Acid-sensitive channel inhibition prevents fetal alcohol spectrum disorders cerebellar Purkinje cell loss

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Maternal ethanol abuse during pregnancy can result in a range of structural and functional abnormalities that include lifelong physical, mental, behavioral, and learning disabilities, now collectively termed fetal alcohol spectrum disorders (FASD) (42, 46). The incidence of FASD is estimated to be as high as 10 per 1,000 live births (28). Each year, in the United States alone, 40,000 babies are born with FASD (46) at an estimated cost of $1.4 million per individual and a total cost of $6 billion (22). The concern over the alarmingly high incidence of FASD in spite of extensive efforts to educate women not to abuse ethanol during pregnancy has stimulated the investigation of ways to prevent or mitigate the effects of prenatal ethanol exposure (9).

Efforts to successfully prevent or ameliorate the teratogenic effects of ethanol have been impeded, at least in part, by a limited understanding of the mechanisms by which ethanol damages the developing brain (9). A series of reports from our laboratory have established in an ovine model system that maternal ethanol consumption results in a decrease in maternal and fetal arterial pH (11, 33, 39). Acidemia was hypothesized to be a mechanism underlying the teratogenic effects of ethanol (16), even before fetal alcohol syndrome was described by Jones et al. (17). Clinically, in humans, ethanol consumption causes a decrease in blood pH that is directly proportionate to the blood ethanol concentration (20, 21, 44, 57). Therefore, we hypothesized that a pH-sensitive pathway is involved in ethanol teratogenesis and that interference with this pathway may prevent fetal cerebellar neuronal loss, a brain structure that is extremely sensitive to ethanol (33, 38, 53). Abnormal cerebellar development is reported to be the most sensitive morphological indicator of prenatal ethanol exposure in humans (1), and the developing cerebellum and the differentiating cerebellar Purkinje neurons have been established as the most vulnerable brain region and cell type, respectively, in animal model systems (24, 25).

To test the hypothesis that pH is mechanistically important in mediating the fetal cerebellar damage in response to prenatal ethanol exposure, we included in this study an ethanol exposure group, a group in which the ethanol-mediated pattern of pH alteration was created independent of ethanol exposure and a group where ethanol-induced alterations in pH were prevented by the controlled infusion of a nonselective blocker of the newly identified TWIK-related acid-sensitive potassium channels (TASK 1 and TASK 3). The TASK 1 isoform is an exquisitely pH-sensitive channel with a pK of 7.3–7.4 (13, 15) and is responsible for chemoreceptor responses to extracellular pH (28). These channels are expressed in chemoreceptors, as well as throughout the central nervous system, in both respiratory and nonrespiratory areas, and they exhibit a standing outwardly rectifying potassium current that is inhibited principally by decreases in extracellular pH (13, 30). The TASK 3 isoform has a pK of 6.0–6.7 and, in humans, is only expressed in the cerebellum (28, 42). Finally, although TASK channels are principally inhibited by pH, their activity is reported to be modulated by decreases in oxygen levels, and because ethanol induces both a fall in pH as well as a mild transient decrease in the maternal arterial partial pressure of oxygen (PaO2), we also

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tested the role of oxygen on the differentiating fetal cerebellar Purkinje cells (11).

These experiments were performed in a sheep model system, where the brain growth spurt occurs in utero as in humans (12, 41), using a 3 days/wk binge drinking pattern (a common drinking pattern among women who drink during pregnancy) (6, 14, 25) throughout the third trimester equivalent of human brain development.

METHODS

Subjects. The experimental procedures were approved by the Institutional Animal Care and Use Committee at Texas A&M University. Suffolk ewes, aged 2–6 years of age, were mated and pregnancies of known date of conception were confirmed, as previously described (37). The day of mating was designated as gestational day (GD) 0. Ewes were maintained in shaded outdoor pens with herdmates from before mating until GD 90. On GD 90, the ewes were relocated to an environmentally regulated facility (22°C and a 12:12 light-dark cycle), where they remained for the duration of the experiments. Animals in all treatment groups were fed 2 kg/day of a “complete” ration (Sheep and Goat Pellet, Producers Cooperative, Bryan, TX). All animals consumed all of the feed offered (37).

Treatment groups. Eight treatment groups were used in this study: 1) an untreated normal control group, 2) a saline control group (received 0.9% saline at a rate and volume equivalent to that of the ethanol group on a per-kilogram basis) that served as a control for nutrition, instrumentation and the volume of infusion delivered, 3) an ethanol group that received ethanol at a dosage of 1.75 g/kg body wt, as a 40% wt/vol solution diluted in 0.9% saline, 4) an “ethanol-normal PaO2” group, where a transient decrease in the maternal arterial partial pressure of oxygen (PaO2) produced at the end of each ethanol infusion was abolished by providing increased inspired fractional concentration of oxygen (FiO2) to the ewe at 0.9% saline, 5) a “low PaH-normal PaO2” group, where the arterial pH (pHa) pattern produced by ethanol was mimicked for the whole of the third-trimester equivalent, independent of ethanol (received saline), 6) a “low PaH-low PaO2” group, where the maternal fall in pHa and the transient decrease in maternal PaO2 were both mimicked, 7) an ethanol-TASK inhibitor (ethanol-TI) group, in which TASK channels were inhibited pharmacologically, and 8) a saline-TASK inhibitor (saline-TI) group that served as a control for the ethanol-TI group. A total of 56 pregnant ewes were enrolled in this study. The final number of fetal brains used for quantitative analysis was 40. Losses occurred due to loss of pregnancy, catheter failure, the elimination of subjects for minor ailments (inappetance, fever, or hoof ailments), or mechanical damage to the fetal brain during processing. In ewes with twin pregnancies, the fetus with the larger body weight was selected for neuronal counting. A group of pregnant ewes and fetuses, in addition to the eight treatment groups, were chronically instrumented on GD 113 to obtain fetal blood gas responses to acute administration of maternal treatments (saline, ethanol, low pHa-normal PaO2, or low pHa-low PaO2).

Maternal and fetal surgery. A week before the start of the experiment, on GD 102, the ewes underwent surgery (except the normal control and fetal instrumented groups) to chronically implant maternal femoral arterial and venous catheters (0.050-in. inner diameter, 0.090-in. outer diameter polyvinyl chloride). Details of the surgery protocol have been described earlier (11).

In the fetal instrumented group, surgery was performed on GD 113 to implant chronic indwelling catheters, as previously described (11). In brief, a ventral midline laparotomy was performed and the uterus and fetal membranes were incised. A catheter (0.030-in. inner diameter, 0.050-in. outer diameter polyvinyl chloride) was passed from the cranial tibial artery into the abdominal aorta. Catheters were passed through the flank of the ewe and were stored in a pouch attached to the skin.

**Experiment protocol.** The treatment conditions were created on three consecutive days beginning on GD 109 followed by 4 days without treatment with the weekly pattern being repeated until GD 132. In all treatment groups, the infusion solutions were delivered intravenously over an hour by peristaltic pump (Masterflex, model 7014-20, Cole Parmer, Niles IL) through a 0.2-μm bacteriostatic filter. Pumps were calibrated before each infusion.

On the day of an experiment, ewes in all treatment groups were placed in a modified metabolism cart, so that the animal’s head was inside a Plexiglas chamber. A vinyl diaphragm attached to the open side of the chamber was drawn around the animal’s neck to isolate the atmosphere in the chamber from ambient air. In the low pHa-normal PaO2 and low pHa-low PaO2 groups, subjects were exposed to increased inspired fractional concentrations of carbon dioxide for 6 h, to create a matching magnitude and pattern of reduction in the pHa compared with that produced by ethanol in previous studies (11) and in the present study (Fig. 1). The rate at which CO2 was introduced into the chamber in the low pHa groups was determined by monitoring maternal arterial pHa (ABL 5, Radiometer, Cleveland, OH); the CO2 inflow rate was adjusted so that maternal pHa in the low pHa and ethanol groups were matched over the duration of the 6-h experimental period on all 12 experimental days. The percentage of oxygen and carbon dioxide in the chamber was measured using a gas monitor (oxygen, model S-3A; carbon-dioxide, model CD-3A, Applied Technologies, Pittsburgh, PA). Normoxic conditions were maintained throughout the experiment in the low pHa-normal PaO2 group. In the low pHa-low PaO2 group, in addition to replicating the low pHa, the mild transient reduction in the maternal PaO2 (~4 mmHg) observed at the 1st h in response to ethanol (the end of ethanol infusion) (11) was mimicked by increasing the inspired fractional concentration of nitrogen (FiN2). This increased FiN2 would not result in hypocapnea, as the inspired CO2 concentration was experimentally controlled. For instance, the PaCO2 values in the low pHa-normal PaO2 and the low pHa-low PaO2 groups were 34 ± 0 mmHg and 34 ± 1 mmHg at 0 h and 41 ± 0 mmHg and 42 ± 1 mmHg at 0.5 h, respectively. In the ethanol-normal PaO2 group, the transient reduction in maternal PaO2 was abolished by increased inspired fractional concentration of oxygen. Subjects in the ethanol and the saline control groups had their heads inside the Plexiglas chamber, but the chamber bottom was removed to allow breathing of room air. In the ethanol-TI and
saline-TI groups, a TASK 1/3 channel blocker (doxapram hydrochloride, Dopramp-V, Fort Dodge, IA) (56) was administered; the rate of administration was adjusted to 0.5 mg/min, 1 mg/min, 2 mg/min, 2.67 mg/min, 4 mg/min, 4.67 mg/min, 3.33 mg/min, 2.67 mg/min, 1 mg/min, and 0.5 mg/min at 0, 5, 10, 15, 30, 45, 75, 90, 120, and 135 min, respectively. The rates of TI administration were further fine-tuned in the ethanol-TI group to prevent any ethanol-induced decreases in pHi based on the immediate feedback data obtained from blood gas analyzer measurements.

Measurement of arterial pH, blood gases, and blood ethanol concentrations. Blood (1 ml) was drawn from the femoral artery catheter at 0, 0.5, 1, 1.5, 2, 3, 4, 5, and 6 h for blood gas analysis on all experiment days. Samples were collected in heparinized 3-ml syringes, capped and immediately analyzed using a blood gas analyzer (ABL 5; Radiometer Westlake, OH).

Blood ethanol concentration was measured at 0 and 1 h. A 20-μl aliquot of blood was collected into microcapillary tubes and transferred into vials that contained 0.6 N perchloric acid and 4 mM N-propyl ethanol (internal standard) in distilled water. The vials were tightly capped with a septum-sealed lid and were stored at room temperature until analysis by headspace gas chromatography (Varian Associates, model 3900; Palo Alto, CA) at least 24 h after collection. The basic gas chromatographic parameters were similar to those reported by Penton (35), with the exception of the column (DB-wax, Megabore; J&W Scientific, Folsom, CA) and the carrier gas (helium) used (53).

Fetal cerebellar tissue processing. On GD 133, the ewes were euthanized using pentobarbital sodium (75 mg/kg iv), and the fetuses were removed from the uterus and 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 five slabs. The slabs were dehydrated through increasing concentrations of ethanol (70, 95, 100%) and then infiltrated with increasing concentrations of infiltration solution (25, 50, 75, 100% methyl methacrylate; Technovit 7100 Embedding kit; Leica, Wetzlar, Germany). The tissue in each slab was embedded in a solution containing 1 ml DMSO (hardener) per 15 ml of 100% infiltration solution and allowed to harden. After hardening, the tissue was sectioned into 30-μm sagittal sections by microtome (model RM2255; Leica, Nussloch, Germany). Every twentieth section was saved, mounted on a glass slide, stained with cresyl violet, and coverslipped.

Stereological cell counting. The total number of fetal cerebellar Purkinje cells was estimated using unbiased stereological cell-counting techniques, as described previously (39). In brief, the microscope (Nikon Optiphot; Garden City, NY) used in this study had a 40× objective lens with a 1.4 numerical aperture condenser. The microscope had a motor-driven stage to move within the x and y axes and an attached micrometer to measure the z axis. The image was transferred to a personal computer (Millennium, Micron; Boise, ID) via a color video camera (model 2040; Jai, Copenhagen, Denmark). The reference volume (Vref) was estimated using the Cavalieri’s Principle and was calculated by the equation Vref = Σp × A(p) × t where Σp is the total number of points (p) counted, A(p) is the known area associated with each point, and t is the known distance between two serial sections counted. The GRID software (Medicosoft, Nussloch, Germany) provided templates of points in various arrays that were used in point counting for reference volume estimation. The Purkinje cell density was determined by following the optical dissector method, which was calculated using the formula, Nv = ΣQ/(Σdisector × A(fr) × h), where ΣQ is the sum of the Purkinje cells counted from each dissector frame, Σdisector is the sum of the number of dissector frames counted, A(fr) is the known area associated with each dissector frame, and h is the known distance between two dissector planes. The placement of the dissector frames was determined by the GRID software in a random manner. The estimated total number of Purkinje cells in the cerebellum was then calculated by multiplying the reference volume of the cerebellum and the numerical density of cells within this reference volume, as described before (53).

Immunohistochemistry. On GD 133, the ewes were euthanized using pentobarbital sodium (75 mg/kg iv), and the fetuses were removed from the uterus and perfused with saline followed by cold fixative solution containing 4% formalin (pH 7.4). The brains were removed and stored in additional fixative until processed for immunohistochemistry.

The fetal cerebellum was dissected, paraffin embedded, and cut by microtome (model RM2255; Leica, Nussloch, Germany) into 4-μm sagittal sections. Sections were deparaffinized and rehydrated, and antigen retrieval was achieved by microwave heating for 15 min on low power in 0.01 M citrate buffer (pH 6.0), as described previously (54). Endogenous peroxidase activity was blocked by applying 3% hydrogen peroxide. After 30 min of blocking with goat serum, rabbit anti-TASK 1, rabbit anti-TASK 3 antibodies (Sigma, St. Louis, MO) at dilution of 1:50, or the control isotype IgG was applied and incubated overnight at 4°C. Slides were washed 3 times with PBS for 5 min. The biotinylated secondary antibody and the streptavidin-biotin complex conjugated with HRP (Invitrogen, Carlsbad, CA) were applied, each for 30 min at room temperature with interval washings. After rinsing with PBS, the slides were immersed for 10 min in 3,3’-diaminobenzidine (Sigma) at 0.4 mg/ml, rinsed with distilled water, counterstained with hematoxylin, and dehydrated; a cover-slip was then applied. The results were visualized on an Olympus (AH-3; Olympus, Tokyo, Japan) microscope equipped with a Spot Insight Color digital camera (Diagnostic Instruments, Sterling Heights, MI), and images were obtained using Spot Digital Camera Systems software.

Data analysis. The stereology data, including the estimated total number of cells, cell density, and tissue volume for all eight treatment groups, were analyzed using a one-way ANOVA with “treatment” as the sole independent variable. When treatment significance was established by the initial analysis, pairwise comparisons were performed using Fisher’s protected least significant difference test. Statistical significance was established a priori at P < 0.05.

RESULTS

The effect of ethanol on the fetal cerebellar Purkinje cell number. The maternal blood ethanol concentration (258 ± 10 mg/dl) peaked at the end of each ethanol infusion (1 h) in the ethanol-treated ewes. Quantitative analysis demonstrated significantly lower cerebellar Purkinje cell number in ethanol-exposed fetal brains compared with pair-fed saline-infused or normal control fetal brains (Fig. 2). Ethanol resulted in a 45% reduction in the total number of Purkinje cells compared with the saline controls. Cerebellar Purkinje cell density also was lower in ethanol-exposed subjects compared with controls (Figs. 3 and 4A, 4B).

The effect of decreases in pH on the fetal cerebellar Purkinje cell number. Maternal pHi was reduced for 6 h with every bout of ethanol infusion (Fig. 1) to a degree known to inhibit the TASK 1 but not the TASK 3 isofrom (36). Mimicking the pHi throughout the chronic infusion period produced a significant reduction (~24%) in the number of fetal cerebellar Purkinje cells compared with that in the two control groups (Figs. 2, 4C). However, the estimated number of Purkinje cells in the low pHi-normal PaO2 group was significantly higher compared with the ethanol-infused subjects.
The effect of transient maternal decreases in oxygen on the fetal cerebellar Purkinje cell number. In addition to the significant fall in pH, a mild transient decrease in the maternal PaO2 (≈4 mmHg) was observed at the end of each ethanol infusion. Because TASK channels respond to decreases in oxygen levels in addition to changes in extracellular pH, we tested the role played by these decreases in maternal PaO2 by supplementing the ethanol-infused ewes with increased inspired fractional concentration of oxygen (ethanol-normal PaO2 group) to abolish any decreases in maternal PaO2. However, supplementation with oxygen did not have an exclusive effect on the fetal cerebellar Purkinje cell number. The treatments under each horizontal line are not different from one another, while each group of treatments beneath a horizontal line are significantly (*) different from all other groups of treatments. Values are expressed as means ± SE.

DISCUSSION

Ethanol reduces fetal cerebellar Purkinje cell number. The present study used a 3 day/wk binge drinking pattern. This is a common drinking pattern among women who drink during...
pregnancy (6, 14, 25). Consistent with previous FASD literature, the current study demonstrates a significant reduction in the cerebellar Purkinje cell number in ethanol-exposed fetal brains. The dysgenesis of cerebellum is one of the most common abnormalities in human prenatal ethanol exposure studies and is considered to be the most sensitive morphological indicator of FASD (1, 7, 34). Complementing these human studies, quantitative stereology studies conducted using the rat have demonstrated the developing cerebellum to be one of the most vulnerable brain structures to prenatal ethanol exposure and the Purkinje cells to be the most susceptible cerebellar cell type (3, 23, 26).

Role of pH. Alteration in fetal pH was suggested as a mechanism underlying the teratogenic effects of ethanol even before fetal alcohol syndrome was described by Horiguchi et al. (16) and Jones et al. (17). In humans, ethanol consumption results in acidosis, and the change in blood pH is directly proportional to the blood ethanol concentration (20, 21, 57). In this study, we found that mimicking the pH pattern created by ethanol throughout the third-trimester equivalent of gestation resulted in fetal cerebellar Purkinje cell loss. These changes in pH are comparable to fetal cerebellar interstitial pH (19, 45, 48). Multiple explanations might account for the role of ethanol-induced fall in pH in fetal brain injury. Alterations in pH

![Fig. 4. Photomicrographs of sagittal sections of the fetal cerebellar vermis, all taken from a similar location in the uvula, represent the density of Purkinje cells (arrows) in saline control (A), ethanol (B), low pH-normal PaO2 (C), and ethanol-TI treatment group (D) subjects. Note the lower Purkinje cell number in response to ethanol (B) and low pH (C) compared with saline control (A) and ethanol-TI (D) treatment group subjects.]

Fig. 5. Fetal sheep cerebellar TASK 1 and TASK 3 channel distribution. A and B: TASK 1 channels are exclusively present in the cerebellar Purkinje cells (brown). TASK 1 immunoreactivity was not detected in the granule cells while TASK 3 channels (C and D) were detected only in the granule neurons (brown). TASK 3 immunoreactivity was not detected in the Purkinje cells.
like those created in this study decrease the TASK 1 channel rectifying current, resulting in Purkinje cell depolarization (13, 36, 56). In adult humans, TASK 1 channels are expressed in the central nervous system, including the cerebellum and some peripheral tissues, whereas significant expression of TASK 3 channels occurs only in the cerebellum (29, 43). In fetal sheep, we found that TASK 1 channels are expressed predominantly in the Purkinje neurons within the cerebellum. While it is possible that repeated decreases in pH and consequent TASK channel-mediated Purkinje cell depolarization during fetal brain growth spurt may be responsible for reduced Purkinje cell numbers in response to prenatal ethanol, the finding that the nonspecific TASK 1/3 channel inhibitor prevented the ethanol-induced reduction in Purkinje cell number argues against this conclusion. Therefore, mechanisms not involving TASK channel manipulations that include pH-mediated inhibition of granule cell NMDA receptors (EC50 = 7.3) (50), decreases in maternal glutamine levels (18, 27), increases in ACTH and cortisol (2, 10), decreases in growth factors (4, 5), increases in oxidative stress (55), and actions on ultrasensitive protein switches (47) could have played a role in the observed fetal brain injury.

Protection offered by nonspecific TASK channel inhibition. The most salient finding in this study is that the pharmacological inhibition of the novel tandem two-pore domain acid-sensitive potassium channels (TASK 1 and TASK 3) effectively protects the fetal cerebellum from Purkinje cell loss in response to 3 days/wk ethanol bingeing throughout the third-trimester equivalent of human brain development. In this study, we found that TASK 1 channels are expressed in the fetal sheep cerebellar Purkinje neurons, whereas TASK 3 channels are expressed in the granule cells. Cerebellar granule cells (neurons that synapse on the Purkinje cells) are one of the most populous cell types in the mammalian brain, and they express a standing outwardly rectifying potassium current (TASK current) that does not inactivate and is responsible for the large negative resting membrane potential in these cells (15, 30, 52). These channels control the firing threshold and the firing frequency. They are insensitive to the classical broad-spectrum potassium channel blocking drugs 4-aminopyridine and tetraethylammonium ions (30, 52). Ethanol exposure in the perinatal period is known to induce arrest of cell differentiation through inhibition of granule cell NMDA receptors (32, 51). During brain development, modest NMDA receptor activation has trophic effects and has a permissive effect on the action of other neurotrophic factors (49). Therefore, TI-induced depolarization of the cerebellar granule cell, leading to increased excitability (30) and NMDA-elicited calcium signals (31) may act to prevent ethanol-induced cerebellar Purkinje cell death. It should also be noted that a similar protection could not have been offered by a fall in pH, as the magnitude of fall would have effectively inhibited only TASK 1 channels, which were not expressed in the granule cell. On the contrary, decreases in pH are known to inhibit granule cell NMDA receptors with an EC50 of 7.3 (50). Second, TASK channels are abundantly present in the peripheral and central chemoreceptors and, upon inhibition of these channels, they prevent maternal and fetal decreases in pH by increasing maternal ventilation (56). In summary, these findings suggest that nonspecific TASK 1/3 pharmacological blockade may prevent the cerebellar injury seen in FASDs.

**Hyposia and neuronal damage.** While ethanol causes a reduction in maternal PaO2, these decreases are small, of questionable biological significance, and do not result in changes in fetal PaO2 (11, 33, 40). Further, abolishing any mild decreases in maternal PaO2 with oxygen supplementation in ethanol-treated ewes produced no effect on cerebellar Purkinje cell number. Finally, these data support previous reports that ethanol does not result in fetal histotoxic, anemic, ischemic, or hypoxic hypoxia (33). Therefore, this study provides conclusive evidence that hypoxia is not responsible for the cerebellar damage in response to moderate doses of ethanol exposure during the third-trimester equivalent of human brain development.

**Perspectives and Significance**

In summary, the present findings demonstrate, in a whole animal model that effectively models human brain development during the third trimester, a selective, mechanism-based intervention that prevents the fetal cerebellar Purkinje cell loss mediated by prenatal ethanol exposure. This study demonstrates that direct pharmacological blockade of TASK 1 and TASK 3 channels protects the most sensitive target of fetal alcohol exposure, cerebellar Purkinje cells. In addition to these beneficial effects, it is suggested that pH and TASK channels may actually form the basis for a number of other therapeutic interventions that are currently being investigated to prevent and/or mitigate the effects of maternal drinking on the offspring. For example, investigators have proposed choline supplementation during pregnancy as a way to prevent or mitigate prenatal ethanol exposure injury (8, 49), but the mechanism is unknown. One possibility is that choline supplementation increases ACh, which blocks TASK channels through its action on M1 muscarinic receptors (30). Others have demonstrated that developmental ethanol exposure results in the arrest of cell differentiation through the inhibition of NMDA receptors (32); the underlying mechanisms are again unknown. We suggest that this effect of ethanol may be mediated by the action of pH (50). Therefore, we propose that pH-related mechanisms may form the basis for a number of therapeutic strategies that have been hypothesized to prevent specific damaging effects of prenatal ethanol exposure and that inhibition of the novel tandem two-pore domain potassium channels (TASK 1 and TASK 3) shows a promising direction for preventing the damaging consequences of maternal drinking during pregnancy.

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