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Research article

# Ethanol exposure during development reduces GABAergic/glycinergic neuron numbers and lobule volumes in the mouse cerebellar vermis

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## HIGHLIGHTS

- Used transgenic mice expressing fluorescent inhibitory neurons.
- Exposed to ethanol during 2nd and 3rd trimester equivalents.
- Inhibitory interneurons were reduced in lobule II at postnatal day 16.
- Purkinje cells were reduced in lobules II, IV–V and IX.
- Volume of lobules II, IV–V, VI–VII, IX and X was also decreased.

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## ABSTRACT

Cerebellar alterations are a hallmark of Fetal Alcohol Spectrum Disorders and are thought to be responsible for deficits in fine motor control, motor learning, balance, and higher cognitive functions. These deficits are, in part, a consequence of dysfunction of cerebellar circuits. Although the effect of developmental ethanol exposure on Purkinje and granule cells has been previously characterized, its actions on other cerebellar neuronal populations are not fully understood. Here, we assessed the impact of repeated ethanol exposure on the number of inhibitory neurons in the cerebellar vermis. We exposed pregnant mice to ethanol in vapor inhalation chambers during gestational days 12–19 and offspring during postnatal days 2–9. We used transgenic mice expressing the fluorescent protein, Venus, in GABAergic/glycinergic neurons. Using unbiased stereology techniques, we detected a reduction in Venus positive neurons in the molecular and granule cell layers of lobule II in the ethanol exposed group at postnatal day 16. In contrast, ethanol produced a more widespread reduction in Purkinje cell numbers that involved lobules II, IV–V and IX. We also found a reduction in the volume of lobules II, IV–V, VI–VII, IX and X in ethanol-exposed pups. These findings indicate that second and third trimester-equivalent ethanol exposure has a greater impact on Purkinje cells than interneurons in the developing cerebellar vermis. The decrease in the volume of most lobules could be a consequence of a reduction in cell numbers, dendritic arborizations, or axonal projections.



## 1. Introduction

It is well-established that exposure to ethanol during fetal development causes birth defects, with the developing brain being particularly sensitive to the teratogenic effects of ethanol [38]. Among the brain regions affected by developmental ethanol exposure is the cerebellum, which is involved, not only in motor coordination, but also in higher cognitive processes [22,37]. Frequent problems in individuals with Fetal Alcohol Spectrum Disorder (FASD) include deficits in fine motor control, motor learning and balance, which have been linked to cerebellar damage [5,14]. Moreover, studies suggest that alterations in timing, sensorimotor processing, and mathematical ability are also a consequence of cerebellar circuit dysfunction [10,26]. Neuroimaging studies have demonstrated structural and functional alterations in cerebella of children with FASD [3,9]. Although the precise mechanisms responsible for these effects are presently unknown, studies with

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rodent models suggest that ethanol disrupts neuronal migration and survival both in the developing cerebellar cortex and deep cerebellar nuclei [13,15,28,30,31]. Longer-term deficits in neuronal function, synaptic transmission, and plasticity have also been observed in Purkinje and granule cells of rodents exposed to ethanol during development [8,19,20,29,40,43]. However, little is known regarding the impact of developmental ethanol exposure on other cerebellar neuronal populations.

The molecular layer of the cerebellar cortex contains two populations of specialized GABAergic interneurons that control the function of Purkinje cells [22,42]. Stellate cells, located in the outer portion of the molecular layer, make synaptic connections with Purkinje cell dendrites. Basket cells are located in the inner portion of the molecular layer and provide inhibitory input to the soma and axonal initial segment. The granule cell layer also contains inhibitory interneurons: the Golgi cells, which release GABA onto granule cells, other Golgi cells and unipolar brush cells (they also release glycine onto the latter) and Lugaro cells which release GABA and glycine onto Golgi cells and molecular layer interneurons. Other less abundant subtypes of granule cell interneurons are globular and candelabrum cells [2,18]. During the equivalent to the 2nd trimester of human pregnancy, cerebellar interneurons are born and gradually begin to migrate to their final destinations [27,39]. This process continues during the first 2 weeks of postnatal development in rodents, which is, in part, equivalent to the 3rd trimester of human pregnancy. Specifically, between postnatal day (P) 4 and P7, undifferentiated molecular layer interneurons migrate toward the developing molecular layer and by P12 they begin to form synaptic connections with Purkinje cells [43]. This process is generally completed by P21. Golgi cells are present at birth and start to make synaptic connections with granule cells by P7 [43]. In sum, cerebellar interneurons undergo profound developmental changes during the equivalent to 2nd and 3rd trimesters of human pregnancy, suggesting that they can be susceptible to damage during these critical developmental periods.

The purpose of this study was to compare the impact of repeated ethanol exposure on the number of inhibitory neurons in the cortical region of the cerebellar vermis. We hypothesized that ethanol exposure during the mouse equivalent to the 2nd and 3rd trimesters of human pregnancy results in a similar decrease in the number of molecular layer interneurons, granule cell layer interneurons, and Purkinje cells. We used transgenic mice expressing a fluorescent protein in these neurons and unbiased stereology techniques to test this hypothesis.

## 2. Methods

All animal procedures were approved by the University of New Mexico Health Sciences Center Institutional Care and Use Committee. For the studies described here, we used the vesicular GABA transporter (VGAT)-Venus mouse line expressing Venus in GABAergic and glycinergic neurons throughout the brain, including the cerebellum where virtually all inhibitory neurons expressed this fluorescent protein [44]. The Venus fluorescent protein (a variant of enhanced yellow fluorescent protein) was developed by Dr. Atsushi Miyawaki at RIKEN (Wako, Japan). Animals were housed in standard cages at a room temperature of 22C. Lights were on at 6 am and off at 6 pm. We mated wild-type C57BL6 females with VGAT-Venus heterozygous males for 6 days, after which males were removed from the cage. The first day of mating was considered as the first day of gestation. At gestational day 12, females were transferred to custom built vapor inhalation chambers [34]. Pregnant dams were exposed for 4 h/day (10 a.m. to 2 p.m.) to ethanol between gestational days 12 and 19. Food was changed daily at the end of the 4 h ethanol exposure. After pups were delivered on gestational days 19-20, the VGAT-Venus pups were identified during the first 2 postnatal days via transcranial illumination with fluorescence goggles containing a 480/40 nm excitation filter, and a long pass 520 nm emission filter (BLS ltd. Budapest, Hungary). Pups that did not express Venus were euthanized; the number of pups/litter was $9.2 \pm 0.8$ ($n = 5$) and $8.5 \pm 1.0$, ($n = 4$) for the air and ethanol groups, respectively ($t(7) = 0.54$, $p = 0.6$); the number of Venus positive pups was $4.8 \pm 0.74$ ($n = 6$) and $4.5 \pm 0.6$, ($n = 6$) for the air and ethanol groups, respectively ($t(10) = 0.33$, $p = 0.7$). Pup weight at the time of birth was not significantly different between the air ($1.55 \pm 0.2$ g; $n = 7$) and ethanol ($1.39 \pm 0.07$ g; $n = 5$) groups ($t(10) = 0.61$, $p = 0.55$). Ethanol vapor exposure for 4 h/day was then resumed between P2–9. Pups remained with their mothers during exposure. Ethanol vapor chamber levels were gradually increased to allow the animals to adapt to the exposure paradigm and minimize toxicity. Ethanol chamber levels were 3–5 g/dl at gestational days 12–14, 6–7 g/dl at gestational days 15–17, 7–8 g/dl at gestational days 18–19, 3–4 g/dl at P2-3, 5–6 g/dl at P4-5 and 7–8 g/dl at P6-9. Blood alcohol levels were measured using an assay based on the activity of alcohol dehydrogenase [11]. In the dams, these were determined to be $0.1 \pm 0.02$ g/dl in tail blood obtained at gestational days 12–18 and $0.16 \pm 0.06$ g/dl in trunk blood obtained 7–8 days after giving birth ($n = 3$ dams). The ethanol concentration in trunk blood from P7-8 pups was $0.33 \pm 0.02$ g/dl ($n = 13$ pups from 3 litters). For reference, the legal intoxication limit in the U.S. is 0.08 g/dl. At P9, average pup weights were $7.18 \pm 0.2$ ($n = 7$) and $5.12 \pm 0.23$ ($n = 5$) for the air and ethanol groups, respectively ($t(10) = 6.68$, $p < 0.0001$). At P16, average pup weights were $9.8 \pm 0.3$ ($n = 7$) and $7.4 \pm 0.35$ ($n = 5$) for the air and ethanol groups, respectively ($t(10) = 4.5$, $p < 0.001$).

At P16, male pups were transcardially perfused with 4% paraformaldehyde under deep anesthesia with ketamine (500–700 mg/kg intraperitoneally). Cerebella were cut from the forebrain and kept in 4% paraformaldehyde for 48 h and then stored in phosphate buffered saline (PBS), all at 4C. Each cerebellar vermis was blocked and sectioned at 50 μm thickness in the parasagittal plane using a vibrating tissue slicer (Vibratome 1000, The Vibratrome Company, St. Louis, MO). We chose to focus on the cerebellar vermis because studies indicate that this area is an important target of developmental ethanol exposure [7]. Fifteen consecutive sections of the vermis (see Fig. 1 for representative images) were mounted sequentially on Superfrost plus slides (VWR Micro Slides, Radnor, PA), incubated for 20 min with 4′,6-diamidino-2-phenylindole (DAPI; 1:4000 in PBS), washed with PBS 5 times, with each wash lasting 5 min, coverslipped with Vectashield mounting media (Vector Laboratories, Burlingame, CA), and sealed with clear nail polish. The cell body numbers of molecular and granule cell layer interneurons, as well as Purkinje cells (Fig. 1) was estimated using the optical fractionator method with the aid of an Olympus DSU spinning disk confocal microscope (Olympus, Melville, NY) and Stereo Investigator software (Version 9, MBF Bioscience, Williston, VT). Venus positive cells (of smaller size than Purkinje cells) located in the outer and inner halves of the Purkinje cell layer were considered to be molecular and granule cell layer interneurons, respectively. The contours of each lobule (Fig. 1A–B) were manually traced under 4 x magnification. Starting with the third section from each vermis, Venus positive somata were counted in every 5th section using a 40x objective (i.e., a total of 3 sections per vermis were counted). If any of the sections was torn, the closest intact section was counted. The guard zone was set at 5 μm and the dissector height was set at 15 μm. The section thickness was measured while counting at every sampling site. The counting frame size was set at $50 \times 50$ μm and 15% of the area of each lobule was sampled. The volume of each of the cerebellar vermis lobules was estimated according to the Cavalieri principle [32]. Every 5th section, starting with the 3rd section (total