No Effect of Prenatal Ethanol Exposure on Number or Distribution of Islet-1 Expressing Cells in the Lateral Ganglionic Eminences of Swiss Webster Outbred Mice

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ABSTRACT

Fetal Alcohol Spectrum Disorders (FASD) is the label given to birth defects causing impairment in motor movement, behavior, cognition, and facial morphology to the fetus due to alcohol consumption during pregnancy. In animal studies, prenatal ethanol exposure was found to cause apoptosis of basal ganglia cells, causing a reduction in overall size. The basal ganglia are responsible for control of voluntary movements, cognition, procedural learning, among other functions. The decrease in size of the basal ganglia could relate to the motor impairment and cognitive problems that are observed as a phenotype of FASD. We are using a Drinking in the Dark (DID) paradigm to model FASD in mice and to mimic moderate human binge drinking. In this paradigm, for 2-4 hours during the dark period when mice are most active, tap water and a 20% ethanol solution were given to the control and experimental group respectively. An islet-1 antibody was used to label striatal cells within the area of the basal ganglia. We hypothesized that within the lateral ganglionic eminences (LGE), the location of developing striatum within the basal ganglia, that the number of islet-1 cells would decrease and there would be a difference in the distribution in the cells of embryonic day (E) 14 mice with prenatal ethanol exposure. The data suggests that the hypothesis was not supported and no significant difference between groups for islet-1 cell count or distribution of the cells within the LGE was found. This study suggests that there is no difference in striatal cell count, at this time in development, with prenatal ethanol consumption using the DID model in Swiss Webster mice.

Abbreviations: FASD- Fetal Alcohol Spectrum Disorders; BAC - Blood alcohol concentration; DID - Drinking in the Dark; LGE - Lateral ganglionic eminences; E - Embryonic day; MGE - Medial ganglionic eminence; PBS - phosphate buffered saline; TUNEL - Terminal deoxynucleotidyl transferase dUTP nick end labeling.

Keywords: Fetal Alcohol Spectrum Disorder, Basal Ganglia, Ethanol Exposure, Drinking in the Dark

Introduction

Alcohol consumption during pregnancy can cause a wide range of developmental problems for the offspring that are classified as Fetal Alcohol Spectrum Disorder (FASD) (Mattson, 1998; Archibald, 2001). Morphological and structural changes that can occur as a result of FASD include low birth weight, reduced growth, craniofacial abnormalities, microcephaly, or abnormalities in brain structures, (Stratton, 1996). Neurological problems can include seizures, impairments in motor skills and sensory processing, while functional problems include developmental and cognitive disabilities, including learning delay, impulse control, memory and attention problems (Stratton, 1996). This is a spectrum disorder in which the more ethanol that is consumed prenatally usually corresponds to a greater severity of impairments for the offspring, affecting their quality of life (Middaugh, 1995). By gaining a greater understanding of the effects of prenatal ethanol exposure during fetal development, we can better predict the negative consequences and spread awareness of the dangers of drinking during pregnancy.

It is believed that the basal ganglia is responsible for motor function (Packard, 2002). However, the dorsal striatum of the basal ganglia is believed to be linked to learning and memory (Packard, 2002). In humans, this decrease in the size of the basal ganglia can be the origin of the motor
Impairments as well as cognitive delays that are exhibited by patients with FASD. In human studies, previous research discovered a decrease in size of only the basal ganglia in embryonic brains exposed to ethanol (Norman, 2009; Riley, 2005). Additionally, other studies found that the caudate nucleus, which is part of the basal ganglia, also appears significantly smaller after ethanol exposure (Archibald, 2001; Mattson, 1992). There are different ways in which to mimic prenatal ethanol exposure in mouse models. In a study by Sari (2009), pregnant dams were given a continuous liquid diet of 25% ethanol solution from embryonic day (E) 7 to 13 (Sari, 2009). The findings from the study included decreased brain size, but an inverse increase of Terminal deoxynucleotidyl transferase dUTP nick end labeling, or TUNEL, positive cells. TUNEL is a stain that is able to detect DNA fragments, which suggests that the decrease in brain size was from cell apoptosis (Sari, 2009). Another method of introducing the ethanol prenatally is through intraperitoneal injection (Schambra, 1990; Dunty 2001). Both of these studies did a series of injections in order to maintain a blood ethanol concentration of 500-600 mg/dl (Schambra, 1990). What they found in this mouse study was that there were deficits to the neostriatum, including forebrain deficiencies in both the lateral and dorsal region and loss of midline structures in both E14 embryos and E18 fetuses (Schambra, 1990). Apoptosis of the basal ganglia, diencephalon, pons, and developing cerebellum were also found in E11 mice following a 2.9 g/kg dosage of ethanol at 0.5 day increments at varying stages of development from E6.5 to 11 in mice (Dunty, 2001). Overall, prenatal ethanol exposure has been linked to a decrease in size of the forebrain structures, including the basal ganglia, which is attributed to a decrease in neurons caused by apoptosis (Schambra, 1990; Sari, 2009; Dunty, 2001; Norman, 2009; Riley, 2005).

Within the basal ganglia are the ganglionic eminences, which are a transitory brain structure present in embryonic and fetal brain stages that help to guide axon migration (Encha-Razavi, 2003). The ganglionic eminences are made up of two main components, the lateral ganglionic eminences (LGE) and the medial ganglionic eminences (MGE). The LGE is also the origin of cells that are committed to creating striatal cells in the developing brain (Deacon, 1994). During development, the LGE comprise the striatal primordium. In the adult brain, the basal ganglia is made up of the caudate, putamen, and the globus pallidus. Islet-1 immunostaining is able to label the striatal primordium of these developing brains (Wang, 2001). There are two neuronal populations called the striatonigral and striatopallidal neurons, and both are involved in controlling movement-related functions in the basal ganglia circuits (Lu, 2014). Islet-1 is an LIM homeodomain transcription factor that is specifically expressed in striatonigral neurons during development (Lu, 2014). In this way Islet-1 is involved in cell survival, differentiation, and axonal projections of striatonigral neurons within the basal ganglia of developing brains (Lu, 2014). We stained for islet-1 to see if ethanol causes a change in the size of the striatum within the LGE of mice.

In this study we used the Drinking in the Dark (DID) model that was created by Boehm et al. (2008), which allows voluntary drinking to mimic binge drinking, which is most similar to human drinking during pregnancy. The Center for Disease Control defines binge drinking as a pattern of drinking that brings a person’s blood alcohol concentration (BAC) to 0.08 grams percent or above (National Institute of Alcohol Abuse and Alcoholism). This type of drinking typically happens when men consume five or more drinks, and when women consume four or more drinks, in about two hours (National Institute of Alcohol Abuse and Alcoholism). Binge drinking is one of the most common drinking models of mothers who produce FASD offspring with more severe symptoms (Wechsler, 1995). Other ways to administer ethanol include intraperitoneal injection (Shamba 1990; Dunty 2001) and intragastric intubation with an ethanol-liquid diet (Sari, 2009). These methods, which were used in the previously mentioned studies, resulted in decreased basal ganglia size, most likely by apoptosis, as well as changes in the neurostriatum. However, because the methods of introducing ethanol can produce stress that could alter the development of the offspring, this can
also mask the negative effects of the ethanol exposure. In this study the voluntary drinking paradigm would potentially reduce the amount of stress present for the pregnant female, and reducing this extraneous stress would hypothetically allow the ethanol exposure to be the main contributor to any neurodevelopmental changes. For this investigation the region of interest within the basal ganglia is the LGE of E14 mice brains prenatally exposed to ethanol compared to water exposed controls. An islet-1 antibody was used to stain the developing striatum within the LGE, and the focus was not only on the number of islet-1 stained cells present in the LGE, but also the distribution of the cells across the LGE overall. In this study we hypothesized that by using the DID model of ethanol exposure, the resulting pups would have a decrease in the count and distribution of islet-1 labeled cells within the LGE of the basal ganglia. Following previous research the embryos were expected to have a decreased size of the basal ganglia, and therefore LGE, from apoptotic cell death (Dunty, 2001; Schambra, 1990; Sari, 2009). If cell death were to occur, the average number of islet-1 counted cells would decrease, confirming the hypothesis. If cell death were to occur, then it is probable that the distribution of the islet-1 stained cells within the LGE would change, where one or more areas could experience more or less cell death more than others. When looking at the distribution across the whole LGE, if there was a difference either related or not to the amount of cell death, then this could also confirm the hypothesis of a change in the distribution of cells within the LGE resulting from prenatal ethanol exposure. This shift in distribution of the striatum at this time in development could also impact the resulting development of the basal ganglia overall. These changes in development of the basal ganglia both in cell count and distribution could contribute to the neurological and phenotypic behaviors associated with Fetal Alcohol Spectrum Disorders (FASD).

Materials and Methods

Mouse Breeding

Swiss Webster mice from Ace Animals (Boyertown, PA) were used in this experiment. To get E14 embryos, female mice were 8-12 weeks old when introduced to males by relocating individual females to a cage that contained a single male. The female mouse was allowed one night for breeding then they were then placed back into their original cages. Females were considered pregnant when the females gained 2 grams of weight by E7. All mice were on a reverse twelve hour light/dark cycle with lights off at 10 am.

Ethanol Exposure

All pregnant females were exposed to the Drinking in the Dark (DID) model, eight females were randomly assigned to receive a sipper tube of either tap water (control, N=4 embryonic brains, 1 from each female) or 20% ethanol (experimental N=4 embryonic brains, 1 from each female) for 2-4 hours at the beginning of the dark cycle (Boehm, 2008). This ethanol exposure paradigm is reported to produce average blood ethanol levels of 115-182 mg/dl in C57BL6 mice (Boehm, 2008). Each sipper tube was measured at the beginning and end of the drinking period. Both the experimental and control females were given access to food without limit. At E14 the embryos were extracted from the females through caesarean section after females were euthanized with CO2. The different specimens were named after what female they came from. Embryo brains were fixed in 4% paraformaldehyde diluted in
phosphate buffered saline overnight. Embryo brains were embedded in paraffin wax and cut into 5 µm thick coronal sections, and then stored until further examination. All animal procedures were carried out according to protocols approved by the Ursinus College Institutional Animal Care and Use Committee, permit number A4347-01.

Islet-1 Immunostaining of Mouse Brain Sections

Parafilm sections were dewaxed. Two changes of Histo-Clear (National Diagnostics, Atlanta, GA) 5 min each, 5 min 100% Ethanol (EtOH), 2 min 95% EtOH, 2 min 70% EtOH, 2 min distilled water (dH2O), and 2 min Phosphate Buffered Saline (PBS). Blocking solution (PBS + 0.1% TritonX-100 + 2% normal goat serum) was pipetted onto each slide in order to block nonspecific antibody staining. Slides were then incubated in a humidified chamber for 30 minutes. Mouse α-islet-1 primary antibody was diluted in 200 µL blocking solution at a final concentration of 1:100 (Favero et al., 2013), coverslipped with parafilm, placed in the humidified chamber, and left to incubate overnight at 4°C.

On the second day, parafilm coverslips were removed and the slides were put into 1X PBS and washed three times for 5 minutes each. When PBS washes were completed, 1:100 dilution of goat anti-mouse secondary antibody, which was used to recognize the primary antibody, was pipetted onto the slide and incubated at room temperature for 30 minutes in a humidified chamber. Vectastain ABC peroxidase (Vector Labs, Burlingame, CA) was made according to manufacturer’s instructions. Slides were washed again for three times for 5 minutes with PBS before adding 200 µL ABC solution onto each slide, covered with paraffin, and left to incubate in a humidified chamber for 30 minutes. When incubation was donen slides were washed again with PBS (three times for 5 minutes) and then placed into dH2O for 2 minutes. Then 200 µL of 3,3’Diaminobenzidine peroxide solution was added onto each slide and incubated at room temperature for 2 minutes and then slides were put into fresh dH2O. Finally, the slides were dehydrated by using exchanges of 70% EtOH (2 min), 95% EtOH (2 min), 100% EtOH (100 min), Histo-Clear I (5 min), and Histo-Clear II (5 min) and then coverslipped using Cytoseal.

Biotinylated goat anti-mouse secondary antibody, Vectastain Elite ABC kit, and Vector DAB substrate kit came from Vector Labs, Burlingame, CA. Islet-1 primary antibody came from the Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, Iowa. Ethanol was obtained from Pharmco in Brookfield, CT. Paraformaldehyde, paraffin wax, histo-Clear, triton X-100, and Cytoseal were obtained from Electron Microscopy Sciences in Hatfield, PA. Phosphate buffered saline and parafilm came from Fisher Scientific in Pittsburgh, PA. Normal goat serum and goat anti-mouse biotin secondary antibody came from Jackson Immunoresearch Laboratories, Inc. in West Grove, PA.

Analyzing the islet-1 brain sections with ImageJ

Islet-1 immunostained E14 brain sections were viewed using a Nikon Eclipse 80i Compound Microscope at 4X magnification (Figure 1A) and 20X magnification. The 20X magnification images were compiled together to form one continuous montage of the area (Figures 1B and C) using Adobe Photoshop. This compiled image was then imported into ImageJ software program and
the image was analyzed by adding a grid of ten rows and five columns to cover the LGE, where each row is separated by a different color code (Figure 1C). Then the islet-1 stained cells in each row were counted using the ImageJ software cell counter plugin. The results of the counted cells were then put into a Microsoft Excel spreadsheet and used for statistical analysis and graphing.

![Image of islet-1 immunostaining](image)

**Figure 1.** Islet-1 immunostaining of whole brain and cell count in LGE. Islet-1 stained cells are the dark brown cells highlighted in the red box. 4X picture taken with Nikon Eclipse 80i Microscope (A). 20X picture of islet-1 stained cells in the LGE (B). Cell count of the islet-1 stained cells in LGE, different color represent the different rows (C).

**Statistical Analysis and Graphing**

The total islet-1 stained cell counts and the cell counts for each row were recorded and processed in Microsoft Excel in order to run descriptive statistics and a two sample t-test. An ANOVA was used in order to test for a significant difference between the distribution of islet-1 cells between treatments groups. Data are presented as mean +/- standard deviation. Graphs were then created and used to interpret the data. The percent of cells in each row was calculated by taking the total number of cells in the row, divided by the total number of cells counted for all ten rows (Figure 3).

**Results**

**Cell Count**

Overall the mean water intake was 36.67 g/kg +/- 4.45 and the ethanol solution intake 28.48 g/kg +/- 1.58. However, because the ethanol solution was made up of 20% ethanol, then the amount of pure ethanol consumed was 4.49 g/kg.

In order to test the hypothesis that there would be a decrease in the number of islet-1 cells present with prenatal ethanol exposure, the total cell counts per row (Table 1) and the average cell counts for all the sample brain slices for each treatment group brains were found (Figure 2). The highest average cell count was found in brain sample Control 3, which came from a female given tap water, while the lowest average cell count was found in brain sample Experimental group 4 (Table 1). The mean cell count of the treatment group was 1319.88 +/- 24.751 for the control group and 1238.88 +/- 24.18 for the ethanol group (Figure 2).

It appears that there is a slightly higher total average islet-1 cell count for the control group when compared to the ethanol group.

<table>
<thead>
<tr>
<th>Brain Sample</th>
<th>Control 1</th>
<th>Control 2</th>
<th>Control 3</th>
<th>Control 4</th>
<th>Experimental 1</th>
<th>Experimental 2</th>
<th>Experimental 3</th>
<th>Experimental 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cell Count</td>
<td>1251</td>
<td>1271</td>
<td>1088</td>
<td>1669.5</td>
<td>1569</td>
<td>1159</td>
<td>1372</td>
<td>855.5</td>
</tr>
</tbody>
</table>

*Table 1: The total islet-1 stained cell count within the LGE for each brain sample. Total number of islet-1 stained cells within the LGE of water-control brains and ethanol-experimental brains within the DID experimental model.*
The distribution of the islet-1 cells within the LGE for each treatment group was examined. There was no significant difference between the treatment groups for the distribution of islet-1 cells across the rows. The p-values from the resulting t-tests in row order are 0.074, 0.6826, 0.130, 0.262, 0.855, 0.984, 0.628, 0.680, 0.804, 0.701, which are all greater than the significance value of 0.05. An ANOVA was also conducted to see if there was a significant difference between the experimental groups and the mean distribution of islet-1 cells per row. There was no significant difference between the experimental groups with a p-value for the reaction of 0.897. The results also indicate that there is a significant difference between the row distribution
values (p-value 8.77e-15), which confirms that across the LGE there was a difference in the distribution of cells. However, there was no significant difference in the distribution between two treatment groups (p-value of 5.27e-1).

Discussion

Summary of findings and limitations of study

Our hypothesis being tested was that there would be a significant decrease in the number or distribution of islet-1 stained cells in the LGE of E14 embryos after prenatal ethanol exposure. After running a T-test and an ANOVA comparing the means between the two groups, there was no significant difference in cell count between the control brains and the brains that were exposed to ethanol (Figure 2). Similarly the overall distribution within the LGE did not vary significantly between samples (Figure 3). We had hypothesized that there would be a difference in the distribution of the cells because of expected apoptotic cell loss that would decrease the size of the area (Schambra, 1990). Since there was no change in distribution of cells, or the overall total cell count, our hypothesis was not supported.

Some limitations of the study include a small sample size (four brains per treatment group), our use of outbred mice, and the variable drinking of the mothers. In our study we used Swiss Webster mice, which are very different from the C57B6 mice that Boehm and others (2008) used. It

Figure 3. Average percentage of islet-1 stained cells in LGE per row for each sample brain. Binning was done using ImageJ, a grid consisting of 10 blocks per column covering the LGE sample area. There was no significant difference between the treatment group and the number of islet-1 cells per row are a using a T-Test. The resulting p-values, in row order, 0.074, 0.6826, 0.130, 0.262, 0.855, 0.984, 0.628, 0.680, 0.804, 0.701 (alpha value of 0.05).
has been suggested that the C57B6 mice drink more ethanol than Swiss Webster mice, and therefore there would have been a greater impact in the cells of the C57B6 mice with the higher ethanol consumption (Metten, 2010). Other limitations include the use of islet-1 stain, which is able to label other cells besides just the basal ganglia primordium within the entire brain section. Although the stain is effective in that area, because it stains striatal cells that are present and crucial in the developing basal ganglia, an islet-1 stain is unable to determine if apoptosis occurred in that area. This is why within the study that it was hypothesized that, if there was a decrease of the number of islet-1 cells present, that this occurred through apoptosis following the evidence found in previous research. However, a TUNEL staining to detect DNA fragments would be more accurate in detecting if apoptosis was occurring.

Although there were no significant results that would suggest prenatal ethanol exposure has an effect on the islet-1 cells at this time in embryonic development, it could be due to the focus of the cells within the LGE. The LGE is a transitory brain structure present in embryonic and fetal brain stages, which is responsible for helping to guide axon migration (Encha-Razavi, 2003). Previous studies have found that the LGE contain the origin of cells that are committed to striatal phenotypes in the developing brain (Deacon, 1994). It was for this reason that the LGE was chosen as the region of interest in our study. One of the striatal phenotypes that the LGE commits to is the caudate later in development. Previous research indicated that there was a decrease in the size of the caudate from prenatal ethanol exposure, and so we wanted to see if this change was present early in development. However, because the LGE is also responsible for the initiation and migration of developing striatal cells, then it is possible that, if cell death was present, then the cells that could have died in the LGE were the ones that would have migrated to the MGE. For this reason we found no change in the number of cells at this embryonic day in the LGE, but further investigation is needed in the MGE to see if a significant reduction of islet-1 cells is present at the same point in embryonic development. If a reduction of islet-1 cells is found within the MGE, then this could be an indicator that prenatal ethanol exposure could affect the migration of neurons from the LGE to the MGE during fetal development.

One of the main limitations of this study was that we did not actually measure the BAC of the female mice. The sipper tubes were measured at the beginning and end of the drinking period with a mean ethanol solution intake of 28.48 g/kg +/- 1.58, meaning an average pure ethanol intake of 4.49g/kg. It was seen that the females who were exposed to ethanol had been drinking, but because the BAC was not measured, there is no definitive way to know the level of ethanol in the females during pregnancy. It can be expected that following the DID paradigm of Bohem et al. (2008) that the expected BAC would be 115-182 mg/dl. By not knowing the exact BAC then it is harder to compare the ingested amounts of ethanol with the effect that it could have on the offspring. The variation in dose amount of ethanol is important in FASD because it is a spectrum disorder in which the greater amount of ethanol that is consumed prenatally will cause more severe changes in development (Mattson, 1998; Archibald, 2001; Stratton, 1996). For example, a teratogenic dose amount of 2.9 g/kg in the Dunty et al. (2001) study produced significant changes in development due to apoptosis of the basal ganglia and forebrain structures in mice. This dose comparison in humans would be
extremely large and not compatible with the average drinking of pregnant women. In the study by Schambra et al. (1990), their levels varied in order to maintain a BAC of 500-600 mg/dl, which is comparable to the drinking habits of an alcoholic or recovering alcoholic. Both of these studies showed changes in development, but their levels of alcohol consumption are not realistic with human binge drinking. Our DID model, which models human binge drinking, has a lower expected blood alcohol, and, therefore, because of the spectrum nature of FASD, we did not see as dramatic changes in brain development.

Comparing Current and Previous Research

Previous research found that apoptotic cell death is due to the embryo being exposed to moderate to high levels of ethanol prenatally (Dunty et al., 2001). In our experimental procedure using the DID model we were exposing the mice to moderate ethanol levels, so we expected to see similar results. Independent of concentration, the method in which the alcohol is administered can also have an impact on how the pregnant dam is affected, as well as the offspring. For example, in a previous experiment the mice were only provided with a liquid diet that had a 25% ethanol concentration (Sari, 2009). This method of feeding is also known to result in malnutrition of the ethanol feeding mothers and stress of the control females because they are only allowed the caloric intake of the ethanol feeding mothers (Middaugh, 1995). The strain and malnutrition from the liquid diet can cause stress and therefore can increase apoptotic death, which is why the voluntary and stress-free drinking of the DID model was chosen (Boehm, 2008). This might be one of the reasons that the Sari et al. (2009) study may have seen results even with such low concentrations of ethanol.

Another method that is used in animal studies is intraperitoneal injection (Shambra, 1990; Dunty, 2001). This method of administering the ethanol by injections can cause stress to the mother and then can influence the developmental changes in the offspring that can mask the effects of ethanol only (Boehm, 2008). These studies also had a high BAC, and so by using the method of intraperitoneal injections it is possible that both the stress from the injections as well as the ethanol exposure could have caused the observed developmental changes, but it is almost impossible to see the effect independent of each other. This may be an explanation as to why these studies found a significant difference in brain structures following ethanol exposure, but our study did not. According to Boehm et al. (2008), who also used the DID model and mouse studies, there was an overall decrease in weight of the pups; they suggest that brain weight should parallel this. Since in this study we did not specifically measure the area of the LGE, but instead focused on the number of islet-1 cells within the LGE, we do not know if the LGE is smaller in size. In human children FASD studies there is previous research that does suggest that the actual size of the LGE is what is affected by being exposed to ethanol prenatally (Archibald, 2001; Mattson, 1992).

Future Work

For this specific study, only the number and distribution of islet-1 stained cells was looked at in the LGE. For future work we can compare the MGE to the LGE at this time in development (E14), which might produce more significant results regarding the cell count and distribution of cells. This comparison between the MGE and LGE might also a determination of whether there is an impairment in the
migration of cells from the LGE to the MGE and if this process is affected with prenatal ethanol exposure. In addition, other components of the basal ganglia, like the globus pallidus, are found in the MGE and may be more affected by prenatal ethanol exposure, but are not labeled by an islet-1 stain. In future work we should look at the caudate and putamen primordium specifically; in this study we only looked at all the islet-1 stained cells in the LGE. Future work can also include a focus on the overall size and area of the LGE, either through Nissl staining or finding the area of the islet-1 stained region. We could use a different method to do a density analysis of the cells in the LGE, which could possibly be done by using ImageJ. Also a TUNEL stain could be used, as in in previous studies, so that any apoptotic cell death within the regions of interest could be monitored. In previous research there was an overall size reduction of the LGE, as well as in the basal ganglia (Norman, 2009; Riley, 2005). This suggests that the cell death might occur after these regions of the brain have already been formed, instead of earlier on in their development. Studying mouse brains at a later time in development might provide significant results regarding the size and distribution of the islet-1 stained cells in the basal ganglia. To get a higher concentration of ethanol we could alter our model and use an injection or intubation, however this could cause extra stress. Within our mouse model we could also try and use different mice breeds, like the C57Bl/6 mice used in Boehm et al. (2008), in order to determine if there is a large different in ethanol consumption between mouse strains.

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