily. It was assumed that the consumption was approximately equal between different mice within each cage in order to estimate individual alcohol consumption. Offspring were weaned at 20 days and group-housed in same-sex, littermate cages with free access to food and tap-water. **The offspring have not been exposed to alcohol since weaning. Thus, ethanol exposure was from pre-conception, throughout gestation and throughout lactation. It must be remembered that there is evidence of a detrimental effect of ethanol on the fetus in the very early stages of pregnancy, but also in the last trimester in humans, which equates to the first few post-natal days in rodents. This animal model of PAE was previously described by Allen et al.(Allan et al., 2003a)**

### 2.3 Blood alcohol concentration determination

Blood alcohol concentration was determined by gas chromatography. Briefly, blood was collected by cardiac puncture post mortem from a small sample of male and female ex-breeders between 1100h and 1300h. Plasma samples were deproteinated with 10% trichloroacetic acid spiked with 1 % ethanol and centrifuged at 9400 g for 10 minutes. The supernatant was filtered before gas

1 chromatography analysis. Standards and samples, both with an internal standard (1% propan-1-ol),  
2 were analysed on a Perkin Elmer Clarus 500 gas chromatograph with a Zebron Phase (ZB-waxplus  
3 column), equipped with a flame ionization detector. The optimal operating conditions were as  
4 follows: oven temperature 35°C and flame ionisation detector temperature 150°C, with the injection  
5 temperature 250°C and the capillary temperature 250°C; hydrogen and air pressure were set at  
6 optimal conditions of 58psi and 60psi, respectively.  
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## 10 11 12 **2.4 Tissue sampling and analysis**

13 Colonic mucosa and LMMP tissue samples were harvested from the progeny of the breeding harem  
14 under alcohol exposed and control conditions at 3-6 months of age and stored at -80 Celsius.  
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16 To measure levels of tryptophan, 5-HT and 5-HIAA, tissue samples were placed in 0.1M perchloric  
17 acid. The mixture was then centrifuged at 13,200 g at 4 C for 10 min and the resultant supernatant  
18 was analyzed using HPLC. HPLC apparatus consisted of a Jasco HPLC pump (Model: PU-980) and  
19 Rheodyne manual injector equipped with a 20 µl loop. A Kinetic ODS 2.6 mm 100 mm x 2.1 mm i.d.  
20 analytical column with an in-line guard column (Phenomenex, Macclesfield, UK) was employed. The  
21 HPLC system was run at a flow rate of 100 mL min<sup>-1</sup>. CHI630B potentiostat (CH Instruments, Austin,  
22 TX, USA) was used to control the detector voltage and record the current. A 3-mm glassy carbon  
23 electrode (flow cell, BAS) served as the working electrode and was used with a Ag|AgCl reference  
24 electrode and a stainless steel block as the auxiliary electrode. Amperometric recordings were  
25 carried out, where the working electrode was set at a potential of +950 mV vs. Ag|AgCl reference  
26 electrode. Control and data collection/processing were handled through the CHI630B software. The  
27 stock buffer for the mobile phase was comprised of the following: 0.1 M sodium acetate, 0.1 M citric  
28 acid and 27 mM disodium ethylene-diamine-tetra-acetate (EDTA). This was then buffered to pH 3.0.  
29 The mobile phase was prepared with the stock buffer mixed with methanol in the ratio of 8 : 2 (v/v)  
30 and degassed after mixing.  
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## 44 **2.5 Data Analysis**

45 The peak areas for the responses for 5-HT, 5-HIAA and tryptophan were measured from the  
46 chromatograms and converted to concentration using calibration plots as previously shown (Parmar  
47 et al., 2011). The concentration of tryptophan, 5-HT and 5-HIAA in colonic LMMP and mucosa from  
48 male and female offspring were analysed using 2-way analysis of variance followed by Tukey post-  
49 hoc analysis. P values less than 0.05 were deemed to indicate significant differences.  
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## 54 **3. Results**

### 55 **3.1 Alcohol intake and blood alcohol levels**

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Seven breeding cages were monitored: three control cages and four PAE cages. Daily fluid consumption (per animal) was recorded over 10 weeks and plotted as a daily average over a weekly period and a measure of alcohol intake per weight of animal was calculated for female breeders; the average ethanol intake for the female breeders was  $8.9 \pm 0.4$  g/kg/24hours. The total volume of fluid ingested was significantly lower in the alcohol group compared to control ( $p < 0.0001$ , unpaired t-tests). Only 3 of the 5 blood samples tested had measurable blood alcohol concentrations, with values of 1.885 mg/dL, 6.549 mg/dL and 4.793 mg/dL, giving a mean value ( $\pm$  s.e.m.) of  $2.65 \pm 1.31$  mg/dL. Progeny weight was determined prior to sacrifice: overall there was no difference in weight between control and PAE animals. **Data from other publications using similar ethanol dosing schedules have measures blood alcohol concentrations at the following the period of maximum intake. For example Brady et al. (Brady et al., 2012a) and Allan et al. (Allan et al., 2003b) report peak blood alcohol concentrations of 80-120 mg/100ml using similar doses of alcohol. Our quoted values were taken at the time of lowest expected blood alcohol concentration and are given to illustrate that there is constant low-dose exposure on the fetus.**

### 3.2 Mucosa

Tissues were harvested from 4 male and 4 female mice of the saccharine control group and 4 male and 3 females of the prenatal alcohol exposure group (??)

#### Synthesis of 5-HT

The highest mean concentration of tryptophan in mucosa was observed in the group of female mice exposed to alcohol ( $67.78 \mu\text{mol/l} \pm 6.74$ ) followed by females from the saccharine group ( $35.99 \mu\text{mol/l} \pm 11.09$ ), males exposed to alcohol ( $31.25 \mu\text{mol/l} \pm 16.86$ ) and males from the saccharine group ( $25.45 \mu\text{mol/l} \pm 16.00$ ). There was a statistically significant difference in mean mucosal tryptophan concentration between females from alcohol and control group ( $p < 0.05$ ) and males and females within the alcohol exposed group ( $p < 0.05$ ) [Figure 1A].

A difference in 5-HT/tryptophan ratio was observed in both sexes. The mean ratios in alcohol exposed group and saccharine group were  $0.0018 (\pm 0.0015)$  vs.  $0.078 (\pm 0.046)$ , respectively in females ( $p < 0.001$ ) and  $0.0017 (\pm 0.0008)$  vs.  $0.12 (\pm 0.048)$  in males, respectively ( $p < 0.01$ ) [Figure 1D].

#### Levels of 5-HT

Among the female mice there was a significant ( $p < 0.05$ ) decrease in mean mucosal 5-HT concentration in the ethanol-exposed group in comparison to control group ( $0.13 \mu\text{mol/l} \pm 0.10$  vs.  $3.06 \mu\text{mol/l} \pm 1.99$ ). The same effect was observed in male mice, the mean 5-HT concentration in the

1 exposed group ( $0.044 \mu\text{mol/l} \pm 0.01$ ) was more than 100-times lower than in the control group ( $3.15$   
2  $\mu\text{mol/l} \pm 2.29$ ) [Figure 1B].

### 3 **Turnover of 5-HT**

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6 There was no significant difference in mean 5-HIAA concentration in the mucosa between alcohol  
7 exposed and control group or between male and female mice in both groups [Figure 1C].

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10 In both sexes there was a significant difference in 5-HIAA/5-HT ratio. In males the mean 5-HIAA/5-HT  
11 ratio was  $25.2 (\pm 7.32)$  in the alcohol group and  $0.525 (\pm 0.37)$  in the control group,  $p < 0.001$  while in  
12 females the ratios were  $31.27 (\pm 13.28)$  and  $0.97 (\pm 0.87)$ , respectively ( $p < 0.05$ ) [Figure 1E].

## 16 **3.3 LMMP**

### 17 **Synthesis of 5-HT**

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20 The difference in tryptophan concentration in muscle tissue between the alcohol exposed and the  
21 saccharin group was only observed in females ( $81.26 \mu\text{mol/l} \pm 19.15$  vs.  $37.48 \mu\text{mol/l} \pm 16.66$   
22 respectively  $p < 0.05$ ). The concentrations in males were:  $41.86 \mu\text{mol/l} \pm 20.99$  and  $14.1 \mu\text{mol/l} \pm 8.02$ ,  
23 respectively [Figure 2A].

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26 A 5-HT/Tryptophan ratio was significantly lower in alcohol exposed groups in both sexes. In females  
27 the ratios were  $0.0003 \pm 0.0002$  and  $0.042 \pm 0.02$ , respectively ( $p < 0.01$ ). In males the ratios were  
28  $0.0003 \pm 0.0002$  and  $0.054 \pm 0.02$ , respectively ( $p < 0.05$ ) [Figure 2D].

### 29 **Levels of 5-HT**

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32 Similarly, the difference in 5-HT concentration in muscle between alcohol and control group was  
33 noted only in females ( $0.02 \mu\text{mol/l} \pm 0,0$  vs.  $1.3 \mu\text{mol/l} \pm 0.72$ , respectively,  $p < 0.05$ ). The  
34 concentrations observed in males were:  $0.01 \mu\text{mol/l} \pm 0.01$  and  $0.05 \pm 0.02$ , respectively [Figure 2B]

### 35 **Turnover of 5-HT**

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38 There was no difference in 5-HIAA concentration in muscle between sexes or exposure groups.  
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42 The 5-HIAA/5-HT ratio was significantly higher in exposed females than in control females ( $37.96$   
43  $\pm 23.22$  vs.  $0.81 \pm 0.39$ , respectively,  $p < 0.001$ ). Interestingly, there was also a difference between  
44 alcohol exposed females and alcohol exposed males ( $37.96 \pm 23.22$  vs.  $>50.86 \pm 22.36$  respectively,  
45  $p < 0.05$ ) [Figure 2E].

## 46 **4. Discussion**

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1 The dose of alcohol used in the current study is lower than that used in many prenatal alcohol  
2 exposure models. The average ethanol intake per breeding female was approximately 9g/kg/day.  
3 Taking into account the different apparent volumes of distribution for ethanol (total body water) in  
4 female humans and mice (0.63 l/kg v. 0.8 l/kg) and the greater rate of ethanol elimination in mice  
5 (Cederbaum, 2012), the ethanol intake in the current study equates to a human daily ethanol intake  
6 of approximately 8g, the equivalent of less than one bottle of table wine. Similar daily consumptions  
7 in mice have been reported as resulting in blood alcohol concentrations averaging 80 to 120  
8 mg/100ml (Allan et al., 2003b; Brady et al., 2012b). Our results identified concentrations significantly  
9 lower than these, which could be explained by rodents drinking primarily during the dark phase  
10 (which in the current would be 1900-0700h) compared to blood sampling some 5 hours later. Allan  
11 et al. (Allan et al., 2003b) monitored blood alcohol concentrations of B6SJL/F1 mice over a 24 hour  
12 period (with lights on 0700-1900) and found that administration of 14 g/kg/day of ethanol, resulted  
13 in peak blood alcohol concentrations of 140 mg/100ml at approximately 0100h and minimum  
14 concentrations of 50 mg/100ml between 0900-1200h. Our blood collection was conducted at the  
15 time of lowest blood alcohol and it revealed very variable blood alcohol concentrations, although a  
16 mean of approximately 0.265 mg/100ml clearly indicates that our 9g/kg/day average-model results  
17 in lower-dose exposure to alcohol than the 14g/kg/day.  
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30 This is the first study to evaluate the influence of low-dose prenatal alcohol exposure on the  
31 serotonergic system in colonic tissue  
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35 We observed a decrease in 5-HT in the mucosa among prenatally exposed male and female mice  
36 together with an increased concentration of tryptophan in the mucosa of the females. In the LMMP,  
37 we observed a significant decrease in 5-HT in females but not in males albeit the trend was similar.  
38 There were increased tryptophan levels in female mice only. The decreased 5-HT synthesis in both  
39 mucosa and muscle could result from decreased tryptophan hydroxylase and/or amino acid  
40 decarboxylase function and result in further tryptophan accumulation. If this decrease is due to  
41 tryptophan hydroxylase, this would suggest that both tryptophan hydroxylase 1 and 2 are both  
42 altered due to prenatal exposure to alcohol. This finding is in contrast with previous reports which  
43 demonstrated a decrease only in tryptophan hydroxylase 1, in the brain. Moreover, in our study we  
44 observed no decrease in 5-HIAA, which suggests that serotonin transporter SERT in the intestinal  
45 tissue is not affected by prenatal alcohol exposure, while in previous reports (Zafar et al., 2000) the  
46 function of SERT was decreased in the brain. This finding might be explained by the differences in  
47 development and maturation of serotonergic system in the brain and in the intestinal tissue  
48 (Gershon & Erde, 1981).  
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1 That the observed decrease in 5-HT concentration in mucosa is not a result of increased monoamine  
2 oxidase activity is demonstrated by the fact that there is no increase in 5-HIAA concentrations nor an  
3 increase in 5-HIAA/5-HT ratios.  
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6 In muscle the same trend was observed, although the difference in 5-HT concentration between  
7 male mice prenatally exposed to alcohol and male controls is not statistically significant. However the  
8 tryptophan accumulation is observed in both sexes.  
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11 Again the decreased gastrointestinal muscle 5-HT is probably due to decreased tryptophan  
12 hydroxylase activity rather than increased release and metabolism of 5-HT. The results therefore  
13 indicate that prenatal alcohol exposure results in decreased synthesis of 5-HT in gastrointestinal  
14 muscle and mucosa at 3-6 months of age (young adult), several months after the last exposure to  
15 alcohol.  
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19 Previous work by Clausning et al. (Clausning et al., 1996) indicated a sex difference in the effects of  
20 prenatal alcohol exposure with a decrease in striatal 5HT and 5HIAA seen in females only. On the  
21 other hand, Asghari et al. (Asghari et al., 2011) reported differences in tryptophan hydroxylase 1  
22 activity in the brain throughout estrous cycle and López-Contreras et al. reported sex differences in  
23 dihydroxyphenylalanine decarboxylase activity in the intestine between the sexes (López-Contreras  
24 et al., 2008). **There is also evidence for sex differences in serotonergic gastrointestinal system among**  
25 **humans. Viramontes et al.(Viramontes et al., 2001) documented different responsiveness to 5-HT3**  
26 **antagonist (aloseptron) among females and males. Houghton et al. studied 5-HT concentration in**  
27 **platelet-depleted plasma of both IBS and healthy male and females (Houghton et al., 2009).** The  
28 authors documented the influence of oestrous cycle on 5-HT levels. Due to ethical reasons, direct  
29 measurement of mucosal and muscle 5-HT and its metabolites have not been performed on humans.  
30 The sex difference observed in the current study may therefore reflect a sex difference consequent  
31 to variations in female reproductive hormones.  
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45 In conclusion, clinical evidence suggests that children with fetal alcohol spectrum disorders (FASD)  
46 are more likely to suffer from disorders associated with decreased gut motility such as constipation  
47 or pseudoobstruction syndrome. The results of the current study in a mouse model of FASD  
48 indicates that low-dose prenatal alcohol exposure results in decreased conversion of tryptophan to  
49 5-HT, and therefore reduced tissues storage of 5-HT. Such transmitter depletion might be expected  
50 to be associated with decreased gut motility. Strategies to overcome the 5-HT deficit, such as  
51 monoamine oxidase inhibitors, may therefore be of use in the treatment of these children.  
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3 **Declaration of interest:**

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5 The authors report no conflict of interest.  
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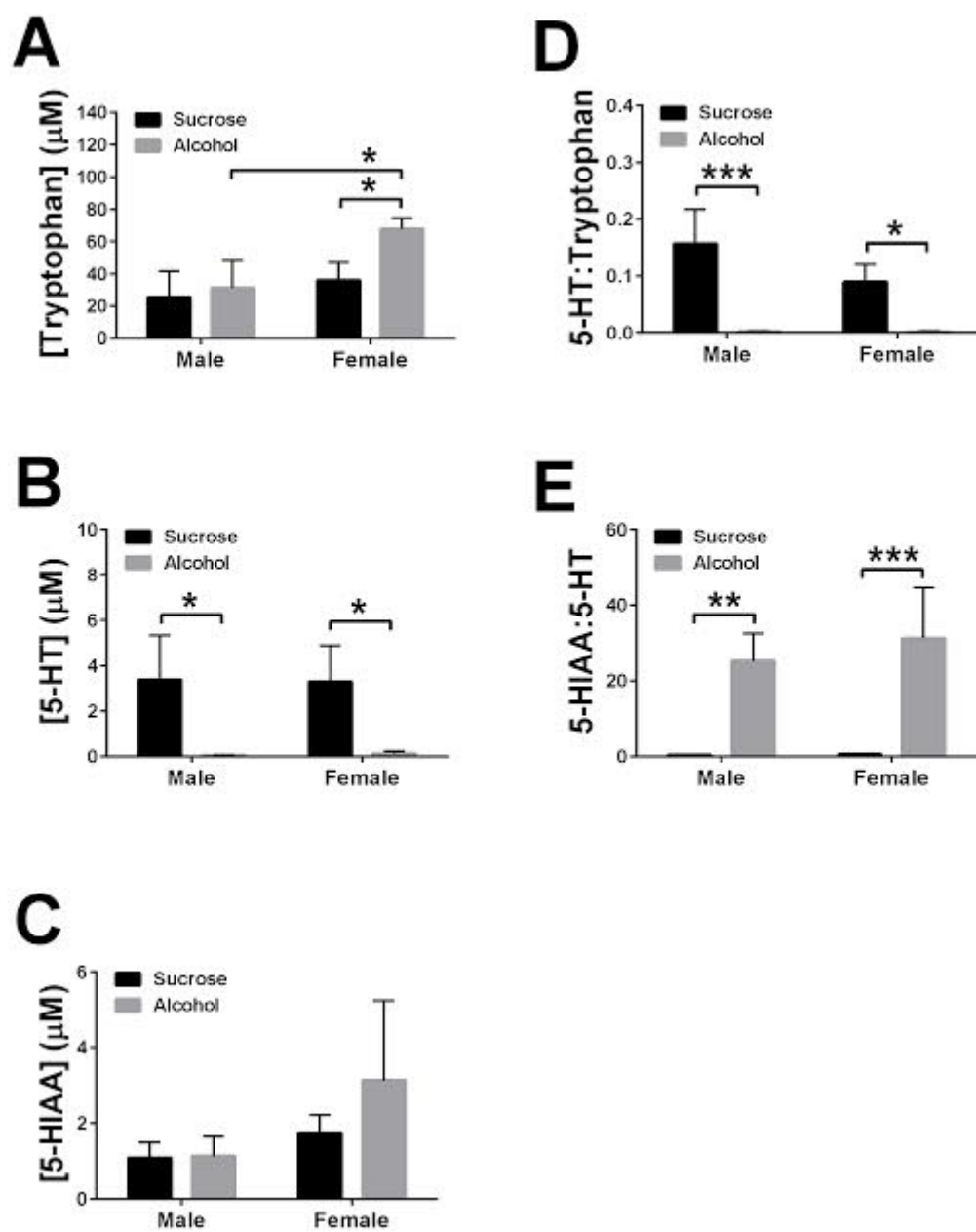
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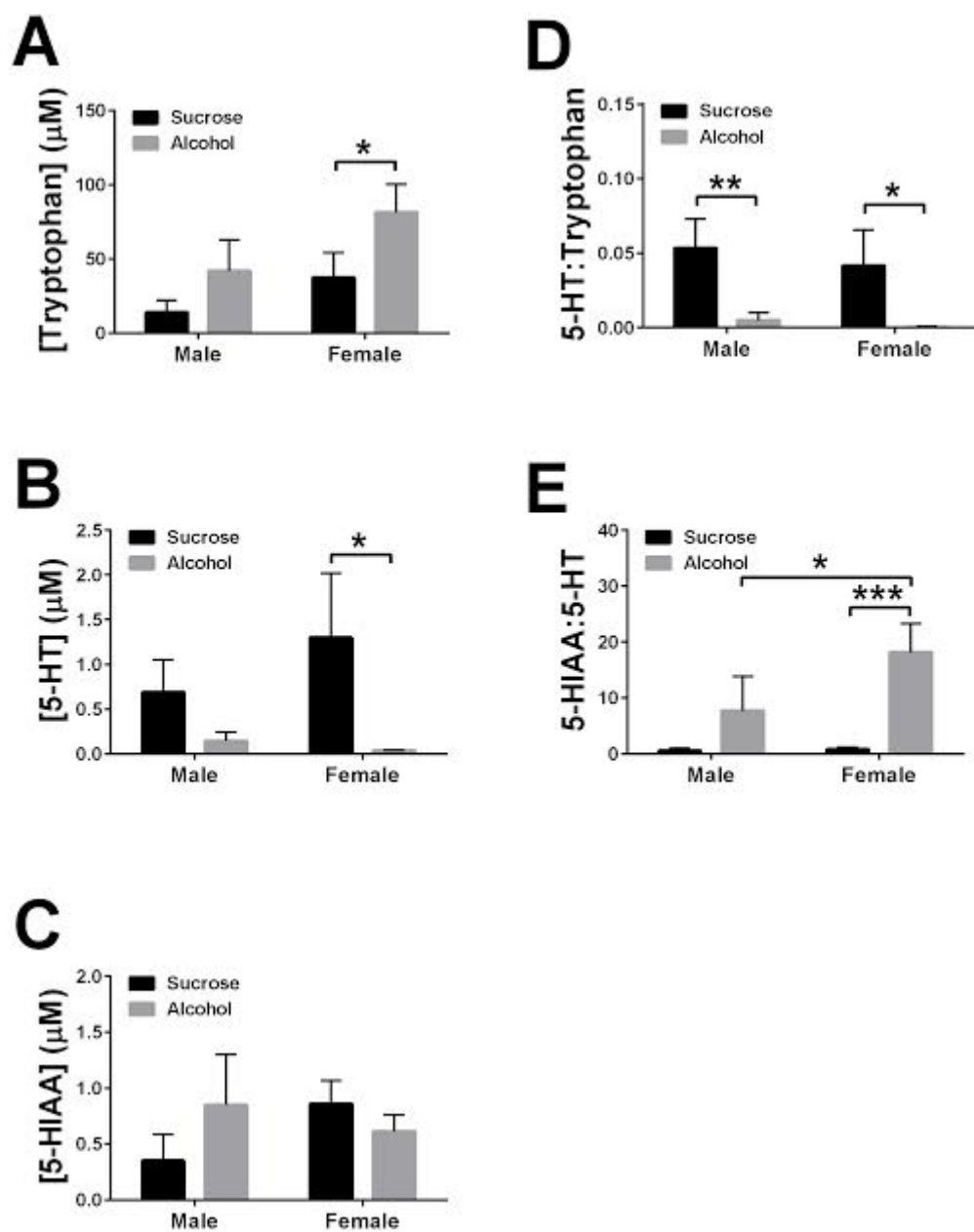
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**Figure 1.** Tryptophan, 5-HT, 5-HIAA concentration and 5-HT/Tryptophan and 5-HIAA/5-HT ratio in intestinal mucosa of mice prenatally exposed alcohol in comparison to control group.



**Figure 2.** Tryptophan, 5-HT, 5-HIAA concentration and 5-HT/Tryptophan and 5-HIAA/5-HT ratio in intestinal muscle of mice prenatally exposed alcohol in comparison to control group.