

# Prenatal ethanol exposure affects calbindin expression in an FASD mouse model

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Alcohol consumption during pregnancy produces a wide range of birth defects classified as Fetal Alcohol Spectrum Disorders (FASD), including motor defects. Motor functions are regulated in part by Purkinje cells in the cerebellum, which are particularly susceptible to ethanol exposure and contain high levels of calbindin, a calcium-binding protein essential to their function and survival. Previous research has demonstrated that alcohol consumption during pregnancy can produce motor defects in a newborn, but the underlying mechanisms are poorly understood. We investigated the effects of low dose prenatal ethanol exposure on calbindin expression in the cerebellum by examining the number and relative optical density of cells expressing calbindin in the cerebella of mouse offspring exposed to ethanol from gestational day 7.5 until birth. Calbindin expression was visualized using immunostaining. ImageJ software was used to count and measure the relative optical density of cells expressing calbindin. Calbindin expression was interpreted along a continuum of intensity (0=high stain intensity, 1=low stain intensity). The cerebella of mice prenatally exposed to ethanol demonstrated a relative optical density of calbindin expression (0.431) significantly higher than control mice exposed to water (0.369), and thus a lower expression of calbindin ( $p=0.003$ ). No differences in the number of calbindin immunostained cells in mice exposed to tap water and ethanol were observed. Our results suggest that intracellular calcium-binding proteins in Purkinje cells are reduced following low levels of ethanol exposure during prenatal development. These observations may lead to valuable insights regarding the mechanisms underlying the impairment of motor function observed in mild cases of FASD in humans.

## INTRODUCTION

While high levels of alcohol consumption during pregnancy are often associated with the development of Fetal Alcohol Syndrome (FAS), moderate alcohol consumption during pregnancy can result in a variety of birth defects in offspring including physical, mental, behavioral, and learning disabilities. The severity of these deficits falls along a continuum that is classified as Fetal Alcohol Spectrum Disorders (FASD), inducing a variety of motor deficits such as problems with small muscle movements, ataxia, tremors, and gait disturbance (Miki et al., 1999).

One major target of ethanol toxicity in the developing central nervous system is the cerebellum (Servais et al., 2007). Located in this region are Purkinje cells, which constitute the sole output of the cerebellum. They are involved in motor function and coordination and are particularly susceptible to ethanol exposure (Miki et al., 1999; Servais et al., 2007).

Calbindin is a calcium-binding protein highly concentrated in Purkinje cells in the cerebellum (Garcia-Segura, et al., 1984). Calbindin regulates motor coordination and sensory integration, vital functions of Purkinje cells in this region (Arnold & Heintz, 1997; Barski et al., 2003). Mice with null mutations for calbindin D-28k exhibited impaired limb motor coordination and compensatory eye movements (Barski et al., 2003). Reduction in calcium-binding proteins are also purported to alter neural cell firing (Barski et al., 2003; Bastianelli, 2003). Thus alterations in calbindin protein levels directly affect Purkinje cell function.

Previous research has reported that rodents prenatally exposed to high levels of ethanol demonstrate a reduced number of Purkinje cells expressing calbindin D-28k and reduced intensity of calbindin immunostaining suggesting that calbindin expression may be implicated in regulating migration, differentiation, and survival of Purkinje cells (Wierzba-Bobrowicz et al., 2011). Consequently, motor deficits often observed in FASD may result from developmental changes in protein expression in Purkinje cells (Mameli et al., 2008; Miki et al., 1999; Servais et al., 2007; Wierzba-Bobrowicz et al., 2011).

Much research exploring the effects of ethanol exposure on the cerebellum has shown Purkinje cell loss late in development. A 20% loss of Purkinje cells was observed in three and seven-week-old mice prenatally exposed to ethanol, and the loss of Purkinje cells is correlated with impaired motor function (Thomas et al., 1998; Servais et al., 2007). Proposed mechanisms underlying Purkinje cell loss suggest that postnatal ethanol exposure may act directly or indirectly, by affecting other early developmental events such as synaptogenesis or granule cell generation and/or migration (Miki et al., 1999). It is also suggested that a combination of impaired signaling of various receptors and growth factors in response to postnatal ethanol exposure may lead to cellular death (Kumar et al., 2013). Finally, reduced calbindin protein leads to impaired migration, differentiation, and survival of Purkinje cells and thus, may be a mechanism underlying the loss of Purkinje cells in response to prenatal ethanol exposure (Wierzba-Bobrowicz et al., 2011). Based on these previous reports, we hypothesized that prenatal ethanol exposure would result in reduced calbindin expression and/or reduced number of Purkinje cells in the cerebella of postnatal day 0 (P0) mice.

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## MATERIALS AND METHODS

### Animal treatment

Approximately eight-week-old male and female out-bred Swiss Webster mice were purchased from Ace Animals for breeding. During mating, a single female mouse was placed in a single male's home cage just prior to lights out. Females were removed the next morning (designated as gestational day (G) 0.5), checked for plugs as a sign of intercourse, weighed, and put back in their home cage. On G7.5, female mice were weighed again to determine if they were pregnant. Female mice that gained two or more grams were presumed pregnant and were chosen for the experimental procedure. Food was freely available to all mice at all times. Prior to experimental procedure, water was freely available.

### Ethanol administration

On G7.5, pregnant dams were given either tap water or 20% ethanol diluted in tap water as their sole drinking fluid. On the day of birth, postnatal day (P0) pups were removed from the cage, weighed, and euthanized by decapitation. Brains were dissected, weighed, and stored at 4°C for further analysis. For each treatment, two mice each from two different litters were used for analysis.

### Calbindin immunostaining of mouse brain sections

Cerebellar sections of P0 mice that were prenatally exposed to tap water and ethanol were immunostained with calbindin antibody. Using a procedure similar to Kim et al. (2009), free floating 50  $\mu\text{m}$ -thick, sagittally-cut mouse brain sections were stained in 24-well plates with calbindin primary antibody. Non-specific antibody binding was blocked with 200  $\mu\text{L}$  of blocking solution (Phosphate buffered saline (PBS) + 0.1% TritonX-100 + 2% normal goat serum). 200  $\mu\text{L}$  of 1:500 primary antibody diluted in blocking solution was added into each well. Wells containing only blocking solution served as a negative control. Plates were incubated overnight at 4°C. The next day, primary antibody solution was removed from the wells and tissue was washed with PBS three times for five minutes. PBS from the last wash was removed and 200  $\mu\text{L}$  of 0.5%  $\text{H}_2\text{O}_2$  in PBS was transferred into each well. The tissue was incubated for 30 minutes then, the  $\text{H}_2\text{O}_2$  solution was removed, followed by another set of three five-minute PBS washes. 200  $\mu\text{L}$  of 1:200 dilution of anti-rabbit secondary antibody in blocking solution was added to all experimental wells. Plates were incubated at room temperature for one hour on a shaker.

Vectastain ABC peroxidase complex solution was made according to manufacturer instructions. Secondary antibody solution was removed followed by another three five-minute PBS washes. 200  $\mu\text{L}$  ABC solution was added to each well and incubated on a shaker for 30 minutes. Sections were washed with PBS, three times for five minutes. Diaminobenzidine (DAB) peroxide solution from the Vectastain DAB Substrate kit was made according to manufacturer instructions. PBS was removed from each well and 200  $\mu\text{L}$  of the DAB-peroxide solution was added. Sections were allowed to incubate at room temperature

for 10 seconds to one minute. Once the sections turned brown, DAB solution was removed from the wells, which were then washed with distilled water. Stained tissue was mounted onto glass slides and allowed to dry for 24 hours. Slides were incubated in 100% ethanol for one minute followed by xylene for five minutes. Slides were then coverslipped using Cytoseal (Kim et al., 2009).

### Analyzing calbindin stained brain sections with ImageJ

Calbindin immunostained P0 mouse brain cerebella from four mice prenatally exposed to tap water ( $n=2$ ) and ethanol ( $n=2$ ) were imaged using a Nikon Eclipse 80i Compound Microscope at 4X (Figure 1A-B) and 40X (Figure 1C-F) magnification and analyzed (at 40X only) using the ImageJ software program and according to ImageJ User Guide instructions (Ferreira & Rasband, 2011).

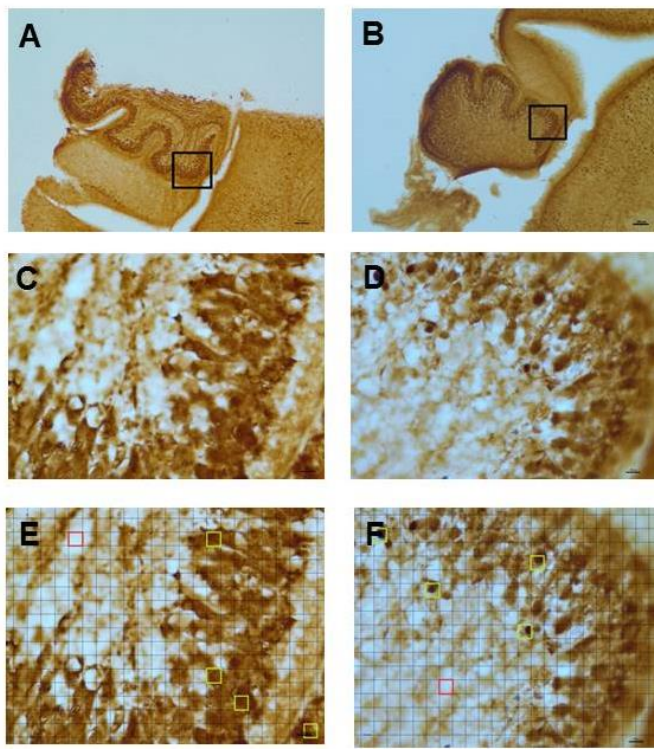
Images were calibrated with a 100 micron scale bar. To analyze the intensity of the calbindin immunostaining in Purkinje cells, a black 90 microns<sup>2</sup> grid was superimposed over each image. All procedures were performed on all 40X images (Ferreira & Rasband, 2011).

### Relative optical density measurements

Calbindin levels were analyzed by comparing the intensity of calbindin immunostaining visualized with (DAB) to an unstained reference to calculate the relative optical density (ROD) using the ImageJ computer software program (Kim et al., 2009; Ferreira & Rasband, 2011). In each calibrated and gridded 40X image of P0 mouse cerebella ( $n_{\text{tap water}}=6$ ,  $n_{\text{ethanol}}=8$ ), grid squares were selected and outlined for analysis in ImageJ (Ferreira & Rasband, 2011). Only grid squares containing stained cells that were presumed to be Purkinje cells based on morphology and size were selected for analysis. One unstained grid square was selected as a control reference and four grid squares containing calbindin stained Purkinje cells were selected as regions of interest (ROIs) (Figure 1E-F). This procedure was completed for all 14 images producing a total of 24 and 32 ROIs for tap water and ethanol exposure, respectively. A histogram of the pixel intensity values for all 255 pixels within each selected grid square as well as the average intensity and standard deviation were extracted (Ferreira & Rasband, 2011).

The ROD of each grid square was calculated using the values extracted from ImageJ. Using Microsoft Excel, the ROD for all pixel values for one grid square was calculated and converted from a grayscale value to a value relative to the unstained cell. A value of 1 indicates that the pixel value in the ROI is the same as the average optical density of the unstained grid. Since the pixels were expressed relative to the unstained cell, the automated ROD Excel spreadsheet weighted them to obtain the representative relative pixel intensity (weighted value of relative pixel intensity = weighted observations = counts  $\times$  relative pixels). This was applied to all pixels for the grid square. The average ROD for the ROI was then calculated ( $\Sigma$ weighted observations/ $\Sigma$ counts). ImageJ quantifies pixel intensity along a continuum with 0 representing the highest (darkest) intensity and 1 as the lowest (lightest) intensity. The smaller the average ROD

value of the grid square the darker the stain. We concluded that the darker the stain, the greater the calbindin expression.



**Figure 1. Decreased calbindin immunostaining in cerebellum of P0 mice exposed to ethanol (A-F).** Images of slices of whole mouse cerebella taken at 4X magnification (A-B) are labeled with an inset indicating the lobule section imaged at 40X magnification (C-F) for data collection and analysis. Calbindin immunostained P0 mouse cerebella exposed to tap water and ethanol, respectively at 4X magnification (A-B). Calbindin immunostained P0 mouse cerebellum exposed to tap water and ethanol, respectively at 40X magnification (C-D) and superimposed with 90 micron<sup>2</sup> grid with unstained reference (highlighted in red) and regions of interests (ROIs) (highlighted in green) containing calbindin immunostained Purkinje cells (E-F). Based on visual observation, sections of P0 mouse cerebella prenatally exposed to ethanol (B, D, and F) appear to exhibit less intense calbindin immunostaining than controls exposed to tap water (A, C, and E). No differences in Purkinje cell number are

#### Automated cell counting using ImageJ

The number of cells exhibiting size and morphology characteristic of Purkinje cells were counted. Automated cell counting was performed on calbindin immunostained sections of P0 mouse cerebella in ImageJ according to the ImageJ User Guide instructions (Ferreira & Rasband, 2011). Each image was then analyzed for area, mean gray value, and perimeter. The minimum cell area to count as a cell was set to 100 and outlines of cells in the image was selected for display. A results window and new image appeared displaying a drawing with outlines of detected and numbered particles. This drawing was compared to the original image. When groups of distinct cells were detected

as clumps by the program, the watershed function was applied to break them apart. Data displayed in the results window were extracted for analysis (Ferreira & Rasband, 2011).

#### Statistical analysis and graphing

Descriptive statistics including the means and standard deviations of the ROIs and cell count of calbindin immunostained sections of P0 mouse cerebella prenatally exposed to tap water and ethanol were performed. All data samples were tested for normality using NormQuant (Cedar Crest College). Because of small sample size, non-directional Mann-Whitney U tests with a 0.05 alpha level were performed comparing tap water (control) and ethanol exposed groups for calbindin immunostaining intensity and cell number. All statistical analyses were performed using a calculator provided by Vassar Stats and graphs were generated by Microsoft Excel.

### RESULTS

#### Relative optical density

Images of calbindin immunostained P0 mouse cerebellar brain sections observed in the ImageJ software program showed that sections of P0 cerebella of mice prenatally exposed to ethanol (Figure 1, right column) appeared to demonstrate lighter staining compared to control mice that were exposed to tap water (Figure 1, left column).

NormQuant analysis indicated that all samples were normally distributed. The non-directional Mann-Whitney U test performed on the relative optical density data collected from cerebella of P0 mice prenatally exposed to tap water and ethanol from each litter (Table 1) indicated that the ROD of calbindin immunostaining in P0 mouse cerebella exposed to tap water

|  | Treatment |         |
|--|-----------|---------|
|  | Tap Water | Ethanol |
| <b>Mean ROD</b>  | 0.369     | 0.431   |
| <b>Standard Deviation</b>  | 0.075     | 0.073   |
| <b>Sample Size</b><br>(Number of grid squares containing calbindin immunostaining) | 24        | 32      |
| <b>P-value</b>   | 0.0083    |         |

**Table 1. Mean relative optical density (ROD), standard deviation (SD), sample size, and p-value of calbindin immunostaining of P0 mouse cerebella exposed to tap water and ethanol from each litter.**

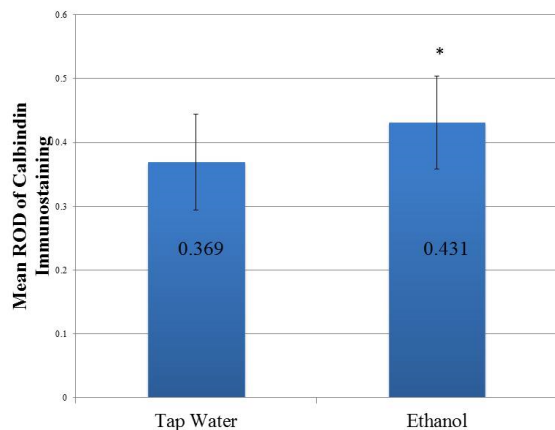
Table of mean relative optical density (ROD), standard deviation, sample size, and p-value for each of the four calbindin immunostained P0 mouse cerebella observed. The ImageJ software program quantifies the light intensity of pixels along a continuum from 0 to 1. Darker pixels are represented by lower values with 0 representing the darkest intensity. The ROD of each grid square was calculated using the values extracted from ImageJ. The intensity of the calbindin immunostaining was determined based on the assumption that the darker the intensity of the stain, the greater the calbindin protein expression.

(mean= 0.369, sample size (n)=24 grid squares containing calbindin immunostaining) was significantly lower than that of mice who were exposed to ethanol (mean= 0.431, n= 32 grid squares containing calbindin immunostaining) at the 0.05 alpha level (p=0.0083) (Figure 2).

**Cell count**

To determine cell count, we visually inspected calbindin stained cells exhibiting size and morphology characteristic of Purkinje cells in sections of P0 mouse cerebella. Sections of P0 cerebella of mice prenatally exposed to ethanol (Figure 1, right column) appeared to have similar amounts of Purkinje cells compared to the cerebella of control mice that were exposed to tap water (Figure 1, left column).

To confirm our findings by visual inspection, we counted the cells using the automated cell counting task in ImageJ. Only cells exhibiting the characteristic size and morphology of Purkinje cells were counted. A non-directional Mann-Whitney U test indicated no statistically significant differences between the number of calbindin immunostained cells in P0 mouse cerebella exposed to tap water (mean=27.00, sample size (n)=6 images of calbindin immunostained sections) or ethanol (mean=39.375, sample size (n)=8 images of calbindin immunostained sections) (p=0.0819) (Table 2, Figure 3).



**Figure 2. Increased relative optical density (ROD) of calbindin immunostaining in cerebella of P0 mice exposed to ethanol.** Graph of mean relative optical density (ROD) of combined calbindin immunostained samples of P0 mouse cerebella prenatally administered tap water and ethanol. The ImageJ software program quantifies the light intensity of pixels along a continuum from 0 to 1. Darker pixels are represented by lower values with 0 representing the darkest intensity. The ROD of each grid square was calculated using the values extracted from ImageJ. The intensity of the calbindin immunostaining was determined based on the assumption that the darker the intensity of the stain, the greater the calbindin protein expression. A non-directional Mann-Whitney U test indicated that ethanol exposed mice had a significantly higher mean ROD of calbindin immunostaining (mean=0.431, sample size (n)=32 grid squares containing calbindin immunostained Purkinje cells) than controls (mean=0.369, (n)=24 grid squares containing calbindin immunostained Purkinje cells; p-value=.0083). Error bars indicate standard deviation. \*Indicates statistical significance.

**DISCUSSION**

**Current findings and limitations of study**

In support of our hypothesis, calbindin immunostaining in the cerebella of P0 mice prenatally exposed to low doses of ethanol appeared lighter in stain intensity compared to those exposed to tap water (Figure 1). This was confirmed by a non-directional Mann-Whitney U test which revealed that cerebella of P0 mice prenatally exposed to ethanol exhibited significantly higher relative optical densities compared to those exposed to tap water at the 0.05 alpha level (p=.0083) (Table 1; Figure 2). Because higher values represent lighter staining, we suggest that ethanol-exposed cerebella contained reduced levels of calbindin protein. Although we expected a loss of calbindin immunostained cerebellar cells in response to ethanol exposure, the lack of reduced numbers of cells suggests that the reduced calbindin immunostain intensity that was observed is the result of the loss of calbindin protein in these cells and not the mere loss of cells expressing this protein.

Other studies have shown that reduced calbindin staining intensity correlates with reduced calbindin protein levels. Similar to our study, Wallace et al. (2010) observed reduced calbindin expression in sections of rat cerebella upon visual inspection and confirmed that this was the result of reduced calbindin protein using Western blotting. Based on this study, our results likely indicate that reduced calbindin intensity was the result of reduced calbindin protein in Purkinje cells prenatally exposed to ethanol. Furthermore, Wallace et al. (2010) also demonstrated that reduced calbindin protein correlates with impaired motor coordination and balance analysis. Although we did not test the behavior of mice prenatally exposed to ethanol, we would expect similar motor impairments.

| Stain Type                       | Calbindin Immunostaining |         |
|----------------------------------|--------------------------|---------|
|                                  | Tap Water                | Ethanol |
| Mean Cell Count                  | 27.00                    | 39.375  |
| Standard Deviation               | 6.450                    | 14.020  |
| Sample Size (n=number of images) | 6                        | 8       |
| P-Value                          | 0.0819                   |         |

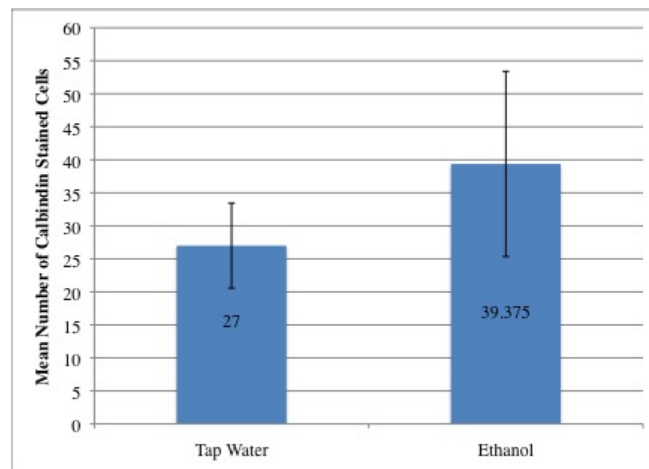
**Table 2. Mean cell count, standard deviation (SD), sample size, and p-value of calbindin immunostaining of P0 mouse cerebella exposed to tap water and ethanol from each litter** Table of mean cell count, standard deviation, sample size, and p-value for each of the four calbindin immunostained P0 mouse cerebella observed. Data was collected using automated cell counting in the ImageJ software program and analyzed using Microsoft Excel and Vassar Stats.

Studies investigating the effect of ethanol exposure on Purkinje cell number have consistently yielded results suggesting that cell loss occurs at a rate of a 20-50% (Miki et al., 1999; Servais et al., 2007). In our study, we were unable to replicate these results using calbindin immunostaining. In fact, our results suggested an insignificant increase in calbindin stained Purkinje

cells (Table 2, Figure 3). The lack of reduced numbers of Purkinje cells in the current study may be the result of developmental timing. In mice, Purkinje cells begin to develop between embryonic days 10-13 (Apps & Hawkes, 2009; McKay & Turner, 2006). It is not until between the third to fourth postnatal weeks that these cells develop their characteristic anatomy, connectivity, and morphology (Apps & Hawkes, 2009; McKay & Turner, 2006). In conjunction, much of the research investigating Purkinje cell number in response to ethanol exposure has examined cell count during postnatal development of mice and rats. During the first two weeks after birth, the brain undergoes significant development (Miki et al., 1999). It is during this period that the brain is most vulnerable to the effects of ethanol exposure (Miki et al., 1999). Postnatal days 4 through 9 appear to be crucial for Purkinje cell generation in mice and rats, particularly around the 7<sup>th</sup> postnatal day (Volk et al., 1981; Thomas et al., 1998; Miki et al., 1999). Volk et al. (1981) state that it is on the 7<sup>th</sup> postnatal day that Purkinje cells mature. Thomas et al. (1998) indicate that the survival of Purkinje cells in response to ethanol exposure is dependent upon its maturation. As a result, the Purkinje cell population prior to maturation is left highly vulnerable to ethanol exposure. In our study, we examined Purkinje cell number on postnatal day 0. As a result, the lack of reduced Purkinje cells in offspring prenatally exposed to ethanol in this study may have been because they were examined prior to this vulnerable period.

In their study, Wierzba-Bobrowicz et al. (2011) observed reduced calbindin D-28k immunoreactivity in Purkinje cells and complete loss in Golgi cells in response to prenatal ethanol exposure (Wierzba-Bobrowicz et al., 2011). Therefore, the reduction in staining we observed could be due to loss of calbindin expression in Golgi cells alone with no difference in Purkinje cells. We feel this is unlikely because when selecting regions of interest containing calbindin staining for our study, we selected regions with cells that were presumed to be Purkinje cells based on morphology and size. Furthermore, Wierzba-Bobrowicz et al. (2011) seem to suggest that Golgi cells are immunoreactive to calbindin D28k. Several studies, however, indicate the specificity of calbindin D-28k for Purkinje cells in both mice and humans (Bastianelli et al., 2003; Kim et al., 2009; Whitney et al., 2008). In our study, we visually inspected the calbindin immunostained sections of P0 mouse cerebella exposed to tap water and ethanol. In these sections, the only calbindin immunostained cells observable exhibited large size and formed a layer within the cerebellum that is consistent with the location of the Purkinje cell layer. Based on the observations, we strongly believe that the cells counted were Purkinje cells and not other cerebellar cells.

Altogether, the lack of reduced cell number in calbindin immunostained sections suggests that the reduced calbindin stain intensity we observed is not due to fewer Purkinje cells but rather the intracellular loss of calbindin protein. This, however, does not rule out the possibility of the loss of other cerebellar cells reducing calbindin expression. To confirm this definitively, future experiments would require co-staining the cerebellum with



**Figure 3. No difference in cell count in calbindin stained cerebella of P0 mice exposed to ethanol.** Graph of mean cell count of calbindin immunostained sections of P0 mouse cerebella prenatally administered tap water and ethanol. Values were determined using automated cell counting in ImageJ. A non-directional Mann-Whitney U tests indicated that ethanol exposed mice did not have significantly different numbers of calbindin stained cells (mean=39.375, sample size (n)=8 images containing calbindin stained cells) than controls (mean=27.00, (n)=6 images containing calbindin stained cells; p=0.0819). Error bars indicate standard deviation.

calbindin and specific immunomarkers for Purkinje cells, followed by Western blot analysis for confirmation.

### Relevance of timing and dosage of ethanol exposure on cerebellar development and function

We observed that prenatal ethanol exposure reduces calbindin protein in Purkinje cells of P0 mouse cerebella. In mice, Purkinje cells begin to express calcium-binding proteins such as calbindin D-28k by embryonic day 14 (Enderlin et al., 1987). These intracellular proteins contain regions that allow them to bind calcium and regulate its intracellular levels and neuronal functions such as presynaptic neurotransmitter release (Barski, et al., 2003; Enderlin et al., 1987, Bastianelli, 2003). Pregnant mice in the current study were administered ethanol on embryonic day 7.5, before Purkinje cells are purported to begin to express calcium-binding proteins. Our results suggest that ethanol exposure during such an early gestational period may have altered the normal development of calbindin resulting in reduced expression and thus, stain intensity. In turn, this may alter the normal development of cerebellar cells and their function.

While high levels of alcohol consumption and FAS have been widely investigated, less is understood about the effects of mild to moderate alcohol consumption on the development of the central nervous system and symptoms of FASD. Our study is only the second to demonstrate that prenatal ethanol exposure alters the expression of calcium-binding proteins in the cerebellum. Wierzba-Bobrowicz et al. (2011) observed reductions in calbindin D-28k immunostaining in Purkinje and Golgi cells of ten-day old rats exposed to 12% ethanol (6g/kg

body mass) during the entirety of gestation. The ethanol dosage used in the study conducted by Wierzba-Bobrowicz et al. (2011) would result in a blood alcohol level (BAL) of about 250-300 mg/dL (Kaiser et al., 2009). In our study, we administered 20% ethanol diluted in tap water. Using similar ethanol administration techniques to our study, Servais et al. (2007) observed that pregnant female mice given 18% ethanol solution possessed a BAL of  $72.7 \pm 20.7$  mg/dL. In humans, this BAL is about five to six times lower than what would be expected according to Wierzba-Bobrowicz et al.'s (2011) exposure paradigm and just below what the National Institute on Alcohol Abuse and Alcoholism qualifies as intoxication (80 mg/dL). The similarity of our findings to that of Wierzba-Bobrowicz et al. (2011), with regard to cerebellar calbindin expression is particularly compelling because of the low dose of ethanol used.

Several studies have investigated the functional effects of reduced or absent calcium-binding protein expression. For instance, reductions in calcium-binding proteins alter neural cell firing (Barski et al., 2003; Bastianelli, 2003). Mice with null mutations for calbindin exhibited deficits in the precision of motor coordination as well as processing of vestibular information (Barski et al., 2003). Barski et al. (2003) suggests that the reduction of synaptically-mediated calcium signaling in Purkinje cells as well as changes in the timing of the release of postsynaptic calcium after deletion of the calcium-binding protein calbindin D-28k may produce motor coordination impairments (Barski et al., 2003). Our results together with those of previous studies suggest that reduced calbindin protein as a result of mild doses of ethanol prenatally can affect normal cerebellar development, potentially resulting in motor deficits observed in FASD. To confirm this, future studies should conduct behavioral tests to investigate motor activity in mice prenatally exposed to low doses of ethanol.

### Relevance to human FASD

According to the Human Genome Project, mice and humans share the gene for the calcium-binding protein calbindin D-28k (U.S. Department of Energy Genome Programs, 2011; Eppig et al., 2012). Unfortunately, there are no studies examining the expression of calcium-binding proteins in cerebella of humans with FASD. Based on neuroimaging of the human brain, however, we can speculate about the changes in the human cerebellum during prenatal development in response to alcohol exposure. Prenatal ethanol exposure in humans decreases volume, surface area and number of cerebellar cells (Norman et al., 2001; Autti-Rämö et al., 2002). Calbindin protein develops in humans early on, between the first four to five weeks of gestation and its stain intensity increases with development (Laure-Kamionowska & Masilinska, 2009). In humans, calcium-binding proteins are involved in a variety of processes including cell division, outgrowth, and movement and homeostasis (Laure-Kamionowska & Masilinska, 2009). In Purkinje cells, these proteins are responsible for producing normal motor coordination (Arnold & Heintz, 1997; Barski et al., 2003). Through reduced calcium concentration and signaling and in turn, Purkinje cell loss, the reduction of cerebellar calbindin expression may act as a

potential mechanism for the motor deficits observed in FASD.

As a result, alcohol consumption during pregnancy leads to loss of cerebellar cells and the cells that remain exhibit reduced calcium-binding protein expression resulting in improper cell division, migration, and function (Miki et al., 1999; Servais et al., 2007; Wierzba-Bobrowicz et al., 2011). Together, these developmental anomalies may contribute to the various motor deficits observed among individuals with FASD.

Our study explored cellular differences in ethanol-exposed mice. Future research will need to examine the functional outcomes of these findings in terms of motor function. We observed that mild ethanol exposure is sufficient to reduce calbindin protein expression in the cerebellum during prenatal development, indicating it as a potential mechanism underlying the various motor deficits observed in mild FASD. Although the principal cause of this reduction is not yet understood, these observations may lead to more valuable insights regarding the mechanisms underlying the various motor deficits observed in the less severe range of the FASD spectrum as well as possible therapies. For instance, mice with spinocerebellar ataxia type 1 who were treated with Insulin-like Growth Factor-1 (IGF-1) were observed to have partial recovery of calbindin D-28k expression in Purkinje cells as well as improved rotarod performance (Vig et al., 2006). Future studies could investigate whether IGF-I treatment recovers calbindin protein expression in this model system to give evidence for its use as a potential therapy for humans with FASD.

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