Published in final edited form as:

Neurotoxicol Teratol. 2013 ; 35: 7–13. doi:10.1016/j.ntt.2012.11.001.

Different Patterns of Regional Purkinje Cell Loss in the Cerebellar Vermis as a Function of the Timing of Prenatal Ethanol Exposure in an Ovine Model

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Abstract

Studies in rat models of fetal alcohol spectrum disorders have indicated that the cerebellum is particularly vulnerable to ethanol-induced Purkinje cell loss during the third trimester-equivalent, with striking regional differences in vulnerability in which early-maturing regions in the vermis show significantly more loss than the late-maturing regions. The current study tested the hypothesis that the sheep model will show similar regional differences in fetal cerebellar Purkinje cell loss when prenatal binge ethanol exposure is restricted to the prenatal period of brain development equivalent to the third trimester and also compared the pattern of loss to that produced by exposure during the first trimester-equivalent. Pregnant Suffolk sheep were assigned to four groups: first trimester-equivalent saline control group, first trimester-equivalent ethanol group (1.75 g/kg/day), third trimester-equivalent saline control group, and third trimester-equivalent ethanol group (1.75 g/kg/day). Ethanol was administered as an intravenous infusion on 3 consecutive days followed by a 4-day ethanol-free interval, to mimic a weekend binge drinking pattern. Animals from all four groups were sacrificed and fetal brains were harvested on gestation day 133. Fetal cerebellar Purkinje cell counts were performed in an early-maturing region (lobules I-X) and a late-maturing region (lobules VIc-VII) from mid-sagittal sections of the cerebellar vermis. As predicted, the third trimester-equivalent ethanol exposure caused a significant reduction in the fetal cerebellar Purkinje cell volume density and Purkinje cell number in the early-maturing region, but not in the late-maturing region. In contrast, the first trimester-equivalent ethanol exposure resulted in significant reductions in both the early and late-maturing regions. These data confirmed the previous findings in rat models that third trimester-equivalent prenatal ethanol exposure resulted in regionally-specific Purkinje cell loss in the early-maturing region of the vermis, and further demonstrated that first trimester ethanol exposure caused more generalized fetal cerebellar Purkinje cell loss, independent of the cerebellar vermal region. These

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findings support the idea that prenatal ethanol exposure in the first trimester interferes with the genesis of Purkinje cells in an unselective manner, whereas exposure during the third trimester selectively kills post-mitotic Purkinje cells in specific vermal regions during a vulnerable period of differentiation and synaptogenesis.

Keywords

Cerebellum; ovine; ethanol; teratogenicity; Purkinje cells

1. Introduction

Fetal alcohol spectrum disorders (FASD) is an umbrella term encompassing the full range of effects that can occur in an individual whose mother consumed ethanol during pregnancy. These include effects on physical, behavioral or cognitive development that can persist as lifelong disabilities. The most severe end of the spectrum is fetal alcohol syndrome (FAS) [2,49,51,52], for which facial abnormalities, growth deficits and central nervous system (CNS) abnormalities are the defining diagnostic features. Despite efforts to educate the public about FASD, the prevalence of ethanol consumption in women of child-bearing age has remained essentially the same [1,11,12,35]. Ethanol is a neurotoxic teratogen and has the potential to cause damage in many regions of the brain [8,11,18,46,49]. Postmortem reports and neuroimaging studies in children clinically affected by the heavy prenatal ethanol exposure have shown smaller head and brain size [4,13,25,34,37,50]. Human studies have reported structural abnormalities and physiological dysfunction in several brain regions, including the cerebral cortex, basal ganglia, thalamus and hypothalamus, hippocampus, and corpus callosum [4,34,45,47,48,50], but based on both human and animal studies the cerebellum appears to be particularly vulnerable to ethanol exposure during development [5-7,10,20,21,23,28,40,41,53].

The timing of ethanol exposure during pregnancy is an important determinant of the type and extent of fetal brain damage that occurs [13,14,44,46]. Human and animal studies have indicated that the developing cerebellum is vulnerable throughout gestation. Experimental studies in neonatal rats have shown that daily binge-like patterns of ethanol exposure, during the early postnatal period of brain development that is comparable to that of the human third trimester, produced dose-dependent loss of post-mitotic cerebellar Purkinje cells when peak blood ethanol concentrations (BECs) exceeded 150 mg/dl [15,19,22,23,27,29-32,36,38]. In contrast, when daily binge-like ethanol exposure was given throughout gestation in rats (when brain development is equivalent to that of the human first and second trimesters) in doses of 2.25, 4.5, or 6.5 g/kg/day (producing mean peak BECs of 136, 290, and 422 mg/dl, respectively), only the high dose (6.5 g/kg) resulted in a Purkinje cell loss [31]. In addition, direct comparisons of binge-like ethanol exposure in rats during the second trimester-equivalent versus the third trimester-equivalent, in which efforts were made to match the resulting BECs across the two periods, showed significantly greater reductions with the third trimester-equivalent exposure [32]. In contrast, studies in a sheep model and a nonhuman primate model of binge-like prenatal ethanol exposure reported significant reductions in cerebellar Purkinje cell numbers regardless of whether exposure was during the period of brain development equivalent to that of the human first trimester, third trimester, or all three trimester-equivalents [9,40,41].

The findings in the sheep model, in which brain development over all three trimesters equivalent occurs *in utero*, raise interesting questions as to why comparable Purkinje cell loss was observed with different exposure periods or durations across pregnancy. The stages of cerebellar development and the mechanisms of Purkinje cell loss resulting from ethanol

exposure are markedly different between the first- and third trimester-equivalent. In terms of Purkinje cell development, the first trimester exposure occurs prior to Purkinje cell genesis and so will affect the precursor cells, whereas the third trimester exposure occurs after the genesis of Purkinje cells, during their post-mitotic differentiation. Hence, the specific nature of temporal windows of vulnerability to ethanol-induced cerebellar cell loss across different stages of brain development has not been resolved, including whether the temporal and regional profiles of cerebellar vulnerability to ethanol may vary across species.

The possibility that the regional differences in the fetal cerebellar Purkinje cell loss in response to ethanol exposure may be related to the chronology of lobular development was first proposed by Bauer-Moffett and Altman [6,7]. Experimentally, Pierce and colleagues showed that postnatal ethanol exposure from day 4 to 10 in Sprague-Dawley rats resulted in more severe Purkinje cell loss in lobule I than in lobules V, VII, VIII, and IX of the cerebellar vermis [38]. Bonthius and West then demonstrated that the Purkinje cells that were more mature at the time of ethanol exposure were more vulnerable than the less mature Purkinje cells [10], which could account for a substantial regional variation in the vermis in the extent of alcohol-induced Purkinje cell loss in the third trimester-equivalent rat model. A single episode of binge-like ethanol exposure on postnatal day 4 caused Purkinje cell loss in lobule I to V, IX and X but not in lobules VI-VII, and this differential vulnerability was correlated with differences in the timing of Purkinje cell dendritic outgrowth, which is initiated in lobules VI-VII significantly later than in lobules I-IV and IX-X [20,21]. The relatively greater vulnerability of the “early-maturing” lobules of the cerebellum in the neonatal rat model is consistent with the findings by Hamre and West (1993) who showed that when binge-like exposure was limited to two days starting on different days between postnatal days 4-9, the greatest Purkinje cell loss was observed when ethanol exposure occurred on postnatal days 4-5 and no significant cell loss was observed when the exposure began after postnatal day 7 [23].

The goal of the present study was to use the sheep model to better resolve the extent to which vulnerability to prenatal ethanol-induced Purkinje cell loss depends both on the developmental timing of exposure and on region within the vermis. A binge ethanol exposure paradigm was restricted to either the entire first trimester-equivalent or the entire third trimester-equivalent, and mid-sagittal sections of the cerebellar vermis were divided into the early-maturing region, defined as lobules I and X, and the late-maturing region, defined as portions of lobules VIc and VII (see Figure 1). We hypothesized that both the early- and late-maturing cerebellar regions would be vulnerable to prenatal ethanol exposure but that the third trimester exposure would produce the Purkinje cell loss in the early-maturing but not in the late-maturing lobules, whereas the first trimester exposure would produce a generalized Purkinje cell loss irrespective of the vermal region.

2. Methods

2.1 Animals and Breeding

The experimental procedures were approved by the Institutional Animal Care and Animal Use Committee at Texas A & M University. Suffolk ewes (aged 2 to 6 years) were bred under controlled conditions as described elsewhere [39]. The day of mating (the day that the ewes were marked by the ram) was designated as gestational day (GD) 0. Ewes were then maintained in an environmentally regulated facility (22°C and a 12:12 light/dark cycle) where they remained for the duration of the experiments. Animals were fed 2 kg/day of a “complete” ration (Sheep and Goat Pellet; Producers Cooperative, Bryan TX) and they consumed all of the feed offered.

2.2 Treatment Groups

There were 4 treatment groups (total n=29) in this study: first trimester-equivalent saline control group (T1Sal) (n=7), first trimester-equivalent ethanol group (1.75 g/kg/day) (T1Eth) (n=7), third trimester-equivalent saline control group (T3Sal) (n=7), and third trimester-equivalent ethanol group (1.75 g/kg/day) (T3Eth) (n=8). This study modeled intermittent binge drinking over the first trimester-equivalent and the third-trimester-equivalent, respectively,

2.3 First Trimester-Equivalent Groups

On GD 4, the beginning of the first trimester-equivalent in this species, an intravenous catheter (16 ga., 5.25 inch Angiocath™; BACton Dickinson, Sandy, UT) was placed into the jugular vein of the ewes. Beginning on GD 4, ethanol (1.75 g/kg) or saline was administered intravenously over a 1-hour period via a peristaltic pump (Masterflex, Model 7014-20; Cole-Parmer, Niles, IL). The ethanol solution was prepared by adding 95% ethanol to sterile 0.9% saline to achieve a 40% w/v ethanol solution. Solutions were prepared under aseptic conditions and were administered through a 0.2 µm bacteriostatic filter. The saline control group received an infusion of isotonic saline (0.9%) that was equal in volume to the 1.75 g/kg ethanol infusion. Pumps were calibrated before each infusion. The ethanol infusions were given on 3 consecutive days, followed by 4 days without exposure, and this pattern was repeated until GD 41 (for a total of 18 alcohol exposure days), modeling “weekend binge drinking.” Maternal blood was withdrawn on GD 41, at the end of ethanol infusion for the measurement of BEC.

2.4 Third Trimester-Equivalent Groups

On GD 102 the ewes underwent surgery to chronically implant femoral arterial and venous poly-vinyl chloride catheters (0.05” inner diameter, 0.09” outer diameter) as previously described [16]. In brief, anesthesia was induced by administering diazepam (0.2mg/kg intravenously; Abbott Laboratories, North Chicago, IL) and ketamine (4 mg/kg intravenously, Ketaset®; Fort Dodge, IA). The ewes were intubated and a surgical plane of anesthesia was maintained using isoflurane (0.5% to 2.5% IsoFlo®; Abbott Laboratories) and oxygen. Arterial and venous catheters were advanced into the aorta and vena cava via the femoral artery and vein, respectively. At the end of surgery, the ewe received an injection of flunixin meglumine (1.1 mg/kg intramuscularly, Banamine®; Scherring-Plough, Union, NJ), a prostaglandin synthase inhibitor to reduce postoperative pain. Ewes also received postoperative antibiotics (ampicillin trihydrate, Polyflex® (25 mg/kg administered subcutaneously for 5 days); Aveco, Fort Dodge, IA and gentamicin sulfate, Gentavet® (2 mg/kg administered intramuscularly twice daily for 5 days); Velco, St. Louis, MO). The ethanol infusions were given on 3 consecutive days, followed by 4 days without exposure, modeling “weekend binge drinking,” from GD 109 to 132 (for a total of 12 exposure days), the third trimester-equivalent brain growth spurt in this species [17]. Maternal blood was withdrawn on GD 132, at the end of ethanol infusion for the measurement of BEC.

2.5 Fetal Cerebellar Tissue Processing

On GD 133, the ewes from all four groups were euthanized using sodium pentobarbital (75 mg/kg, intravenously), and the fetuses were removed from the uterus and fetal body weights and crown-rump lengths were measured and recorded. Fetuses were perfused with saline followed by cold fixative solution containing 1.25% paraformaldehyde and 3% glutaraldehyde in phosphate buffer (pH 7.4). The brains were removed and stored in additional fixative until processed for stereological cell counting. The cerebellum was dissected, embedded in 4% agar, and cut sagittally into 5 slabs. These slabs were dehydrated through increasing concentrations of ethanol (70%, 95%, and 100%) and then infiltrated

with increasing concentrations of infiltration solutions (25%, 50%, 75%, and 100% glycol methacrylate, Technovit® 7100 Cold Curing Embedding kit; Heraeus Kulzer; Wehrheim, Germany). The tissue in each slab was embedded in a solution containing 1 ml dimethyl sulfoxide (hardener) per 15 ml of 100% infiltration solution and allowed to harden. After hardening, the tissue was sectioned into 30 µm sagittal sections by using a microtome (model RM2255; Leica, Nussloch, Germany). Each vermal section of each slab was collected and a random number between 1-10 was generated to indicate the first section to save; every 20th and 21st section was mounted on a glass slide, stained with cresyl violet, and coverslipped.

2.6 Three-dimensional Cell Counting

The numerical volume density (N_V) of fetal cerebellar Purkinje cells was determined within both early- and late-maturing regions of a single mid-sagittal section of the cerebellar vermis using the optical disector method [54]. To select a single mid-sagittal section of the cerebellar vermis, each vermal section of each cerebellar slab was collected and a random number between 1-10 was generated to indicate the first section to save; every 20th and 21st section was mounted on a glass slide, stained with cresyl violet, and coverslipped. Mid-sagittal section of the cerebellar vermis did not include any of the deep cerebellar nuclei. As shown in Figure 1, the early-maturing region was defined as lobules I and X, and the late-maturing region was defined as the segments of lobules VIc/VII between the most posterior point of the first declival sulcus and the most anterior point of the prepyramidal fissure [26]. In brief, counts were made using an Olympus BX51 microscope (Olympus, Tokyo, Japan) that had a UPlanSApo 100X objective lens with a 1.4 numerical aperture condenser. The microscope had a motor-driven stage (MicroBrightField, Williston, VT, USA) to move within the x and y axes and an attached microcator to measure the z axis (Hiedenhain Z Encoder, Germany). The image was transferred to a personal computer (Precision T3500, Dell, Round Rock, TX, USA) via a color digital video camera (Retiga 200R, Q Imaging, Surrey, British Columbia, Canada) and analyzed with stereology software (StereoInvestigator 9®, MicroBrightField, Williston, VT, USA). The reference volume of each region was estimated using Cavalieri's Principle and was calculated by the equation $V_{ref} = \sum p_i A(p_i) t$ where p_i is the total number of points (p_i), $A(p_i)$ is the known area associated with each point (3600mm² in this study), and t is the known thickness of the section counted. The StereoInvestigator 9® software provided the template of points in various arrays that were used in point counting for reference volume estimation. Section thickness (t) was measured optically directly from the section at multiple sites; the four groups did not differ significantly in section thickness, with means ranging between 29.3µm and 29.9µm. The Purkinje cell density of each region within the section was calculated according to the formula $N_V = Q / (\sum disectors * A_{(fr)} h)$ where Q is the sum of the Purkinje cells counted, $\sum disectors$ is the sum of the number of disector frames counted, $A_{(fr)}$ is the known area associated with each disector frame (70µm X 111µm = 7770µm), and h is the known z-axis height of the disector (10µm). The placement of the disector frames was determined by the software in a systematic random manner. The estimated number of Purkinje cells per section in both the early- and late-maturing regions was then calculated by multiplying the reference volume of the region and the numerical density of cells within the region [54].

2.7 Statistical analysis

Fetal growth parameters were analyzed using a two-way analysis of variance (ANOVA) with the treatment group (saline control or ethanol) and trimester of exposure (first or third trimester-equivalent) as between group factors. Fetal cerebellar Purkinje cell density, regional volume, and Purkinje cell counts were analyzed using three-way analysis of variance (ANOVA), with the treatment group (saline control or ethanol) and trimester of

exposure (first or third trimester-equivalent) as grouping factors and the cerebellar region (early or late maturing) as a within-subjects factor. When significant main or interactive effects of treatment group were identified, differences between ethanol and saline control groups within each trimester of exposure were assessed by post hoc t-test comparisons within each region. For BEC determinations, comparisons between the two trimesters of exposure were analyzed with Student's t-test. Statistical significance was set at $\alpha = 0.05$.

3. Results

3.1 Maternal BEC

The mean $\pm$ SEM maternal BECs at the end of ethanol infusion (1 hour; the time point when BECs are known to peak), were 198.7 ± 13.1 mg/dl and 166.6 ± 12.4 mg/dl in the first and third trimester-equivalent ethanol treatment groups, respectively. The difference between the first and third trimester exposure groups was not statistically significant ($p=0.11$). The subjects were conscious throughout and after the ethanol infusion, but they appeared ataxic if stimulated to walk shortly after the end of the infusion. Subjects developed tolerance for the ethanol within 3-4 days and showed better motor coordination at the end of the ethanol infusion.

3.2 Fetal Growth Measurements

There were no significant main or interactive effects of the treatment group or trimester of exposure on fetal body weight (weight: T1Sal, 5.16 ± 0.30 kg; T1Eth, 4.77 ± 0.28 kg, T3Sal, 4.71 ± 0.36 kg; T3Eth, 4.86 ± 0.32 kg). There was a main effect of the trimester of exposure ($p=0.039$) on fetal crown-rump length but no significant main or interactive effects of treatment group (length: T1Sal, 49.49 ± 2.14 cm; T1Eth, 47.13 ± 1.98 cm; T3Sal, 54.80 ± 2.34 cm; T3Eth, 51.6 ± 2.34 cm).

3.3. Purkinje cell density

The average fetal cerebellar Purkinje cell numerical volume densities (N_V) as a function of region are shown in Figure 2. A mixed ANOVA confirmed a main effect of treatment group ($F(1,25)=8.26$, $p=0.008$), trimester of exposure ($F(1,25)=7.28$, $p=0.012$), and cerebellar region ($F(1,25)=20.46$, $p<0.001$), along with a significant region X treatment group interaction ($F(1,25)=13.64$, $p=0.001$) and a significant treatment group X trimester of exposure X region interaction ($F(1,25)=4.69$, $p=0.040$). The significant three-way interaction confirmed that the effect of ethanol treatment on the fetal Purkinje cell density depended on the developmental timing of exposure and the cerebellar region.

Follow-up comparisons for the first trimester exposure indicated that the ethanol exposure resulted in significant reductions of fetal Purkinje cell densities in both the early-maturing region ($p=0.021$) and the late-maturing region ($p=0.040$), with reductions of 28% and 21%, respectively, compared to the saline control group. In contrast, for the third trimester exposure, there was a significant reduction (31%) in Purkinje cell density in the early-maturing region ($p=0.009$) compared to the saline control group, but the groups did not differ significantly for the late-maturing region, confirming the differential vulnerability of the early vs. late-maturing vermal regions to the third trimester-equivalent ethanol exposure.

Representative photomicrographs of Purkinje cell densities of each region for the Saline and Ethanol groups from the first and third trimester exposure groups are shown in Figure 3. Note the reduced linear density of Purkinje cells in the early-maturing region for both the first and third trimester exposure groups, but only the first trimester exposure resulted in reduced linear densities in the late-maturing region.

3.4. Reference volume

Table 1 shows the reference volume of the early- and late-maturing regions of the fetal cerebellar mid-sagittal vermal section. A mixed ANOVA confirmed a main effect of the cerebellar region ($F(1,25)=76.21$, $p<0.001$), along with a significant interaction between the cerebellar region and trimester of exposure ($F(1,25)=4.96$, $p=0.035$). No main or interactive effect of treatment group was significant. The interaction between trimester of exposure and region appeared to be due to the larger volumes of the late-maturing region in the third trimester groups.

3.5. Purkinje cell number per section

The fetal cerebellar Purkinje cell counts per vermal section are shown in Figure 4. A mixed ANOVA confirmed a main effect of treatment group ($F(1,25)=14.49$, $p=0.001$), trimester of exposure ($F(1,25)=9.58$, $p=0.005$), and cerebellar region ($F(1,25)=5.395$, $p<0.029$); the interaction between the cerebellar region and treatment approached significance ($F(1,25)=3.955$, $p=0.058$). Follow-up analyses within the first trimester exposure group confirmed significant reductions of fetal cerebellar Purkinje cells both in the early-maturing region ($p=0.001$) and in the late-maturing region ($p=0.034$), with reductions of ~36% and ~23%, respectively, compared to the saline control group. In contrast, for the third trimester exposure group, there was a significant reduction (~37%) of the fetal cerebellar Purkinje cells in the early-maturing region ($p=0.016$), but no significant effect in the late-maturing region ($p=0.396$).

4. Discussion

The third trimester-equivalent ethanol exposure (between GD 109-132) caused a significant depletion of fetal cerebellar Purkinje cells in the early-maturing region (lobules I-X), but not in the late-maturing region (lobules VIc-VII). These findings for the third trimester-equivalent are similar to and consistent with the earlier findings from rodent models [10,19,22,23,31,32,36,53]. Thus, it appears that the sheep cerebellum, like the rodent, has regional differences in the vermis in vulnerability to binge alcohol exposure during the third trimester-equivalent. These are likely due to regional differences in maturation state at the time of alcohol exposure, assuming that the sheep cerebellum has maturational gradients in the vermis that resemble the rodent. In contrast, first trimester-equivalent ethanol exposure (between GD 4-41) resulted in a significant loss of fetal cerebellar Purkinje cells both in the early-maturing region (lobules I-IX) and in the late-maturing region (lobules VIc-VII) of the mid-sagittal vermis. These findings from the sheep model clearly demonstrate under controlled gestational exposure conditions that the regional pattern of cerebellar Purkinje cell loss induced by binge exposure in the first trimester-equivalent is significantly different from that induced by similar exposure in the third trimester-equivalent. However, because the extent of alcohol exposure was greater in the first trimester condition than in the third trimester condition (18 days of exposure vs. 12 days of exposure), we cannot yet determine whether the late-maturing region is more vulnerable to alcohol-induced depletion of Purkinje cells during the first trimester than the third, because the exposure cycles were not the same.

The most likely reason for the similar loss across lobules with the first trimester-equivalent exposure is that it affects the genesis of fetal cerebellar Purkinje cells as a whole, and thus produces reductions in this population independent of the cerebellar region. In sheep brain development, Purkinje cells do not appear in the fetal cerebellum until after the end of the first trimester-equivalent and immature Purkinje cells began to appear at GD 80 [43]. Exposure during the third trimester-equivalent period affects post-mitotic Purkinje neurons that are undergoing differentiation and dendritic and synaptic growth, and studies in rodents

have shown that the timing of these processes differs between the early-maturing regions and late-maturing regions [20]. The Purkinje cell loss with third trimester-equivalent exposure in rodents is due to ethanol-induced apoptosis [27], but it is not known why the late-maturing region shows less Purkinje cell death than the early-maturing region even if comparable ethanol exposure is timed to occur during the period of dendritic outgrowth of the two respective regions [23].

This study in sheep makes the assumption that the maturation of the cerebellar vermis during the third trimester-equivalent in sheep matches the general regional differences in maturation identified in laboratory rodents, i.e. lobules I and X mature earlier than lobules VIc and VII. No systematic studies have been done to document this in sheep, however, Altman (1972) first demonstrated this principle in rats by showing that Purkinje cells are segregated across the cerebellar vermis according to maturational state [3]. During embryonic development, precursors of Purkinje cells may be generated relatively close in time, but the process of differentiation and maturation of Purkinje cells varies upon their regional location in the cerebellum [10,20]. Therefore, the difference between the Purkinje cells in the two different maturational regions is more 'maturational stage' than 'age' [10]. In rats on postnatal day 8, microtubule-associated protein 2 (MAP2) expression marking dendritic outgrowth of the Purkinje cells was more advanced in lobule I, II, IX and X than in lobule VI, VII and VIII [20].

The mechanisms associated with the greater vulnerability of Purkinje cells in the early-maturing regions of the cerebellar vermis during the third trimester-equivalent are not known, but may be associated with their advanced neural connectivity and network function (e.g., with climbing fibers and parallel fibers) compared to the Purkinje cells from later maturing regions [55]. We speculate that Purkinje cells from more mature regions during the third trimester-equivalent that are vulnerable to ethanol-induced apoptosis may be more likely to form networks with local microglia, which play a role in apoptosis of Purkinje cells. Studies in the mouse cerebellum showed that microglia promote the death and subsequent engulfment of Purkinje cells that express activated caspase-3 when they are undergoing synaptogenesis [33]. Similar results were observed in a developing nematode *C. elegans*, where cells in the advanced caspase (CED-3)-dependent stage of degeneration could recover, if they were not engulfed by neighboring "death execution" cells [24,42]. We speculate that differences in regional vulnerability of Purkinje cells may be associated with the regional differences in these Purkinje cell-microglia networks. Alternatively, there may be regional differences in vulnerability to ethanol-induced oxidative stress. Altman's (1972) findings in the rat model showed that during the postnatal developmental period of the cerebellar cortex, complex regional differences were observed in various oxidative enzymes such as cytochrome oxidase, succinate dehydrogenase, and lactate dehydrogenase and this may contribute to differences in oxidative insult [3].

This is the first *in utero* animal study using a sheep model to report differential regional and temporal vulnerability of fetal cerebellar Purkinje cells to prenatal ethanol exposure. The sheep model has key advantages including a longer gestational period (approximately 147 days) and *in utero* third trimester-equivalent fetal brain growth spurt, which helps to build a strong translational bridge between rodent models and the human condition. Prenatal ethanol consumption at any time in pregnancy can have a deleterious effect on fetal Purkinje cell development. This study and previous studies have shown that early exposure causes more generalized Purkinje cell loss, independent of the cerebellar region. Prenatal ethanol exposure during the third trimester period has selective deleterious impact on the early-maturing cerebellar regions. These results are most likely accounted for by the different mechanisms by which Purkinje cells are lost in early and late gestation. Though we do not yet know the molecular mechanisms behind the differential regional vulnerability of

Purkinje cells in the third trimester-equivalent brain growth spurt period, these findings emphasize that the developing cerebellum is vulnerable to binge ethanol exposure throughout the prenatal period, and that different mechanisms of damage will be related to cell loss at different prenatal periods.

Acknowledgments

Supported by NIAAA Grant AA017120 and AA10940 (TAC) and K08AA18166 (SEW). All or part of this work was done in conjunction with the Collaborative Initiative on Fetal Alcohol Spectrum Disorders (CIFASD), which is funded by grants from the National Institute on Alcohol and Alcohol Abuse (NIAAA). Additional information about CIFASD can be found at www.cifasd.org.

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Highlights

- We proposed prenatal ethanol exposure causes differential fetal Purkinje cell loss.
- Fetal cerebellar lobules were divided into early and late maturing regions. Lobule I and X were early maturing and Lobule VI/VII were late maturing regions.
- First trimester exposure caused Purkinje cell loss, independent of cerebellar region.
- Third trimester exposure caused Purkinje cell loss in the early maturing region only.

Figure 1.

Photomicrograph of a mid-sagittal section of the cerebellar vermis, with the highlighted areas illustrating the regions defined as the “early-maturing region” and the “late-maturing region.” Lobules I and X represent the early-maturing region. The late-maturing region extends from the most posterior point of the first declival sulcus (SDS) to the most anterior point of the prepyramidal fissure (PPF) (and thus includes continuous segments of lobules VIc- VII). Purkinje cells in these two regions were counted to estimate the Purkinje cell density and number per section.

Figure 2.

Mean fetal cerebellar Purkinje cell density (N_v) (cells/mm³) in the early-maturing region (lobules I-X) and in the late-maturing region (lobules VIc-VII) of the mid-sagittal section of the vermis for the saline control and ethanol exposed groups during the first trimester (T1) or third trimester-equivalent (T3) of gestation. First trimester-equivalent prenatal ethanol exposure significantly decreased the fetal Purkinje cell density in both the regions; third trimester-equivalent exposure caused a significant decrease in fetal Purkinje cell density in the early-maturing region, but not in the late-maturing region. Data are presented as group mean $\pm$ SEM and * indicates statistically significant differences between groups with $p < 0.05$.

Figure 3.

Photomicrographs of a mid-sagittal section of the fetal cerebellar vermis. The panels depict representative samples of the density of Purkinje cells (arrows) from subjects in each of the following: Early-maturing region of the first trimester-equivalent groups (A and B) and third trimester-equivalent groups (C and D); Late-maturing region of the first trimester-equivalent groups (E and F) and third trimester-equivalent groups (G and H). First trimester-equivalent chronic ethanol exposure affects fetal cerebellar Purkinje cell density in both the regions (B and F) while third trimester-equivalent ethanol exposure caused a significant depletion of Purkinje cells in the early-maturing region (D) but not in the late-maturing region (H). Slides were stained with cresyl violet. Magnification bar (in red in upper left panel) = 50 μ M, and applies to all panels.

Figure 4.

Mean fetal cerebellar Purkinje cell number in vermal section of the early-maturing region (lobules I-X) and in the late-maturing region (lobules VIc-VII) for the saline control and ethanol exposed groups during the first trimester (T1) or third trimester-equivalent (T3) of gestation. First trimester-equivalent prenatal ethanol exposure decreased the fetal Purkinje cell number in both regions; third trimester-equivalent exposure caused a decrease in fetal Purkinje cell number in the early-maturing region, but not in the late-maturing region. Data are presented as group mean $\pm$ SEM and * indicates statistically significant differences between groups with $p < 0.05$.

Table 1

Reference volume (in mm³) of the two regions of the mid-sagittal fetal cerebellar vermal section. Data are presented as group mean $\pm$ SEM

Group	Early Maturing Region (Lobule I-X)	Late Maturing Region (Lobule VI-VII)
T1 Saline	0.64 $\pm$ 0.06	0.79 $\pm$ 0.05
T1 Ethanol	0.57 $\pm$ 0.04	0.77 $\pm$ 0.06
T3 Saline	0.60 $\pm$ 0.04	0.93 $\pm$ 0.05
T3 Ethanol	0.55 $\pm$ 0.02	0.80 $\pm$ 0.04