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Differentially sensitive neuronal subpopulations in the central nervous system and the formation of hindbrain heterotopias in ethanol exposed Zebrafish

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Abstract

Background—A cardinal feature of prenatal ethanol exposure is CNS damage, resulting in a continuum of neurological and behavioral impairments that are described by the term Fetal Alcohol Spectrum Disorders (FASD). FASDs are variable and depend on several factors, including the amount, timing and duration of prenatal ethanol exposure. To enhance interventions for CNS dysfunction, it is necessary to identify ethanol-sensitive neuronal populations and expand the understanding of factors that modify ethanol teratogenesis.

Methods: To investigate the susceptibility of different neuronal subtypes, we exposed transgenic zebrafish (*Danio rerio*) to several ethanol concentrations (0.25%, 0.5%, 1.0%, 1.5% or 2.0%), at different hours post fertilization (hpf) (0 hpf, 6 hpf or 24 hpf), for various durations (0–24hpf, 0–48hpf, 4–24hpf, 6–24hpf, 6–48hpf or 24–48hpf). Following exposure, embryo survival rates were determined, and CNS neurogenesis, differentiation and patterning was assessed.

Results: Embryo survival rates decrease as ethanol concentrations increase and drastically decline when exposed from 0–24hpf compared to 4–24hpf. Abnormal tangential migration of facial motor neurons is observed in *isl1:gfp* embryos exposed to ethanol concentrations as low as 0.25%, and the formation of IVth ventricle heterotopias are revealed by embryos exposed to 1.0% ethanol. Whereas, expression of *olig2:dsred* and *ptf1a:gfp* in the cerebellum and spinal cord are largely unaffected. While levels of *etv4* mRNA are overtly resistant to ethanol, we observe significant reductions in *ptch2* mRNA levels.

Conclusions: These data show differentially sensitive CNS neuron subpopulations with susceptibility to low levels of ethanol. In addition, these data reveal the formation of ethanol-induced hindbrain heterotopias.

Keywords

Fetal Alcohol Spectrum Disorders; CNS; Zebrafish

Introduction:

Ethanol is a potent teratogen capable of generating a constellation of birth defects, as well as life-long cognitive and behavioral impairments. Fetal Alcohol Spectrum Disorders (FASD) is the term used to describe all deleterious consequences of prenatal ethanol exposure. Phenotypes associated with FASD are grouped by several diagnostic categories, including fetal alcohol syndrome (FAS), partial fetal alcohol syndrome (pFAS), alcohol related birth

defects (ARBD) and alcohol related neurodevelopmental disorders (ARND). While the effects of ethanol on the developing fetus are diverse, the central nervous system (CNS) is especially vulnerable to ethanol teratogenesis.

FASDs are estimated to affect 1.1–5.0% of children in the United States (May et al., 2014; May et al., 2018). However, most children with FASD are never diagnosed or are misdiagnosed for other disorders with similar symptoms. ARND is particularly challenging to identify due to the absence of physical abnormalities (Riley, Infante, & Warren, 2011). Individuals with ARND exhibit a variety of learning and behavioral problems, but do not have the dysmorphic craniofacial features that are characteristic of FAS. Moreover, individuals with ARND may be well into childhood before they begin to manifest neurodevelopmental irregularities which are highly variable. The difficulty in identifying and appropriately diagnosing individuals prenatally affected by ethanol is a considerable barrier to the success of interventions for FASD (Streissguth et al., 2004).

Structural and functional damage to the CNS are prominent features of prenatal ethanol exposure that lead to alterations in gross brain morphology and neuronal dysfunction. Studies of humans with FASD show that certain brain structures are highly susceptible to ethanol during development, including the forebrain, corpus callosum and cerebellum (Moore, Migliorini, Infante, & Riley, 2014). Animal models of FASD recapitulate these phenotypes, demonstrating that ethanol-sensitive brain regions are conserved (Murawski, Moore, Thomas, & Riley, 2015). Moreover, several studies using FASD animal models reveal aberrant development affecting cell proliferation, differentiation and survival that occurs differentially in neuronal populations following ethanol exposure (Goodlett & Horn, 2001; Tateno & Saito, 2008; West, Chen, & Pantazis, 1994; Yin et al., 2014). Thus, in addition to being useful for confirming FASD associated phenotypes of the nervous system, animal models are particularly valuable for examining which neuronal populations and cell processes are vulnerable to prenatal ethanol exposure.

Clinical assessments of infants and children with confirmed prenatal alcohol exposure show that CNS damage and associated neurodevelopmental deficits are highly variable between individuals (Mattson, Crocker, & Nguyen, 2011). This phenotypic variability is attributed to several factors such as the amount, timing and duration in which the fetus was exposed to ethanol. Similarly, animal models of FASD demonstrate variable brain and behavior phenotypes as a result of these factors (Cole et al., 2012; Schneider, Moore, & Adkins, 2011). Despite the increasing body of literature demonstrating variable effects of ethanol on different cell types, the extent to which ethanol concentration, duration and developmental stage of exposure influences cell type susceptibility remains to be fully examined. Therefore, it is important to continue investigating the factors that alter the teratogenic capacity of ethanol and further identify differentially sensitive neuronal populations. This will help predict which functions of the brain are most likely to be affected and increase the likelihood of successful interventions for ethanol-exposed individuals with CNS dysfunction.

In this study, we used zebrafish (*Danio rerio*) embryos to examine factors of ethanol known to influence the level of teratogenesis in the CNS including, the concentration, timing and duration of exposure. In wildtypes exposed to varying ethanol concentrations at, or within

hours of, the time of fertilization, we show that embryo lethality increases as concentration increases. Using transgenic lines that label unique CNS cell populations, we assessed the effect of ethanol on multiple cell types. In line with other FASD animal model studies, our data show that neuronal populations are differentially sensitive to ethanol teratogenesis and that factors of ethanol exposure modify the susceptibility of different cell types. In addition, our data show the formation of hindbrain heterotopias following gastrulation stage ethanol exposure. We also examined the effects of ethanol on signaling pathways that play a role in CNS development. We demonstrate that Shh and Fgf signaling are globally dynamic in the developing CNS, but levels of activity are regionally distinct. Furthermore, quantification of signaling activity using downstream targets for both pathways demonstrate that exposure to 1.5% ethanol significantly reduces levels of *ptch2*, while levels of *etv4* are overtly unaffected by 1.0% or 1.5% ethanol. This study demonstrates the utility of zebrafish for investigating the effects of ethanol on specific populations of cells in the developing CNS. Additionally, these data show how specific groups of neuronal cells have varying levels of sensitivity to ethanol teratogenesis.

Materials and Methods:

Animals:

Wild type, AB strain Zebrafish (*Danio rerio*) were used as well as the following transgenic lines: *Tg(elavl3:EGFP)^{knu3}* – a marker of differentiated neurons (Park et al., 2000); *Tg(olig2:dsred2)^{vu19}* – a marker of oligodendrocytes, motor neuron progenitors and eurydendroid cells (Kucenas et al., 2008); *Tg(ptf1a:EGFP)^{jh1}* – a marker of Purkinje cells and spinal cord interneurons (Godinho et al., 2005); *Tg(isl1:gfp)^{rw0}* – a marker of postmitotic motor neurons (Higashijima, Hotta, & Okamoto, 2000); *TgBAC(ptch2:Kaede)^{pa4596}* – a reporter of Hedgehog signaling (Huang, Xiong, Megason, & Schier, 2012); and *Tg(dusp6:d2EGFP)^{pt6}* – a reporter of Fibroblast Growth Factor signaling (Molina, Watkins, & Tsang, 2007). Here forward, transgenes will be referred to as *elavl3:gfp*, *olig2:dsred2*, *ptf1a:gfp*, *ptch2:kaede*, and *dusp6:gfp*, respectively.

Zebrafish embryos were raised and maintained according to established protocols (Westerfield, 2000) and in accordance with protocol #AUP201800002 approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin. Developmental staging of embryos was determined by the identification of well-characterized morphological features (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995).

Ethanol exposures:

Zebrafish embryos were exposed to varying concentrations of ethanol in embryo media containing .015g/L phenylthiourea to inhibit the formation of pigmentation. The ethanol exposure concentrations used include, 0.25%, 0.5%, 1.0%, 1.5% and 2.0% volume/volume (v/v), equating to media ethanol concentrations of 42.75mM, 85.5mM, 171mM, 256.5mM and 342mM, respectively. However, as shown by Lovely et al, the chorion and extraembryonic fluid act as barriers to full ethanol equilibrium (Lovely, Nobles, & Eberhart, 2014). Consequently, the tissue ethanol concentrations of exposed embryos do not equal

media concentrations but are instead about 30% of media concentrations. Thus, embryos exposed to media ethanol concentrations of 42.75mM, 85.5mM, 171mM, 256.5mM and 342mM have tissue ethanol concentrations of 12.83mM, 25.65mM, 51.3mM, 76.95mM and 102.6 mM, respectively. In the context of mammalian blood alcohol content (BAC), a media ethanol concentration of 171–342mM corresponds to embryonic tissue BACs of 0.20–0.40 g/dL, which are levels within the biological tolerance of humans. Moreover, the ethanol exposure concentrations used in our study are within range of concentrations used in several other FASD zebrafish studies (Joya, Garcia-Algar, Vall, & Pujades, 2014; Kalueff, Stewart, & Gerlai, 2014; Pan, Kaiguo, Razak, Westwood, & Gerlai, 2011; Sylvain, Brewster, & Ali, 2011; Yin et al., 2014; Zhang, Boa-Amponsem, & Cole, 2017; Zhang et al., 2014; Zhang, Ojiaku, & Cole, 2013).

To expose embryos to ethanol, ethanol-free media was completely removed from the dish and immediately replaced with 40 mls of ethanol-containing media. Control embryos were raised in 40 mls of ethanol-free media. Within each experiment, equal numbers of embryos were used between treatment groups. To end exposure, ethanol-containing media was completely removed from the dish, embryos were rinsed briefly with ethanol-free media, and placed in 40 mls of fresh ethanol-free media until the desired stage of analysis.

Confocal imaging:

Confocal images of live embryos were acquired using a Zeiss LSM 710 confocal microscope with ZEN imaging software in Z-series or time-lapse mode as described (McGurk, Lovely, & Eberhart, 2014).

Quantitative Reverse Transcription (RT-qPCR):

We examined the expression of *ptch2* and *etv4* in wildtype control and ethanol-exposed embryos at 24 hpf, using RT-qPCR. Experimental groups consisted of four or five biological replicates for *ptch2* and *etv4*, respectively, and were assessed by RT-qPCR in triplicate. Whole embryos were used to examine the expression of *ptch2*, whereas *etv4* expression was examined using only the heads of embryos manually separated from the remainder of the body at the anterior limit of the otic vesicle. Total RNA obtained from individual samples were normalized to 50 ng/ul, then reverse transcribed using SuperScript™ First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific, 11904018) with random hexamer primers. RT-qPCR was performed with Power Sybr Green PCR Master Mix (Thermo Fisher Scientific, 4367659) on the Applied Biosystems ViiA™ 7 Real-Time PCR System. Solute carrier family 25 (*slc25a5*) and Like-Sm protein 12 homolog b (*lsm12b*) were used as endogenous controls based on stable expression across treatments. Beta actin (*actb1*) was also examined for use as an endogenous control but was omitted from the analysis due to significantly altered expression in 1.5% ethanol-exposed embryos. Raw data was obtained with QuantStudio Real-Time PCR Software. The calculated fold changes of *ptch2* and *etv4* are relative to the average of *lsm12b* and *slc25a5* across biological and experimental replicates.

Cell Number Quantification:

Neurons were quantified in control and ethanol-exposed *elavl3:gfp* and *ptf1a:gfp;olig2:dsred2* transgenic embryos using Fluorescent Activated Cell Sorting (FACS) at 24 hpf and 72 hpf, respectively. A total of 50 embryos were collected per experimental group to assess the number of GFP-positive cells in *elavl3:gfp* embryos. For the *ptf1a:gfp;olig2:dsred2* line, GFP-positive, RFP-positive and GFP;RFP double-positive cells were quantified in separate rostral and caudal divisions of the embryo, using a total of 100 embryos per experimental group. The anterior limit of the otic vesicle was used to define the region of separation. Non-fluorescent, wildtype embryos were used to prepare a negative control sample. Similarly, embryos expressing only GFP or RFP, were used as positive control samples to prepare the cell sorter for the detection of GFP and RFP prior to quantifying experimental samples.

Embryos of the desired stage were dechorionated using 2 mg/mL Pronase (Sigma-Aldrich) then washed using fresh embryo medium. Embryos were collected into a 1.5 ml Eppendorf tube and incubated in cold calcium-free Ringer's solution on ice, followed by repeated pipetting to disrupt the yolk sac. Deyolked embryos were washed with cold embryo media and pelleted by centrifugation (310g) at 4°C. Embryos were incubated in FACSmax cell dissociation solution (Genlantis) for several minutes and then pipetted up and down vigorously to dissociate cells. The cell suspension was filtered through a 40-micron cell strainer into a 35 mm petri dish, collected into a fresh 1.5 ml Eppendorf tube and placed on ice until analyzed. To remove any remaining cell aggregates, prepared cell suspensions were re-filtered prior to sorting using polystyrene Falcon tubes with attached cell-strainer caps (Fisher Scientific).

FACS was performed using a BD FACS Aria Fusion SORP Cell Sorter equipped with 100-micron nozzle. Raw data was obtained using DIVA 8 analysis software (BD Biosciences San Jose, CA, USA). Individual cells were gated according to size and granularity. Cells of interest were fluorescently identified using 488 and 561 nm lasers for GFP- and DsRed2-expressing cells, respectively. For each sample, ~100,000 gated events (i.e. individual cells) were sorted based on these parameters.

Statistical analysis:

Statistical analyses were performed using GraphPad Prism 7. The Kaplan-Meier method was used to generate survival curves then compared using the Logrank test. Quantitative analyses of cells obtained by FACS in *elavl3:gfp* and *ptf1a:gfp;olig2:dsred2* embryos were performed using one-way ANOVA. For the RT-qPCR analysis of *ptch2*, the delta delta Ct ($2^{-\Delta\Delta Ct}$) method was used to quantify relative expression for each experimental group and analysis was performed using one-way ANOVA, followed by the Tukey–Kramer post-hoc test.

Results:

Effects of ethanol on embryo survival

To examine the effect of early ethanol exposure concentration, duration and timing on embryo survival, zebrafish (*Danio rerio*) embryos were placed in media containing 0.5%,

1.0%, 1.5% or 2.0% ethanol for 24 hours from 0 hours post fertilization (hpf) until 24 hpf, or for 20 hours from 4 hpf until 24 hpf. We chose these stages to initiate exposure to assess differences in the lethality of zygotes (0 hpf) controlled by maternal mRNAs and proteins compared to sphere stage embryos (4 hpf) following the midblastula transition (MBT) when bulk zygotic transcription is observed (Kane & Kimmel, 1993; Kimmel et al., 1995). For each ethanol concentration and duration examined, the number of surviving embryos was recorded at 6 hpf, 24 hpf, 48 hpf and 72 hpf (Figure 1).

Embryos exposed to ethanol from 0–24 hpf, had drastically lower rates of survival at 72 hpf, compared to embryos treated from 4–24 hpf. For the 0–24 hpf exposure duration, embryo survival declined as concentrations of ethanol increased (Figure 1A). Embryos exposed to 0.5% ethanol had the highest survival rate with 77% of embryos still alive at 72 hpf. Ethanol concentrations of 1.0%, 1.5% and 2.0% induced significant embryo death. Following exposure to 1.0% ethanol, only 54% of embryos survived until 72 hpf and 0% of the embryos exposed to 1.5% ethanol survived beyond 48 hpf. The greatest impact on survival was for embryos exposed to 2.0%, in which no embryos were found alive at 6 hpf.

In contrast, embryos exposed to ethanol from 4–24 hpf had much higher rates of survival compared to 0–24 hpf, regardless of concentration. For this duration, the percent of surviving embryos at 72 hpf was 93%, 85%, 80%, and 42%, for embryos exposed to 0.5%, 1.0%, 1.5% or 2.0% ethanol, respectively. While embryo death increased slightly as ethanol concentration increased, survival rates between most ethanol-exposed groups and control embryos were similar (Figure 1B). Furthermore, exposure to ethanol from 4–24 hpf did not have as great of an impact on embryo survival as exposure to the same concentrations of ethanol from 0–24 hpf, demonstrating that sensitivity to ethanol-induced death decreases as the embryo develops.

Effects of ethanol on neuronal differentiation

During the primary phase of neurogenesis between 10–12 hpf, motor, sensory and interneurons are generated that establish the basic neural network by 24 hpf. At 48 hpf, the secondary phase of neurogenesis occurs which vastly contributes to growth of the maturing brain (Eisen, 1991). The *elavl3:gfp* transgenic line is one of the earliest markers of differentiated neurons in the CNS (Park et al., 2000). By enabling the visualization of how early born neurons are distributed, *elavl3:gfp* is useful for characterizing neurogenesis and identifying altered neuronal development.

To examine the effects of early ethanol exposure during stages of neuronal differentiation, we transiently exposed *elavl3:gfp* embryos to 0%, 0.5%, 1.0% and 1.5% ethanol for 24 hours at the time of fertilization (0–24 hpf) or for 18 hours in gastrulation stage embryos (6–24 hpf) (Kimmel et al., 1995). We then assessed the distribution of *elavl3* at 24 hpf (Figure 2A–E). Exposures initiated at 0 hpf using ethanol concentrations 1.0% and greater, have profound effects on embryo survival (see Figure 1A). Thus, only the 0.5% ethanol-exposed group was examined for this exposure duration, in addition to all groups of embryos exposed to ethanol from 6–24 hpf. In 1.0% and 1.5% ethanol-exposed embryos, the trigeminal nucleus appears reduced compared to control embryos (Figure 2D'–E'). However, the overall distribution of *elavl3*-positive cells in other regions of the CNS is largely unaffected

across all treatment groups, demonstrating that ethanol exposure does not have an overt effect on the global distribution of differentiated CNS neurons at 24 hpf.

In line with our earlier stage assessment, embryos that were exposed to 0.5%, 1.0% and 1.5% ethanol from 6–24 hpf or 6–48 hpf, did not have an observable difference in the global distribution of *elavl3*-positive cells at 48 hpf (Figure 2G–L). This demonstrates that while ethanol is known to affect the differentiation of various cell types, this effect is not observable at 24 hpf or 48 hpf using a pan-neuronal marker such as *elavl3*, for the concentrations and durations of ethanol exposure we examined.

In addition to our stage-specific confocal analyses, we also assessed *elavl3:gfp* expression using time-lapse imaging, to characterize potential alterations to the dynamics of neuronal differentiation. For this analysis, we treated embryos with 1.0% and 1.5% ethanol from 6–24 hpf and imaged the differentiation of *elavl3*-positive neurons from 24–48 hpf. In controls, differentiating neurons were distributed throughout the entire CNS. This pattern of differentiation did not overtly change in ethanol-exposed embryos (Figure 3, Supplemental Movies 1, 2, 3). Thus, the overall patterning and genesis of neurons is not altered by our ethanol treatments.

Next, we quantified the number of GFP-positive cells in untreated and ethanol-exposed *elavl3:gfp* embryos using Fluorescent Activated Cell Sorting (FACS). Embryos were placed in media containing 0%, 1.0% or 1.5% ethanol from 6–24 hpf. At 24 hpf, the embryos were collected and processed for cell sorting. Embryos exposed to 1.0% or 1.5% show a reduction in GFP-positive cell numbers (Figure 2M). Collectively, these results demonstrate while ethanol does not alter the overall distribution of *elavl3*-positive neurons, it does cause a reduction in the number of differentiated neurons.

Ethanol-induced Heterotopia

While assessing ethanol-exposed *elavl3:gfp* embryos, we identified several with an ectopic cluster of *elavl3*-positive cells positioned at the roof of the IVth ventricle (Figure 4B). The ectopic cluster is visible by fluorescence at 24 hpf. In addition, by 48 hpf the ectopic cluster develops into a mass that is detectable not only by fluorescence but also bright field (data not shown). The frequency by which this ectopic mass forms was determined by quantifying the number of *elavl3:gfp* embryos with ectopic masses at 48 hpf following exposure to 1.0% ethanol from 6–24 hpf. Embryos were scored based on the presence of *elavl3*-positive ectopic cells, as well as a visible mass by brightfield microscopy. Of the embryos examined, 9/79 (11.4%) had ectopic clusters at 48 hpf.

To ensure that the ectopic cell mass was not the result of a transgene effect, specific to the *elavl3:gfp* transgenic line, we repeated our assessment to determine the penetrance using *ptf1a:gfp;olig2:dsred2* transgenic embryos. Embryos were exposed to 1.0% ethanol from 6–24 hpf and examined at 48 hpf (data not shown). These embryos were determined to have ectopic masses by visualizing the mass by brightfield only, due to lacking the *elavl3:gfp* transgene. However, it is noteworthy that although *ptf1a* and *olig2* were not found to be expressed by cells within the mass, the expression of *ptf1a* was displaced in the

neuroepithelium lining the IVth ventricle (arrowhead in Figure 6E). The number of embryos determined to have ectopic masses using the *ptf1a:gfp;olig2:dsred2* line was 10/92 (10.8%).

To characterize the growth dynamics of the heterotopia, we utilized time-lapse imaging (Supplemental Movie 4). We exposed *elavl3:gfp* embryos to 1.0% ethanol from 6–24 hpf, then recorded a time-lapse from 24–48 hpf. Prior to acquisition, embryos were prescreened by fluorescence microscopy to identify those with visible ectopic clusters. Our time-lapse data reveal *elavl3*-positive cells migrating into and, primarily, out of the ectopic mass from the neuroepithelium adjacent to the IVth ventricle. Interestingly, the number of cells that can be seen migrating into the mass does not appear to account for the total cell population present in the mass. Therefore, it appears that these cells have the potential to proliferate once they have entered the mass.

Effects of ethanol on specific subsets of CNS neurons

Our analysis of *elavl3:gfp* revealed that differentiating neurons of the CNS are vulnerable to ethanol exposure. However, as a pan neuronal marker, *elavl3:gfp* is not able to define which neurons are specifically affected. We utilized transgenic lines that label unique subsets of CNS neurons including, *isl1:gfp*, *ptf1a:gfp* and *olig2:gfp*, to analyze specific neuron subpopulations. Expression of these markers were examined in control and ethanol-exposed embryos at developmental stages coinciding with the stages of development unique to each of the labeled cell populations.

The *isl1:gfp* transgene labels cranial and spinal motor neurons including their axonal projections (Higashijima et al., 2000). The *isl1:gfp* transgene has been used widely to characterize genes involved in cranial nerve development, particularly for the migration of facial branchial motor neurons (FBMNs) (Bingham et al., 2010; Jessen et al., 2002; Rebman, Kirchoff, & Walsh, 2016; Sittaramane et al., 2009; Yang, Bassuk, Stricker, & Fritzsch, 2014). It has also been used to demonstrate gene-ethanol interactions in *vangl2*^{-/-} embryos, which develop hypoplastic cranial nerve projections following ethanol exposure (Swartz et al., 2014).

FBMNs undergo a highly stereotypic rostral to caudal pattern of migration, called tangential migration, which makes an excellent model system to investigate the effects of ethanol on neuronal migration. Zebrafish FBMNs are born in hindbrain rhombomere (r) 4 and migrate tangentially to r6, followed by radial migration within r6. FBMN migration events are visualized by the *isl1:gfp* transgene, allowing for the recognition of mispositioned neurons that result from aberrant migration.

Embryos were exposed to 0.25%, 0.5%, 1.0%, 1.5% and 2.0% ethanol from 6–24 hpf, spanning the developmental stages of FBMN induction, differentiation and early migration. To determine the effects of ethanol on FBMN migration, confocal microscopy was used to examine the positioning of FBMNs at a stage during migration (30 hpf) and at the end of migration (48 hpf). At both stages, embryos treated with ethanol display a range of tangential migration phenotypes affecting the distribution of FBMNs along the r4–r6 axis (Figure 5A–F). For quantification, these defects were categorized as intermediate and severe, in which migration into r6 is partially or completely blocked, respectively (Figure 5G). We

find a concentration-dependent response. The small percentage of embryos with partial defects observed in control embryos is greatly elevated even by exposure to 0.25% ethanol. At concentrations of 2.0%, ethanol completely blocks migration of all FBMNs. Thus, migration of this population is exquisitely sensitive to ethanol teratogenesis.

The zebrafish cerebellum and spinal cord express *ptf1a:gfp*. In the cerebellum, *ptf1a:gfp* labels progenitors of the ventricular zone that give rise to GABAergic Purkinje cells (Kani et al., 2010). In the spinal cord, *ptf1a:gfp* is a marker for dorsal interneurons. The developing cerebellum and spinal cord also express *olig2:dsred2*. In contrast to *ptf1a*, *olig2:dsred2* labels progenitors in the cerebellar ventricular zone that gives rise to glutamatergic eurydendroid cells and oligodendrocytes (Bae et al., 2009; Kani et al., 2010). In the spinal cord, *olig2:dsred2* is expressed in motor neuron progenitors and oligodendrocytes.

As a structure well-known to be sensitive to ethanol, we examined the effect of ethanol exposure on neurogenesis of the two major neuronal cell types that populate the cerebellum which begins to form at 48 hpf. Double transgenic *ptf1a:gfp;olig2:dsred2* embryos were exposed to 0%, 0.5%, 1.0% or 1.5% ethanol at stages of various developmental events, including fertilization (0–24 hpf and 0–48 hpf), gastrulation (6–24 hpf and 6–48 hpf), and following the primary phase of neurogenesis (24–48 hpf). Control and ethanol-exposed embryos were then examined using confocal microscopy at 24 hpf (Figure 6A–E') and 72 hpf (Figure 6F–L''). A small population of progenitor cells in r1, which gives rise to the cerebellar primordium, express *ptf1a:gfp*. At 24 hpf, the expression of *ptf1a:gfp* in r1 appears reduced in embryos exposed to 1.0% and 1.5% ethanol from 6–24 hpf (Figure 6D'–E'). At 72 hpf, the expression patterns of *ptf1a:gfp* and *olig2:dsred2* in the cerebellar ventricular zone are largely unaffected by the concentrations and durations of ethanol exposure we examined (Figure 6F–H). However, the punctate expression of *olig2:dsred2* in the caudal cerebellar primordium seems to be augmented in embryos exposed to 0.5% ethanol from 6–48 hpf (arrow in Figure 6H'''). This observation includes embryos exposed to >0.5% ethanol for durations that persist through 48 hpf, such as 6–48 hpf and 24–48 hpf (data not shown). In contrast, the expression of *olig2:dsred2* in the spinal cord appears reduced by 1.0% ethanol following the earlier and shorter exposure window of 6–24 hpf (arrows in Figure 6J'–L'). Similarly, the expression pattern of *ptf1a:gfp* in the dorsal spinal cord appears reduced in embryos at 72 hpf, following exposure to 1.5% ethanol from 6–24 hpf (Figure 6L'').

We also quantified the number of *olig2:dsred2* and *ptf1a:gfp* expressing cells in control and ethanol-exposed embryos at 72 hpf using FACS. Since these transgenes are expressed in different cell types, and at different levels in the anterior and posterior CNS, we quantified the cell numbers in those two regions of the embryo separately. In the head of ethanol-exposed embryos, we observed a downward trend for *olig2* expression, although the reduction did not reach statistical significance. The number of *ptf1a*-expressing and double-positive cells appear largely unaffected. In the trunk, we see no alteration to the overall number of cells that express these transgenes, although there was a trend for reduced numbers of *ptf1a*-expressing neurons (Figure 6M–N).

Effects of ethanol on developmental signaling pathways

We continued our analysis by examining the effects of ethanol on Sonic Hedgehog (Shh) and Fibroblast growth factor (Fgf) signaling, which are two major signaling pathways implicated in ethanol teratogenesis of numerous vertebrate species (Ahlgren, Thakur, & Bronner-Fraser, 2002; Aoto, Shikata, Higashiyama, Shiota, & Motoyama, 2008; Hard, Abdoell, Robinson, & Koren, 2005; Li et al., 2007; Loucks & Ahlgren, 2009; Zhang et al., 2014; Zhang et al., 2013). Moreover, these pathways are essential for nervous system development across vertebrates (Gibbs, Chang-Gonzalez, Hwang, Yeh, & Lekven, 2017; Hernandez-Miranda, Muller, & Birchmeier, 2017; Park, Shin, & Appel, 2004; Takamiya & Campos-Ortega, 2006). Transgenic embryos expressing *ptch2:kaede* and *dusp6:gfp*, serve as readouts of Shh and Fgf signaling, respectively. Embryos expressing either transgene were exposed to 0%, 1.0% or 1.5% ethanol from 6–24 hpf, and assessed using confocal analyses

Time-lapse of control and 1.5% ethanol-exposed *ptch2:kaede* embryos were acquired from 24–48 hpf to assess the effect of early ethanol exposure on Shh signaling during secondary neurogenesis and later stages of nervous system maturation. Our analysis shows dynamic Shh signaling in the early developing CNS. In control embryos from 24–48 hpf, *ptch2:kaede* is robustly expressed throughout the rostral-caudal CNS and appears to pulse variably in specific regions such as the developing hindbrain (Supplemental Movie 3). While ethanol-exposed embryos also display dynamic *ptch2:kaede* expression throughout the rostral-caudal CNS, expression within the rostral aspect appears vastly different compared to controls (Figure 7G–L). We quantified the effect of ethanol on Shh signaling with a downstream target of the pathway, *ptch2*, by RT-qPCR using control and ethanol-exposed wildtype embryos at 24 hpf. We find that 1.0% ethanol does not alter the overall levels of Shh activity. At the highest dose of ethanol tested, 1.5%, we find that ethanol significantly attenuates Shh activity (Figure 7M).

Control and ethanol-exposed *dusp6:gfp* embryos were imaged at 24 hpf, when Fgf signaling plays an essential role in formation of the mid-hindbrain boundary, eye, otic vesicle and trigeminal nerve (Molina et al., 2007). Similar to our *elav13:gfp* analyses, we observe perturbations to the trigeminal nerve nucleus in ethanol-exposed embryos at 24 hpf. In control embryos the nucleus is proximally positioned to the eye, while *dusp6*-expressing trigeminal neurons in ethanol-exposed embryos are positioned more posteriorly (arrowheads in Figure 8B–C). Moreover, while the overall distribution of Fgf receptive cells in the developing eye are largely consistent in control and ethanol exposed embryos, the expression of *dusp6:gfp* in the otic vesicle appears specifically reduced in exposed embryos (Figure 8A–C). It is also possible that expression of the transgene is specifically elevated in the mid-hindbrain boundary of ethanol treated embryos (arrows in Figures 8B–C). We addressed this possibility by quantifying the effect of ethanol on Fgf signaling with a downstream target of the pathway, *etv4*, by RT-qPCR in control and ethanol-exposed wildtype embryos at 24 hpf. To avoid quantifying the level of *etv4* in the otic vesicle that appears perturbed in ethanol-exposed embryos, we used only the heads of embryos separated at the anterior limit of the otic vesicle. We find that the level of *etv4* expression is largely unaffected following exposure to 1.0% or 1.5% ethanol (Figure 8D). Collectively, these findings suggest that

while the Shh signaling pathway is sensitive to embryonic ethanol exposure, Fgf signaling within the rostral aspect of the embryo is largely resistant to perturbation by ethanol.

Discussion:

The consumption of alcohol during pregnancy has profound effects on the developing CNS. The brain is particularly vulnerable to ethanol exposure during embryonic development, which causes irreversible morphological damage and dysfunction. Changes in gross brain morphology lead to life-long impairments in behavior and cognitive functioning, many of which will not manifest until well into childhood. The level of damage that occurs and the corresponding neurological deficit that results is highly variable between affected individuals. This phenotypic variation is due to many factors, including the amount and duration of ethanol exposure, and the gestational stage of the fetus during the exposure.

Due to external fertilization, analyses in zebrafish can tightly control for these variables (Fernandes, Buckley, & Eberhart, 2018; Lovely, Fernandes, & Eberhart, 2016). Previous studies have shown that zebrafish exposed to ethanol during embryonic development recapitulate the major phenotypes that are observed in humans with FASD, including craniofacial, brain and behavioral defects. Moreover, FASD studies using zebrafish and other vertebrate species, have shown a variety of CNS defects induced by ethanol all while using different exposure paradigms, including the amount, duration and timing in which the fetus is exposed to ethanol (Bonthius & West, 1990, 1991; Joya et al., 2014; Ramlan et al., 2017; Yin et al., 2014; Zhang et al., 2014). In this study, we focused on these factors and examined the differential effect they have on the development of specific CNS neuronal populations.

Embryo survival is dependent on the stage of ethanol exposure.

Alcohol use by adolescents and young adults increases sexual risk behaviors, including unprotected sex (Weinhardt & Carey, 2000). Thus, it is likely that a vast number of conceptions occur before alcohol has the time to be eliminated from the body. Moreover, nearly half of all pregnancies are unintended and many women of child bearing age report alcohol use (CDC, 2012; Finer & Zolna, 2011). Consequently, most prenatal ethanol exposures occur before a woman is aware that she is pregnant. Interestingly, several studies have found that ethanol consumption during conception and early pregnancy increases rates of spontaneous abortion/miscarriage in humans (Avalos, Roberts, Kaskutas, Block, & Li, 2014; Henriksen et al., 2004; Kesmodel, Wisborg, Olsen, Henriksen, & Secher, 2002; Windham, Von Behren, Fenster, Schaefer, & Swan, 1997).

We find that zebrafish survival rate is affected by the concentration and timing of ethanol exposure during the early stages of development. We find that embryo survival rates decrease as ethanol concentrations increase, regardless of the stage in which exposure is initiated. Moreover, embryos exposed to ethanol immediately after fertilization (0 hpf) are the highly vulnerable to death, even in ethanol concentrations as low as 0.5%. This vulnerability decreases if ethanol exposure is postponed until 4 hpf, however death is still observed for each concentration examined. The reason for this acute sensitivity immediately following fertilization is not known. Later stage embryos have been shown to have a barrier to ethanol accumulation (Lovely et al., 2014). It may be that this barrier is not present

immediately following fertilization. Further analyses will be required to directly test this possibility.

Differentiation of CNS neurons are sensitive to prenatal ethanol exposure

In accordance with previous studies (Joya et al., 2014; Yin et al., 2014), we have found that ethanol exposure leads to a reduced number of differentiated neurons in the zebrafish at 24 hpf. Using the *elavl3:gfp* transgenic we assessed the distribution of neurons by confocal analyses, and quantified neuronal numbers using FACS. Through our confocal analyses, we observe what appears to be a reduction to the trigeminal facial nerve nucleus. As a transgene marker of sensory and motor neurons, this finding in ethanol exposed *elavl3:gfp* embryos is consistent with previous studies demonstrating that sensory neurons of the trigeminal nerve are particularly sensitive to developmental ethanol exposure (Glendinning et al., 2017; Miller, 1995, 1999). However, we were unable to visualize global differences in expression of the pan-neuronal marker *elavl3:gfp*, despite the number of studies that show reduced cell type specific differentiation in ethanol-exposed embryos. Therefore, the broad level of *elavl3:gfp* expression throughout the embryo at 24 hpf and 48 hpf could mask our ability to visualize differences in the number of *elavl3:gfp* expressing cells. To investigate this possibility, we utilized FACS to quantify the number of GFP-positive cells in control and ethanol-exposed embryos. We found a reduction in the number of GFP-expressing cells in embryos exposed to 1.0% or 1.5% ethanol. Our data would suggest that the reduction is not due to a loss of *olig2*-or *ptf1a*-expressing cells, as we did not find a significant decrease in the number of these cells through FACS quantification of these transgenes in ethanol-exposed embryos. It will be of great interest to continue similar analyses of transgenic zebrafish to identify which neural cell type(s) are most sensitive to ethanol.

Ethanol-induced heterotopia

Heterotopias are clusters of displaced differentiated neurons that can be found in the brains of children with FASD (Jarmasz, Basalah, Chudley, & Del Bigio, 2017; Riley & McGee, 2005). When examining *elavl3:gfp* embryos for ethanol-induced differences in neuronal differentiation, we observed embryos with ectopic cells populating the roof of the IVth ventricle. Other animal studies have reported the ectopic growth of cells following prenatal ethanol exposure (Komatsu, Sakata-Haga, Sawada, Hisano, & Fukui, 2001; Mooney, Siegenthaler, & Miller, 2004; Sakata-Haga, Sawada, Ohnishi, & Fukui, 2004). While these studies report the formation of heterotopias in the cortex and midbrain, no studies have reported ethanol-induced heterotopias located within the IVth ventricle. It remains to be explored which cell type(s) gives rise to, and populates, the heterotopic IVth ventricle mass observed in our ethanol-exposed *elavl3:gfp* embryos. Moreover, because ethanol exposure is known to perturb neuronal processes such as migration, it is of further interest to investigate the mechanism underlying the aberrant migration of cells in the IVth ventricle.

Specific populations of CNS neurons are differentially sensitive to ethanol exposure

Phenotypes associated with ethanol-induced brain dysfunction are due in part to exposure concentration and developmental timing. Therefore, we wanted to examine the ethanol-sensitivity of neuronal populations in the CNS and explore how sensitivity was modified by the concentration, duration and timing of ethanol exposure. Using well-characterized

transgenic lines that express fluorescent proteins in different neuronal populations, we examined the effects of ethanol exposure on a diverse set of CNS neurons.

Consistent with previous studies demonstrating cranial nerves as targets of ethanol during development (Dunty, Zucker, & Sulik, 2002; Mooney & Miller, 2007), we show that ethanol impacts the tangential migration of motor neurons of cranial nerve nVII in *isl1:gfp* zebrafish embryos. Surprisingly, we observe these migration defects in embryos exposed to ethanol concentrations as low 0.25%. Ethanol exposures of 0.25% ethanol in zebrafish approximates a BAC level of .05 g/L (Lovely et al., 2014). These data reveal that very low levels of ethanol can disrupt processes of neuronal development.

We also show that ethanol has subtle effects on *olig2* and *ptf1a* expression in the cerebellum and spinal cord. Motor neurons and Purkinje cells that express *olig2* and *ptf1a*, respectively, have been shown to be sensitive to ethanol (Pierce, Williams, & Light, 1999; Sylvain, Brewster, & Ali, 2010). However, our analysis did not detect any significant changes in the numbers of cells expressing either gene. Our FACS assay revealed a downward trend for the number of *olig2*-positive cells in the brains of ethanol exposed embryos at 72 hpf, however in the spinal cord there is no difference or trend. Conversely, *ptf1a*-positive cells in the spinal cord show a slight downward trend in 1.5% ethanol-exposed embryos, while no difference in cell numbers are detected in the brain. The cell numbers obtained from *ptf1a:gfp;olig2:dsred2* expressing embryos are highly variable, even in controls, which may make it difficult to identify ethanol-induced deficits.

Shh, but not Fgf, signaling is sensitive to early embryonic ethanol exposure

In our confocal analyses of *ptch2:kaede* embryos, we were unable to observe differences in the distribution of Shh responsive cells in control versus ethanol-exposed embryos. In zebrafish, *ptch2* (formerly *ptch1*) is a downstream target of Shh signaling and is the standard readout of Hh signaling activity used in the zebrafish model organism (Concordet et al., 1996; Koudijs, den Broeder, Groot, & van Eeden, 2008; Quintana, Hernandez, & Gonzalez, 2017; Shen et al., 2013; Wang et al., 2013; Xu et al., 2006). To investigate if the level of Shh signaling was sensitive to our ethanol exposure paradigms, we quantified the level of *ptch2* signaling using RT-qPCR in wildtype embryos exposed to 1.0% or 1.5% ethanol and stage-matched controls. While *ptch2* expression in 1.0% ethanol-exposed embryos was not altered, those exposed to 1.5% ethanol did have a significant reduction in *ptch2*. These data indicate that Shh signaling is affected by high doses of ethanol and are in line with previous studies implicating Shh as a target of ethanol in zebrafish (Joya et al., 2014; Zhang et al., 2014; Zhang et al., 2013).

We also examined Fgf signaling using *dusp6:gfp* zebrafish. Soon after the mid-hindbrain is formed, *dusp6* begins to downregulate in that area. However, it appears that expression of the transgene is elevated in ethanol-treated embryos. Quantification of *etv4*, a downstream target gene of the fgf signaling pathway, reveals no significant change in the level of expression following ethanol exposure. Therefore, the perceived elevation in expression of the transgene is likely due morphological changes that occurs to the neuroepithelium following ethanol exposure (Grewal, Carmichael, Ma, Lammer, & Shaw, 2008; Leng, Wang, Cao, & Wang, 2016; Muralidharan, Sarmah, Zhou, & Marrs, 2013). Our confocal analyses

of ethanol-exposed embryos revealed that the pattern of *dusp6:gfp* expression was largely normal at 24 hpf, although regionally distinct perturbations to levels of expression were observed. Interestingly, the distribution of trigeminal neurons was altered by ethanol exposure. This finding, along with our analysis of *isl1:gfp* transgenics and the presence of heterotopias in ethanol-exposed zebrafish, point to neuronal migration as being especially sensitive to ethanol teratogenesis. The zebrafish is an excellent model system for analysis of cell behaviors. Our ongoing work will seek to gain mechanistic insight into how ethanol disrupts neuronal migration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Ethanol concentration and timing of exposure influence embryo survival rate. (a) Kaplan-Meier survival curve of embryos following exposure to varying ethanol concentrations from 0–24 hpf (n=74 for each treatment group). (b) Kaplan-Meier survival curve of embryos following exposure to varying ethanol concentrations from 4–24 hpf (n=40 for each treatment group). Embryo survival was assessed at 6 hpf, 24 hpf, 48 hpf, and 72 hpf.

Figure 2.

Embryonic ethanol exposure reduces the number of differentiated neurons. The distribution of differentiated neurons was examined in (A, F) control and (B-E, G-L) ethanol-exposed *elavl3:gfp* embryos at 24 hpf (A-E') and 48 hpf (F-L). Enlarged images demonstrate an apparent reduction in the trigeminal nucleus following ethanol exposure (D'-E'). Embryos exposed to ethanol were treated for various durations. Confocal images were acquired using a 20×/0.5 objective. (M) Quantification of *elavl3*-positive neurons at 24 hpf in control and ethanol-exposed *elavl3:gfp* embryos following exposure from 6–24 hpf. **** $p < .0001$, ** $p = .0017$.

Figure 3.

The distribution of differentiated neurons is largely unaffected by ethanol exposure. Still images extracted from time-lapse of (A) control, (B) 1.0% and (C) 1.5% ethanol-exposed *elavl3:gfp* embryos at 24 hpf. 10×/0.3 objective, maximum projection. Anterior to left.

Figure 4.

Heterotopic cell formation in the IVth ventricle of ethanol-exposed embryos. (A) oblique view of *elavl3:gfp* control embryo at 48 hpf. (B) Oblique view of *elavl3:gfp* embryo at 48 hpf following exposure to 1.0% ethanol from 0–24 hpf. Arrow indicates the heterotopic neuronal mass within the IVth ventricle. 20x/0.5 objective, maximum projection. Anterior to left.

Figure 5.

Ethanol-induced tangential migration defects in *isll:gfp* embryos. Confocal images of *isll:gfp* embryos at (A-C) 30 hpf and (D-F) 48 hpf following exposure to 1.0% ethanol from 6–24 hpf. Confocal images were acquired using a 20×/0.5 objective. (G) Quantification of *isll:gfp* embryos at 48 hpf displaying normal migration (no migration phenotype), intermediate migration defects or severe migration defects following exposure to ethanol concentrations ranging from 0%–2.0% from 6–24 hpf. Number of embryos quantified listed in parentheses above each bar. r, rhombomere.

Figure 6.

Analysis of *ptf1a:gfp;olig2:dsred2* embryos. Control (A, F) and ethanol-exposed (B-E) embryos were assessed for the distribution of *ptf1a*-expressing or *olig2*-expressing neurons at 24 hpf (A-E). Arrows indicate reduced *ptf1a:gfp* expression in r1 (D'-E'). In addition, the distribution of *ptf1a*-expressing (F'-H'', I'-L'') or *olig2*-expressing (F'-H', I'-L'') neurons in the cerebellum (F-H) and (I-L) spinal cord were examined in embryos at 72 hpf. Arrows indicate increased *olig2* expression in cerebellar OPCs (H''), decreased *olig2* expression in spinal cord OPCs (J'-L') and decreased *ptf1a* expression in spinal cord dINs (L''). FACS quantification of GFP-positive, RFP-positive or double-positive cells in the head (M) and trunk (N) of control and ethanol-exposed *ptf1a:gfp;olig2:dsred2* embryos at 72 hpf. r, rhombomere; OPCs, oligodendrocyte progenitor cells; Cb, cerebellum; dINs, dorsal interneurons; pMNs, motor neuron progenitors.

Figure 7.

Time-lapse analysis of *ptch2:kaede* embryos. Still images of embryos at various timepoints during the time-lapse were extracted to show the dynamic changes in *ptch2:kaede* expression that occurs during early development. The expression of *ptch2* was assessed in (A-F) control and (G-L) ethanol-exposed embryos by time-lapse imaging. Ethanol-exposed embryos were treated with 1.5% ethanol from 6–24 hpf and imaged from 24–48 hpf. $t = 24$ hpf. Time-lapse was recorded using a 10 $\times$ /0.3 objective. (M) Relative expression of *ptch2* expression in wildtype control and ethanol-exposed embryos. Note that *ptch2* is modestly reduced in 1.5% ethanol compared to both control and 1.0% ethanol-exposed embryos.

** $p = .0028$, * $p = .0183$.

Figure 8.

Analysis of *dusp6:gfp* embryos. Confocal imaging of 24 hpf (A) control, (B) 1.0% and (C) 1.5% ethanol-exposed embryos treated from 6–24. Arrows point to the apparent elevation in *dusp6:gfp* expression in the mid-hindbrain boundary. Arrowheads denote differences in the positioning of the trigeminal nucleus in ethanol-exposed embryos. (D) Relative expression of *etv4* expression in wildtype control and ethanol-exposed embryos. MHB, midhindbrain; OV, otic vesicle; nV, trigeminal nerve.