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ANALYSIS OF CROSSTALK BETWEEN RETINOIC ACID AND SONIC HEDGEHOG PATHWAYS FOLLOWING ETHANOL EXPOSURE IN EMBRYONIC ZEBRAFISH

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Abstract

BACKGROUND—Ethanol is a teratogen affecting numerous regions of the developing nervous system. The present study was undertaken to ascertain whether ethanol independently disrupts distinct signaling pathways or rather disrupts interactive pathways that regulate development of ethanol-sensitive tissues.

METHODS—Zebrafish embryos were exposed to ethanol in the absence or presence of *aldh1a3* or *Shh* morpholino oligonucleotides (MOs), which disrupt retinoic acid (RA) or sonic hedgehog (Shh) function respectively. Morphological analysis of ocular or midbrain-hindbrain boundary (MHB) development was conducted, and the ability to rescue ethanol and MO-induced phenotypes was assessed. *In situ* hybridization was employed to analyze *Pax6a* expression during ocular development.

RESULTS—Chronic ethanol exposure, or combined ethanol and MO treatment, results in perturbed MHB formation and microphthalmia. While RA can rescue the MHB phenotype following ethanol combined with either MO, *Shh* mRNA is unable to rescue the disrupted MHB with combined ethanol and *aldh1a3* MO treatment. RA also is unable to rescue microphthalmia induced by ethanol and *Shh* MO.

CONCLUSIONS—These studies demonstrate that while reduction of either RA or Shh signaling produces the same disruption of MHB or ocular development, that can be phenocopied using ethanol combined with either MO, RA overexpression can only rescue disrupted MHB, but not microphthalmia, in combined subthreshold *Shh* MO and ethanol. Our data suggest that MHB development may involve crosstalk between RA and Shh signaling, while ocular development depends on RA and Shh signaling that both are targets of ethanol in FASD but do not depend on a mechanism involving crosstalk.

Keywords

FASD; sonic hedgehog; retinoic acid; microphthalmia; zebrafish

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INTRODUCTION

Alcohol exposure during pregnancy often results in deleterious effects on fetal development and subsequent behavior during adolescence and adulthood. The resulting effects of alcohol exposure on fetal central nervous system development have shown that ethanol perturbs ocular, forebrain, and hindbrain development (Clarren et al, 1978; Stromland, 1985; Cragg and Phillips, 1985; Chan et al, 1991; Stromland and Pinazo-Duran, 1994; Mattson and Riley 1996). Studies have begun to use zebrafish as a model of fetal alcohol spectrum disorders (FASD) and demonstrate that ethanol exposure in embryonic zebrafish results in phenotypes comparable to those observed in other vertebrate models (reviewed in Cole et al, 2012; Bilotta et al, 2004; Dlugos and Rabin, 2007; Kashyap et al 2007; Zhang et al, 2011; Zhang et al, 2013; Joya et al, 2014; Swartz et al, 2014). Our previous studies in zebrafish have used chronic and binge ethanol exposure to identify molecular pathways involved in FASD phenotypes in zebrafish, and support the work of other laboratories that implicate sonic hedgehog (Shh) and fibroblast growth factor (Fgf) signaling in the manifestation of FASD (Zhang et al, 2011; 2013; 2014). These studies show that ethanol exposure during defined stages of embryogenesis lead to hallmark features of FASD such as microphthalmia or perturbed differentiation of GABAergic neurons, by a mechanism involving Shh and Fgf crosstalk (Zhang et al, 2011; 2013; 2014). However, studies in rodent, *Xenopus* and zebrafish have also implicated other molecular targets in the etiology of FASD, such as retinoic acid (RA). This raises the question of whether embryonic exposure to ethanol independently alters multiple signaling pathways or whether ethanol perturbation of CNS development results from the disruption of multiple signaling pathways that function via crosstalk to regulate neuronal differentiation.

A wealth of evidence supports RA function as a target of prenatal ethanol exposure (Sulik et al, 1981; Duester, 1991; Pillarkat, 1991; Zachman et al, 1998; Leo and Lieber, 1999; McCaffery et al, 2004; Yelin et al, 2005; Yelin et al, 2007; Kot-Leibovich and Fainsod 2009; Kumar et al, 2010). Of particular relevance to the current studies, in zebrafish embryos RA can rescue FASD phenotypes that include microphthalmia (Marrs et al, 2010; Muralidharan et al 2015). RA also exerts its function via the modulation of multiple other signaling systems, with several studies describing crosstalk between RA, Shh and Fgf signaling pathways. It is well established that RA signaling controls downstream expression and function of Fgfs and Shh in limb development (Diez del Corral and Storey 2004), with ethanol disrupting Fgf and Shh signaling during mouse limb development (Chrisman et al, 2004). Crosstalk between RA, Shh and Fgfs has also been documented during CNS development, with ventralization of spinal cord and telencephalon involving RA modulation of Shh and Fgf signaling (Shinya et al, 2001; Lupo et al, 2002; Appel and Eisen 2003; Lupo et al, 2005; Ribes et al, 2009). RA modulation of Shh and Fgf signaling also regulates ventralization of *Xenopus* retina (Lupo et al, 2005), indicating the key role of crosstalk between RA, Shh and Fgf pathways in multiple regions of the developing CNS. Accordingly, knockdown of retinaldehyde dehydrogenase 2 (Raldh2), the biosynthetic enzyme for RA, leads to ocular defects and diminished Shh and Fgf signaling (Ribes et al 2005). Combined inhibition of Raldh2 and ethanol exposure in *Xenopus* recapitulates ethanol pathology in *Xenopus* and reduces the expression of Shh (Yelin et al, 2007; Kot-

Leibovich and Fainsod, 2009). Furthermore, morpholino knockdown of *aldh1a3* in zebrafish leads to eye phenotypes that are identical to ethanol-mediated eye defects (Yahyavi et al, 2013), as well as optic nerve defects that are similar to Fgf and Shh loss of function phenotypes (Liu et al, 2008). Interestingly, our previous work has shown that the heparan sulfate proteoglycan (HSPG) agrin is a target of embryonic ethanol exposure that involves crosstalk between Shh and Fgf signaling pathways (Zhang et al, 2013; 2014), and RA also increases heparan sulfate biosynthesis and 3-O sulfation of HSPGs (Zhang et al, 1998), suggesting that ethanol-mediated disruption of HSPG synthesis and sulfation could alter Shh and Fgf signaling.

Our laboratory is therefore exploiting the zebrafish model to combine low dose ethanol exposure with manipulation of a range of molecular targets to elucidate whether ethanol may impact multiple distinct molecular pathways that lead to FASD, or whether ethanol affects pathways that through crosstalk lead to the hallmark characteristics of FASD. We also are using partial knockdown of multiple genes, such as both Shh and RA signaling pathways, to determine if this combined targeting will recapitulate the morphological and behavioral phenotypes of FASD in the absence of ethanol exposure. These studies show that either RA (*aldh1a3*) or Shh (*Shha*) morpholino knockdown produce similar morphological phenotypes in zebrafish that are reproduced by ethanol exposure alone. We show that either subthreshold RA or Shh knockdown combined with ethanol induces FASD phenotypes such as microphthalmia. Our studies indicate that the disrupted MHB and microphthalmia phenotypes induced by subthreshold *aldh1a3* knockdown together with alcohol exposure can be rescued by RA, but not Shh mRNA overexpression. However, RA can rescue the disrupted MHB phenotype induced by subthreshold Shh knockdown combined with ethanol exposure, but cannot rescue the microphthalmia phenotype. These data suggest that alcohol-mediated morphological phenotypes, such as disrupted MHB and microphthalmia, may result from ethanol minimally disrupting two distinct signaling pathways, RA and Shh, which exhibit limited crosstalk to regulate specific aspects of CNS development. These studies also suggest a complex interplay between multiple extracellular signaling molecules may be disrupted by prenatal ethanol exposure, leading to the hallmark features of FASD.

MATERIALS AND METHODS

Animals

Zebrafish were obtained from Zebrafish International Resource Center. The AB strain was used in these studies and fish were housed in automatic fish housing systems (Aquaneering, San Diego, CA) at 28.5° C. All procedures using zebrafish were approved by the NCCU IACUC.

Ethanol treatment of zebrafish embryos

Zebrafish embryos in fish water containing a 1:500 dilution of 0.1% methylene blue (to prevent fungal infection) were exposed to 0.5% ethanol during 6-24hpf. Ethanol was diluted with fish water to its final concentration, and at the selected developmental stage embryos were placed in fresh fish water containing ethanol. Embryos were incubated in 100 mm plates with fish water containing ethanol, with 10-30 embryos per plate. Each experiment

was repeated 3 times, and the combined number of embryos used to calculate ratios of embryos showing effects in response to ethanol exposure. At the end of the exposure period fish water containing ethanol was removed, embryos were washed once with fresh fish water, and then transferred to fresh fish water for the remainder of the experimental time-course. 0.003% 1-phenyl-2-thiourea (PTU) was added to zebrafish embryos in Figure 2 and Figure 3 to inhibit pigmentation from 1dpf to 2dpf.

Eye size was measured as previously described (Zhang et al, 2011) and involved measuring the longest axis along the eye, and calculated against a standard 50 μ m ruler under the same magnification. For the 2 dpf eye, we designated a diameter less than 240 μ m as having a small eye phenotype, since untreated eyes were typically at least 250 μ m in diameter. Malformation of the MHB was assessed visually based on absence of the defined border between the midbrain and hindbrain.

Antisense morpholino injection

Antisense morpholino oligonucleotide (MO) (Gene Tools, Philomath, OR) to *Shh* was designed against exon/intron splice sites (CAGCACTCTCGTCAAAAGCCGCATT) (Zhang et al, 2013). MO to *aldh1a3* was designed to target the start codon (TATAGTCCCGTTCTGTGCCATAGC) (Bill et al, 2009; Yahyavi et al, 2013). *Shh* and *aldh1a3* MOs were solubilized in water at a concentration of 0.03-0.5mM before injection into one to two-cell stage embryos. For *Shh* mRNA rescue, embryos were co-injected with 25pg *Shh* N183 mRNA, which encodes the N-terminal 183 amino acids of Shh in zebrafish. Shh N183 contains all of the biologic activity of Shh protein and has been shown previously to rescue ethanol-induced ocular phenotypes in zebrafish (Loucks and Ahlgren, 2009). 0.1mM RA stock was made by dissolving RA in DMSO. 1nM RA was freshly diluted from 0.1mM RA stock in fish water before treatment for RA rescue. DMSO was added as control for RA rescue.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as previously described, with probe hybridization at 65°C (Kim et al., 2007; Liu et al., 2008). Digoxigenin-labeled riboprobes were transcribed from cDNAs encoding *Pax6a* as previously described (Zhang et al., 2011; 2013). Embryos were cleared in 50% glycerol and then viewed using an Olympus MVX-10 stereomicroscope.

Statistical analysis

Differences in percent of embryos displaying disrupted MHB, eye size and percent of reduced *Pax6a* expression as a result of treatment were analyzed for statistical significance using one-way ANOVA.

RESULTS

Effects of ethanol and perturbation of RA and Shh signaling on midbrain-hindbrain boundary formation

Ethanol exposure during zebrafish embryogenesis results in characteristic morphological malformations of FASD that are dependent on the concentration of ethanol exposure, including loss of the midbrain-hindbrain boundary (MHB) (Zhang et al, 2011; 2014). While chronic 6-24 hpf exposure of embryos to 2% ethanol disrupts formation of the MHB (Figure 1E), exposure to low concentrations of ethanol, such as 0.5% ethanol, does not disrupt formation of the MHB (Figure 1C,D). Multiple molecular targets have been implicated as critical to the etiology of FASD and include RA and Shh. Using a transgenic zebrafish line that expresses YFP under control of multiple retinoic acid response elements (Perz-Edwards et al 2001), we observed marked reduction of YFP expression following ethanol exposure from 6-24 hpf, confirming RA synthesis as a target of ethanol (Supplemental Figure 1). YFP expression is also detected in the MHB of the RARE-YFP transgenic zebrafish (Perz-Edwards et al, 2001), and accordingly perturbation of RA signaling by the knockdown of one of its biosynthetic enzymes, retinaldehyde dehydrogenase 3, using the *aldh1a3* morpholino (MO), disrupts MHB formation (Figure 1F). As we have shown previously, using a lower subthreshold dose of MO (Figure 1F,G,H), combined with subthreshold 0.5% ethanol, reproduces the disrupted MHB phenotype (Figure 1I). This combined treatment with subthreshold ethanol and *aldh1a3* MO can be rescued by treatment of embryos with 1 nM RA during the 6-24 hpf treatment window (Figure 1J), with addition of exogenous RA in the absence of alcohol or MO treatment not altering MHB development (Figure 1B). The number of embryos affected by these treatments and the statistical significance of these treatments are shown in Figure 2, and indicate that 0.5 pmol *aldh1a3* MO and 2% ethanol produce significant effects on MHB formation, with exogenous RA rescuing the MHB morphology phenotype.

To determine if crosstalk between the RA and Shh signaling pathways may lead to the disrupted MHB phenotype, the ability of *Shh* mRNA overexpression to rescue MHB formation was examined. *Shh* mRNA overexpression is unable to rescue the MHB phenotype following *aldh1a3* MO and ethanol treatment (Figure 1M), but inclusion of exogenous RA does rescue this phenotype (Figure 1N). Conversely, *Shh* MO treatment combined with ethanol exposure also disrupts MHB formation (Figure 1O-Q), and RA treatment does rescue this phenotype (Figure 1R). Combined *aldh1a3* and *Shh* MO treatment also recapitulates the abnormal MHB phenotype (Figure 1S) and either *Shh* mRNA overexpression or RA treatment can rescue the disrupted MHB phenotype (Figure 1T-V). These data are quantified in Figure 2, showing the statistical significance of the MO and ethanol mediated disruption of the MHB and the rescue by exogenous RA or *Shh* overexpression.

Effects of ethanol and perturbation of RA signaling on ocular development

As shown in Table 1, a common morphological abnormality in FASD is reduced eye size, microphthalmia. Our previous studies have shown that chronic 1.5% or 2% ethanol exposure from 6-24 hpf leads to marked microphthalmia (Figure 3E and Zhang et al, 2011). As there

is a range of eye diameter observed even in untreated zebrafish embryos at 2 dpf, we selected 240 μm diameter as a minimal cutoff for designating a treated embryo as having a small eye phenotype, as untreated embryos typically have an eye diameter $>250 \mu\text{m}$. This also represents at least an approximate 10% decrease in eye diameter when compared to the Mean eye diameter for untreated embryos (Table 1). Treatment with 0.5 pmol *aldh1a3* MO also induces microphthalmia (Figure 3F), while low dose 0.5% ethanol does not result in reduced eye size (Figure 3C,D). Injection of one-cell embryos with concentrations of *aldh1a3* MO that do not affect ocular development (Figure 3G,H), when combined with 0.5% ethanol, results in microphthalmia (Figure 3I). Again this small eye phenotype can be rescued by treatment of embryos with 1 nM RA (Figure 3J) that alone does not affect eye diameter (Figure 3B). As observed with MHB development, *Shh* mRNA overexpression cannot rescue the small eye phenotype induced by combined ethanol and *aldh1a3* MO treatment (Figure 3M), but addition of exogenous RA to this treatment group does rescue the phenotype (Figure 1N). Quantitative analysis of eye diameter and the effect of the various treatments are shown in Table 1, with these data showing the percentage of embryos displaying microphthalmia and the average eye diameter for each treatment group.

Alteration in *Pax6a* gene expression following ethanol exposure and RA perturbation

We have shown previously that *Pax6a* gene expression is strongly correlated to eye size, with ethanol treatment that reduces eye size also decreasing *Pax6a* or *GAD1* gene expression as measured by in situ hybridization or qRT-PCR (Zhang et al, 2014). Accordingly, treatment with 0.5 pmol *aldh1a3* MO markedly reduces *Pax6a* gene expression (Figure 4E). While neither low dose ethanol nor low dose *aldh1a3* MO affects *Pax6a* gene expression (Figure 4C,D,F,G), combined ethanol and *aldh1a3* MO markedly reduces *Pax6a* gene expression (Figure 4H). Treatment with 1 nM RA restores *Pax6a* expression levels (Figure 4I). As shown for rescue of eye size, *Shh* mRNA overexpression cannot restore *Pax6a* expression to normal levels (Figure 4J-L), but addition of exogenous RA to this treatment group restores *Pax6a* expression (Figure 4M). These data are quantitated in Figure 5, showing the statistical significance of the MO and ethanol mediated disruption of *Pax6a* gene expression, the rescue by exogenous RA and lack of rescue by *Shh* overexpression.

Analysis of potential crosstalk between RA and Shh pathways in ocular development

Since in this study we have shown similarity between effects of ethanol and involvement of RA and Shh in MHB and ocular development, this raised the question of whether treatment of embryos with both *aldh1a3* and *Shh* MOs would also disrupt ocular development. Table 2 summarizes the effects of *Shh* and *aldh1a3* MO treatments on eye diameter. While subthreshold *Shh* MO does not decrease eye size (Figure 6E), when combined with 0.5% ethanol microphthalmia does result, showing that Shh signaling is a target of ethanol in ocular development (Figure 6F). Since this same small eye phenotype is observed with 0.5% ethanol combined with either *aldh1a3* MO (Figure 3H) or *Shh* MO (Figure 6F), we anticipated that injection of embryos with subthreshold *aldh1a3* MO and *Shh* MO would also produce microphthalmia. Surprisingly injection of embryos with both MOs does not induce the small eye phenotype (Figure 6I), and accordingly exogenous RA treatment is unable to rescue the phenotype in embryos treated with ethanol and *Shh* MO (Figure 6H).

DISCUSSION

The primary goal of the current study was to assess whether alcohol exerts its actions on a shared molecular signaling pathway in the developing embryo to elicit the morphological abnormalities that are characteristic of FASD. The basis for this question was the numerous studies, utilizing a variety of animal models such as mouse, *Xenopus* and zebrafish, that have been conducted to identify molecular targets of ethanol exposure during embryogenesis, with multiple distinct signaling pathways having been implicated in the etiology of FASD.

While the majority of information regarding FASD molecular mechanisms has been obtained using rodent models, recent studies have begun to employ zebrafish as an FASD model, and demonstrate that embryonic zebrafish ethanol exposure results in phenotypes comparable to those observed in other vertebrate models. Zebrafish embryos exposed to ethanol display eye defects that range from microphthalmia to cyclopia (Arenzana et al, 2006; Bilotta et al, 2004; Dlugos and Rabin, 2007; Kashyap et al, 2007; Loucks et al, 2007; Reimers et al, 2004), depending in part on the concentration of ethanol used for exposure. Zebrafish embryos exposed to ethanol also exhibit perturbed photoreceptor differentiation and optic nerve hypoplasia (Dlugos and Rabin 2007; Matsui et al, 2006; Liu et al 2008). A common phenotype observed in zebrafish as a result of ethanol exposure during embryogenesis is microphthalmia (Bilotta et al, 2004; Reimers et al, 2004; Dlugos and Rabin, 2007; Kashyap et al 2007; Loucks et al, 2007; Ali et al, 2011; Zhang et al, 2011), which appears to be dose-dependent and dependent on the timing of ethanol exposure (Ali et al, 2011; Zhang et al 2014).

Numerous studies identify Shh signaling as a critical molecular target in FASD (Ahlgren et al, 2002; Aoto et al, 2008; Arenzana et al, 2006; Li et al, 2007; Loucks and Ahlgren 2009). Zebrafish embryos exhibit decreased Shh signaling (Li et al, 2007) following chronic ethanol exposure. *Shh* mRNA overexpression rescues cyclopia and skeletal defects associated with ethanol exposure in zebrafish embryos (Loucks and Ahlgren 2009), confirming the critical role for perturbed Shh function in FASD. Perturbation of Shh signaling may be responsible for the craniofacial abnormalities of FAS, which include microphthalmia (Ahlgren et al, 2002; Arenzana et al, 2006; Li et al, 2007; Aoto et al, 2008; Loucks and Ahlgren, 2009; Fan et al, 2009). Prenatal ethanol exposure during the period of gastrulation and early neurulation leads to reduced *Shh* gene expression in mouse and chick embryos, ultimately resulting in phenotypes characteristic of perturbed Shh signaling (Ahlgren et al, 2002; Loucks et al, 2007; Aoto et al, 2008). Despite this evidence for Shh in FASD, Kashyap et al (2011) concluded ethanol-mediated effects on zebrafish ocular development were not Shh-dependent. However, these embryos were exposed to ethanol at later developmental stages than other studies examining Shh function in FASD, which may account for this different experimental result.

Numerous studies also implicate RA function in FASD (Sulik et al, 1981; Duester, 1991; Pillarkat, 1991; Zachman et al, 1998; Leo and Lieber, 1999; McCaffery et al, 2004; Yelin et al, 2005; Yelin et al, 2007; Kot-Leibovich and Fainsod 2009; Kumar et al, 2010). Among the ethanol-induced phenotypes that can be rescued by RA treatment of ethanol-exposed

embryos is microphthalmia (Marrs et al, 2010; Muralidharan et al 2015), indicating similarity with Shh function. RA signaling regulates downstream expression and function of Fgfs and Shh in limb development (Diez del Corral and Storey 2004), and ethanol disrupts Fgf and Shh signaling during mouse limb development (Chrisman et al, 2004). Ventralization of spinal cord and telencephalon during CNS development involves crosstalk between RA, Shh and Fgfs (Shinya et al, 2001; Lupo et al, 2002; Appel and Eisen 2003; Lupo et al, 2005; Ribes et al, 2009). RA modulation of Shh and Fgf signaling also regulates ventralization of *Xenopus* retina (Lupo et al, 2005), indicating the key role of crosstalk between RA, Shh and Fgf pathways in multiple regions of the developing CNS.

This accumulation of evidence for crosstalk between RA, Shh and Fgf signaling pathways provided the rationale for the current study to ascertain whether ethanol only disrupts a single functional pathway, that depends on crosstalk between multiple extracellular ligand mediated signaling pathways, namely Fgf, Shh and RA. Our previous studies in zebrafish have shown that microphthalmia induced by chronic or binge alcohol exposure can be rescued by *Shh* overexpression (Zhang et al, 2011). In addition, other ethanol-induced FASD phenotypes in zebrafish could be rescued by Fgf8, Fgf19 and *Shh* mRNA overexpression (Zhang et al, 2013). Thus, in the case of microphthalmia and MHB formation, we reasoned these would be straightforward FASD phenotypes to assess if RA, Shh and Fgf crosstalk occurs and is disrupted as a consequence of ethanol exposure during embryogenesis. By inference it could be hypothesized that any observed crosstalk between RA and Shh would also impact Fgf signaling, especially in the case of ocular development where crosstalk between Shh and Fgf8 is well established during embryogenesis (Vinothkumar et al 2008). Our current studies do show that both *Shh* and *aldh1a3* MO treatment alone lead to microphthalmia, and that subthreshold MO combined with low dose ethanol produces the same phenotype. These data are consistent with work showing that combined inhibition of Raldh2 and ethanol exposure in *Xenopus* recapitulates ethanol pathology and reduces the expression of *Shh* (Yelin et al, 2007; Kot-Leibovich and Fainsod, 2009). However, unexpectedly we observed that neither RA overexpression nor *Shh* overexpression could rescue the microphthalmia phenotype, suggesting that microphthalmia induced by ethanol is likely the result of perturbation of distinct signaling pathways, RA and Shh, that do not interact to produce the FASD phenotype. Consistent with this observation, injection of embryos with a combination of subthreshold *aldh1a3* and *Shh* MO did not induce microphthalmia. The conclusion of these experiments is that zebrafish ocular development is dependent on RA and Shh signaling that do not exhibit crosstalk, and that ethanol-mediated disruption of ocular development occurs as the result of both RA and Shh signaling being independent molecular targets of ethanol.

In contrast to ocular development, our data from the present study suggest that crosstalk between Shh and RA signaling pathways regulate MHB development. The MHB is a critical signaling center that regulates both midbrain and hindbrain development and is a source of Fgf8 signaling (Reifers et al, 1998; Irving and Mason 2000; Wiellette and Sive 2004). The MHB is lost in *Fgf8* mutant zebrafish (Reifers et al, 1998) and in morphant zebrafish treated with *agrin* MOs that perturb Fgf signaling (Liu et al, 2008). Treatment of mouse embryos with a cholesterol biosynthesis inhibitor also disrupts the equivalent structure in mouse

brain, the isthmus, with a link to disturbed Shh signaling being provided (Lanoue et al, 1997). In preliminary experiments we have observed that *aldh1a3* MO treatment decreases expression of *fgf8* and *pax2a* mRNAs in MHB (data not shown), consistent with expression of these MHB markers being under the control of multiple signaling pathways that include Shh and RA. These results are also in agreement with previous studies showing that excess RA leads to loss of *pax2a* expression in zebrafish MHB (Waxman and Yelon, 2009), suggesting that tightly regulated levels of RA signaling are required for normal expression of *pax2a* and development of the MHB. Prenatal ethanol exposure at gestational day 7 also leads to a malformed isthmus in mouse brain (Godin et al 2010). These data are in agreement with our data presented here, suggesting a direct role for RA and Shh signaling in MHB formation in zebrafish, which is sensitive to ethanol exposure. We also anticipated observing an effect of *aldh1a3* MO on MHB formation as using RARE-YFP transgenic zebrafish expression of YFP is observed in the MHB, indicating synthesis of RA in this important signaling center (Perz-Edwards et al, 2001). Accordingly *aldh1a3* MO treatment perturbs formation of the MHB. Our present study also shows that either *Shh* MO or *aldh1a3* MO disrupts MHB formation, and combined subthreshold MO and ethanol produces the MHB phenotype. Injection of embryos with both *aldh1a3* and *Shh* MO also perturbs MHB formation, and either RA exogenous treatment or *Shh* mRNA overexpression rescues the MHB phenotype as expected. However, while exogenous RA rescues the *aldh1a3* MO/ethanol MHB phenotype and *Shh* MO/ethanol MHB phenotype, *Shh* mRNA overexpression cannot rescue the *aldh1a3* MO/ethanol MHB phenotype. These data suggest that RA must signal downstream of Shh, since RA treatment can rescue both *Shh* MO and *aldh1a3* MO treatments combined with ethanol, but *Shh* cannot rescue the *aldh1a3* MO/ethanol treatment. As the MHB is a critical signaling center for the regulation of midbrain and hindbrain differentiation, it will be of future interest to ascertain how RA and Shh signaling, presumably acting in concert with Fgf signaling, modulate the differentiation of these brain regions and are affected by ethanol exposure.

In conclusion, the main objective of the present study was to ascertain if ethanol perturbs morphological differentiation of specific CNS structures by disrupting signaling pathways that either function independently of each other or rather through crosstalk control CNS development. Our studies show that while ethanol disrupts MHB formation by perturbing RA and Shh signaling that exhibit crosstalk, the well-described effects of ethanol on ocular development appear to occur by independent inhibition of two distinct signaling pathways utilizing RA and Shh. These studies demonstrate the complex nature of ethanol's actions on distinct regions of the developing nervous system, which may occur in other non-neuronal tissues that employ RA and Shh pathways during development and that are also sensitive to developmental exposure to ethanol. As an example, the effect of ethanol on neural crest cell migration during craniofacial development is well-described (Swartz et al, 2014), and can lead to phenotypes, such as microphthalmia, described here (Dee et al, 2012). While it will be of interest in future studies to examine whether ethanol affects neural crest and craniofacial development via similar signaling pathways, thereby contributing to the hallmark phenotypes of FASD such as microphthalmia, recent studies have shown that *Shh* does not synergize with ethanol to mediate craniofacial malformations in zebrafish (McCarthy et al, 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Effect of ethanol and RA or Shh disrupting MOs on MHB formation

All embryos were analyzed 1 dpf for presence or absence of the defined border between the midbrain and hindbrain (indicated by arrows). A,C,G,I,K,M,O,Q,S and U were treated with DMSO, the vehicle for RA administration. B,D,H,J,L,N,P,R,T, and V were treated with 1 nM RA. A,B, wild-type embryos; C,D,I,J,M,N,Q, and R embryos were exposed to 0.5% ethanol from 6-24 hpf; E, exposure to 2% ethanol from 6-24 hpf; F, embryos were injected at the one-cell stage with 0.5 pmol *aldh1a3* MO; G-J, embryos were injected with 0.03 pmol *aldh1a3* MO that does not produce any changes in zebrafish morphology alone; K-N, embryos were injected with both *aldh1a3* MO and 25 pg Shh N183 mRNA; O-R, embryos were injected with 0.3 pmol *Shh* MO that does not produce any changes in zebrafish morphology; S-T, embryos were injected with both *Shh* MO and *aldh1a3* MO; U-V, embryos were injected with both MOs plus *Shh* N183 mRNA. Note that the MHB does not form properly in panels E,F,I,M,Q and S.

Figure 2. Quantitation of changes in MHB formation in embryos exposed to chronic ethanol or RA and Shh disrupting MOs

Data shown as Mean $\pm$ SD for the percentage of embryos displaying MHB disruption analyzed at 1 dpf. The sample size represents the total number of embryos analyzed across all experiments, with at least 3 independent experiments with at least 10 embryos per experimental group. a: Significantly different from WT + DMSO, $P < 0.05$; b: Significantly different from 6-24hpf 0.5% + DMSO, $P < 0.05$; c: Significantly different from 0.03pm *aldh1a3* MO + DMSO, $P < 0.05$; d: Significantly different from 0.03pm *aldh1a3* MO + 6-24h 0.5% + DMSO, $P < 0.05$; e: Significantly different from 0.03pm *Shh* MO + DMSO, $P < 0.05$; f: Significantly different from 0.03pm *Shh* MO + 6-24h 0.5% + DMSO, $P < 0.05$; g: Significantly different from 0.03pm *aldh1a3* MO + 0.03pm *Shh* MO + DMSO, $P < 0.05$.

Figure 3. Ocular development in embryos exposed to chronic ethanol or RA disrupting MO
 Eye diameter was measured in 2 dpf embryos as described in Methods. A,C,G,I,K and M, were treated with DMSO, the vehicle for RA administration. B,D,H,J,L and N were treated with 1 nM RA. A,B, wild-type embryos; C,D,I,J,M, and N, embryos were exposed to 0.5% ethanol from 6-24 hpf; E, embryos were exposed to 2% ethanol from 6-24hpf; F, embryos were injected at the one-cell stage with 0.5 pmol *aldhl1a3* MO; G-J, embryos were injected with 0.03 pmol *aldhl1a3* MO, the low dosage alone that does not produce any changes in eye morphology; K-N, embryos were injected with both *aldhl1a3* MO and 25 pg *Shh* N183 mRNA. The calibration bar is 50 μ m. Note that microphthalmia is observed in E, F, I and M.

Figure 4. *Pax6a* mRNA expression in 2 dpf retina following chronic ethanol exposure and *aldh1a3* MO treatment

A,C,F,H,J, and L embryos treated with DMSO; B,D,G,I,K, and M, embryos treated with 1 nM RA; A,B, wild-type embryos; C,D,H,I,L, and M, embryos were exposed to 0.5% ethanol from 6-24 hpf; E, embryos were injected at the one-cell stage with 0.5 pmol *aldh1a3* MO; F-I, embryos were injected with 0.03 pmol *aldh1a3* MO that does not produce any changes in eye morphology alone; J-M, embryos were injected with both *aldh1a3* MO and 25 pg *Shh* N183 mRNA. Note that decreased *pax6a* mRNA expression is observed in E, H and L.

Figure 5. Quantitation of decreased *pax6a* expression following chronic ethanol exposure and *aldh1a3* MO treatment

Data are shown as Mean $\pm$ SD for percentage of embryos displaying decreased *pax6a* mRNA expression for at least 3 independent experiments, with total number of embryos analyzed shown. a: Significantly different from WT + DMSO, $P < 0.05$; b: Significantly different from 6-24hpf 0.5% + DMSO, $P < 0.05$; c: Significantly different from 0.03pm *aldh1a3* MO + DMSO, $P < 0.05$; d: Significantly different from 0.03pm *aldh1a3* MO + 6-24hpf 0.5% + DMSO, $P < 0.05$.

Figure 6. Lack of crosstalk between RA and Shh signaling pathways as evidenced by lack of RA rescue of Shh/Ethanol treated embryos and inability of combined *Shh* and *aldh1a3* MOs to induce microphthalmia

A,C,E,G and I, embryos treated with DMSO; B,D,F,H, and J; embryos treated with 1 nM RA; A,B, wild-type embryos; C,D,G, and H, embryos were exposed to 0.5% ethanol from 6-24 hpf; E-H, embryos treated with 0.3 pmol *Shh* MO that does not produce any changes in eye morphology alone; I-J, embryos injected with both *aldh1a3* and *Shh* MOs. Note that microphthalmia is only observed in G and H, when 0.5% ethanol is combined with 0.3 pmol *Shh* MO. Calibration bar is 50 μ m.

Table 1

Ratio of embryos exhibiting microphthalmia and quantitation of eye diameter in zebrafish embryos treated with chronic ethanol and *aldh1a3* MO

Treatments	Overall ratio of microphthalmia	Eye size Mean $\pm$ SD (μ m)
WT + DMSO	0/30	265.6 $\pm$ 10.0
WT + 1nM RA	0/30	264.6 $\pm$ 7.6
6-24h 0.5% ethanol + DMSO	0/30	260.0 $\pm$ 9.4
6-24h 0.5% ethanol+ 1nM RA	0/30	263.6 $\pm$ 12.9
6-24h 2% ethanol	30/30	208.1 $\pm$ 15.1 ^a
0.5pm <i>aldh1a3</i> MO	32/32	161.7 $\pm$ 20.6 ^a
0.03pm <i>aldh1a3</i> + DMSO	1/37	257.9 $\pm$ 10.9
0.03pm <i>aldh1a3</i> + 1nM RA	1/31	258.8 $\pm$ 12.7
0.03pm <i>aldh1a3</i> + 6-24h 0.5% + DMSO	26/60	241.8 $\pm$ 14.9 ^{b,c}
0.03pm <i>aldh1a3</i> + 6-24h 0.5% + 1nM RA	7/46	253.2 $\pm$ 14.9 ^d
0.03pm <i>aldh1a3</i> + 25pg Shh N183 mRNA + DMSO	2/31	256.9 $\pm$ 11.9
0.03pm <i>aldh1a3</i> + 25pg Shh N183 mRNA + 1nM RA	1/37	259.3 $\pm$ 9.7
0.03pm <i>aldh1a3</i> + 25pg Shh N183 mRNA + 6-24h 0.5% + DMSO	28/61	239.4 $\pm$ 17.5 ^{b,c}
0.03pm <i>aldh1a3</i> + 25pg Shh N183 mRNA + 6-24h 0.5% + 1nM RA:	11/62	251.4 $\pm$ 15.3 ^d

The sample size represents the total number of embryos analyzed across all experiments, with 3 independent experiments with at least 10 embryos per experimental group. For untreated embryos eye diameter ranged from 247.2 μ m to 278.6 μ m, while for embryos exhibiting the small eye phenotype the eye diameter ranged from 129.7 μ m to 228.6 μ m, and rescued embryos contained eyes with a diameter ranging from 197.4 μ m to 275.8 μ m.

e: Significantly different from 0.03pm Shh MO + DMSO, P< 0.05

f: Significantly different from 0.03pm Shh MO + 6-24h 0.5% + DMSO, P< 0.05

g: Significantly different from 0.03pm *aldh1a3* MO + 0.03pm Shh MO + DMSO, P< 0.05

^a: Significantly different from WT + DMSO, P< 0.05

^b: Significantly different from 6-24hpf 0.5% + DMSO, P< 0.05

^c: Significantly different from 0.03pm *aldh1a3* MO + DMSO, P< 0.05

^d: Significantly different from 0.03pm *aldh1a3* MO + 6-24h 0.5% + DMSO, P< 0.05

Table 2

Ratio of embryos exhibiting microphthalmia and quantitation of eye diameter in zebrafish embryos treated with chronic ethanol and *Shh* MO

Treatments	Overall ratio of microphthalmia	Eye size Mean $\pm$ SD (μ m)
WT + DMSO	0/30	266.3 $\pm$ 9.1
WT + 1nM RA	0/30	260.5 $\pm$ 8.8
6-24h 0.5% ethanol + DMSO	1/30	261.0 $\pm$ 11.7
6-24h 0.5% ethanol + 1nM RA	0/30	257.7 $\pm$ 9.0
0.3pm <i>Shh</i> MO + DMSO	3/30	256.4 $\pm$ 10.1
0.3pm <i>Shh</i> MO + 1nM RA	4/35	257.5 $\pm$ 12.5
0.3pm <i>Shh</i> MO + 6-24h 0.5% + DMSO	20/44	245.7 $\pm$ 11.7 ^{a,b}
0.3pm <i>Shh</i> MO + 6-24h 0.5% + 1nM RA	15/31	244.7 $\pm$ 14.2 ^{a,b}
0.03pm <i>aldh1a3</i> + 0.3pm <i>Shh</i> MO + DMSO	5/54	255.9 $\pm$ 10.6
0.03pm <i>aldh1a3</i> + 0.3pm <i>Shh</i> MO + 1nM RA	3/37	254.8 $\pm$ 11.1

The sample size represents the total number of embryos analyzed across all experiments, with 3 independent experiments with at least 10 embryos per experimental group.

^a: Significantly different from 6-24hpf 0.5% + DMSO, P< 0.05

^b: Significantly different from 0.03pm *Shh* MO + DMSO, P< 0.05