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AGRIN FUNCTION ASSOCIATED WITH OCULAR DEVELOPMENT IS A TARGET OF ETHANOL EXPOSURE IN EMBRYONIC ZEBRAFISH

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Abstract

BACKGROUND—Alcohol (ethanol) is a teratogen known to affect the developing eyes, face and brain. Among the ocular defects in fetal alcohol spectrum disorder (FASD) are microphthalmia and optic nerve hypoplasia. Employing zebrafish as an FASD model provides an excellent system to analyze the molecular basis of prenatal ethanol exposure-induced defects since embryos can be exposed to ethanol at defined developmental stages and affected genetic pathways can be examined. We have previously shown that disruption of agrin function in zebrafish embryos produces microphthalmia and optic nerve hypoplasia.

METHODS—Zebrafish embryos were exposed to varying concentrations of ethanol in the absence or presence of morpholino oligonucleotides (MOs) that disrupt agrin function. *In situ* hybridization was employed to analyze ocular gene expression as a consequence of ethanol exposure and agrin knockdown. Morphological analysis of zebrafish embryos was also conducted.

RESULTS—Acute ethanol exposure induces diminished agrin gene expression in zebrafish eyes and, importantly, combined treatment with subthreshold levels of agrin MO and ethanol produces pronounced microphthalmia, markedly reduces agrin gene expression, and perturbs *Pax6a* and*Mbx* gene expression. Microphthalmia produced by combined agrin MO and ethanol treatment was rescued by sonic hedgehog (Shh) mRNA overexpression, suggesting that ethanol-mediated disruption of agrin expression results in disrupted Shh function.

CONCLUSIONS—These studies illustrate the strong potential for using zebrafish as a model to aid in defining the molecular basis for ethanol's teratogenic effects. The results of this work

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suggest that agrin expression and function may be a target of ethanol exposure during embryogenesis.

Keywords

heparan sulfate proteoglycan; zebrafish; fetal alcohol spectrum disorder; ocular development; sonic hedgehog; Mbx; Pax6

INTRODUCTION

Alcohol is a teratogen that in humans has been shown to result in congenital abnormalities affecting the eyes (e.g. microphthalmia and optic nerve hypoplasia) (Chan et al, 1991; Dangata and Kaufman 1997; Stromland 1985; Stromland and Pinazo-Duran 1994), auditory structures (Church and Kaltenbach 1997; Church and Gerkin 1998) and the brain (e.g., microcephaly, holoprosencephaly and brain region-specific hypoplasia) (Clarren et al, 1978; Mattson and Riley 1996). In rodents, the developmental stage-dependency of many of the ethanol-inducible teratogenic endpoints has been shown (Maier et al, 1997; Olney et al, 2002; Sulik 2005). For example, in mice, ethanol exposure during the period equivalent to the third week of human gestation produces facial, ocular, and median forebrain defects, which are similar to those in fetal alcohol syndrome (FAS) and include holoprosencephaly at the severe end of the spectrum of induced defects (Sulik and Johnston 1982; Sulik et al, 1981, 1984; Godin et al, 2010). Ethanol exposure at a time equivalent to the fourth week of human gestation produces a different pattern of craniofacial malformations, along with forebrain and midbrain hypoplasia, and hindbrain dysmorphogenesis characterized by perturbed development of cranial nerves (Dunty et al, 2002; Sulik et al, 1986; Van Maele-Fabry et al, 1995; Parnell et al , 2009). The brain remains sensitive to ethanol teratogenesis during the remainder of embryogenesis as well as during fetal development. With the rodent third trimester human equivalent occurring after birth, much of the research regarding ethanol's adverse effects on the brain have entailed postnatal treatment paradigms, with results showing the hippocampus and cerebellum to be particularly vulnerable (Cragg and Phillips 1985; Goodlett et al, 1990; Savage et al, 1992; Bonthius et al, 1996; Livy et al, 2003; Dikranian et al, 2005).

Growing experimental evidence indicates that ethanol may exert its effects on central nervous system (CNS) development via disruption of extracellular matrix (ECM) function. Sulfation of heparan sulfate chains of heparan sulfate proteoglycans (HSPGs) is diminished, with concomitant loss of axon-promoting function, in response to ethanol (Dow and Riopelle 1990). Genes encoding ECM proteins that are essential to ECM function and CNS development, such as Fgf2 and Fgf8, are targets of ethanol exposure in prenatal mice (Aoto et al, 2008; Rubert et al, 2006). Sonic hedgehog (Shh) signaling is an apparent key target of prenatal ethanol exposure. Its perturbation may, at least in part, be responsible for the craniofacial abnormalities of FASD (Ahlgren et al, 2002; Aoto et al, 2008; Arenzana et al, 2006; Li et al, 2007; Loucks and Ahlgren 2009). Pertinent to the present studies is evidence that Shh function is regulated by interactions with HSPGs such as glypicans and perlecan (Bornemann et al, 2004; Datta et al, 2006; Giros et al, 2007; Park et al, 2003; Takeo et al, 2005), and that the establishment of functional Shh gradients is mediated by binding and

transport of Shh following binding to HSPGs (Han et al, 2004a, 2004b). Perlecan null mice exhibit diminished Shh signaling and brain morphological defects common to FASD (Giros et al, 2007). Importantly, overexpression of the HSPG agrin in transgenic mice produces alterations in Shh and Pax2a expression in the optic stalk (Fuerst et al, 2007). A similar alteration in Pax2a expression has been observed in zebrafish embryos exposed to ethanol (Loucks et al, 2007).

Zebrafish are becoming more widely used as an FASD model, with these studies demonstrating that embryonic zebrafish ethanol exposure results in phenotypes comparable to those observed in other vertebrate models. Ocular defects are a common finding in FASD and include microphthalmia and optic nerve hypoplasia. Different strains of zebrafish exhibit differential susceptibility to ethanol-induced cyclopia (Arenzana et al, 2006; Bilotta et al, 2004; Blader and Strahle, 1998) and also exhibit microphthalmia in response to ethanol (Dlugos and Rabin, 2007; Kashyap et al, 2007; Loucks et al, 2007; Reimers et al, 2004). Zebrafish also display perturbed photoreceptor differentiation and optic nerve development in response to ethanol exposure (Dlugos and Rabin 2007; Matsui et al, 2006). Zebrafish embryos exposed to ethanol exhibit increased hindbrain cell death (Carvan et al, 2004; Loucks and Carvan 2004), which has also been documented in mice following prenatal ethanol exposure (Dunty et al 2001, 2002). Zebrafish embryos exhibit decreased Shh signaling (Li et al, 2007), characterized by reduced *Gli1* gene expression (Loucks and Ahlgren 2007), as a result of ethanol exposure. Importantly, Shh overexpression rescues cyclopia and skeletal defects associated with ethanol exposure in zebrafish embryos (Loucks and Ahlgren 2009).

Agrin is a large ECM and cell surface HSPG (Tsen et al, 1995) that was originally identified in the electric ray *Torpedo californica* NMJ (Nitkin et al, 1987). Altering agrin expression in both transgenic mice (Fuerst et al, 2007) and zebrafish morphant embryos (Kim et al, 2007; Liu et al, 2008) induces many morphological phenotypes related to ocular development, which include microphthalmia and optic nerve hypoplasia. Studies from our laboratory have also shown that agrin binds Fgfs to modulate optic nerve growth, as well as eye development (Kim et al, 2003; Liu et al, 2008). Likewise, our recent studies show that retinal *Pax6a* and *ath5* gene expression is perturbed following agrin knockdown in zebrafish embryos, and is accompanied by perturbed optic stalk development (Liu et al, 2008). Since *Pax6* and *ath5* gene expression can be regulated by Shh signaling (Ericson et al, 1997; Kay et al, 2005; Miyake et al, 2005; Neumann and Nuesslin-Volhard 2000), these data suggest that agrin function may be required for proper Shh signaling during eye development. Thus, our recent work in zebrafish suggests that agrin may be a critical modulator of ocular development via a molecular mechanism that is sensitive to prenatal ethanol exposure. Our present studies support this hypothesis, with agrin gene expression being impaired following ethanol exposure, and agrin loss-of-function and ethanol acting via a common pathway to regulate ocular development.

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.

Ethanol treatment of zebrafish embryos

Zebrafish embryos in fish water containing a 1:500 dilution of 0.1% methylene blue (to prevent fungal infection) and 0.003% 1-phenyl-2-thiourea (PTU, to inhibit pigmentation) were exposed to 0.5%-1.5% ethanol from 6-24 hpf or 6-48 hpf. Ethanol was diluted with fish water to its final concentration, and at the selected developmental stage for ethanol treatment embryos were placed in fresh fish water containing ethanol. 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. We noticed no difference in ocular phenotypes between embryos exposed to ethanol for either time-course, and thus after initial studies using 6-48 hpf exposures all remaining studies were conducted using 6-24 hpf exposures. For morphological analyses of ocular development embryos were exposed to ethanol, but not PTU, to allow normal pigmentation and better visualization of embryonic morphology. Statistical analysis of morphological changes in embryos as a result of experimental treatments was determined using Graphpad Prism software (La Jolla, CA).

Antisense morpholino injection

Antisense morpholino oligonucleotides (MOs) (Gene Tools, Philomath, OR) were designed against exon/intron splice sites. The LG2 agrin MO, which we have shown previously to produce mild to severe phenotypes depending on the concentration of MO injected into one-cell embryos (Kim et al, 2007), was employed for these studies. We have shown previously that all agrin MO-induced defects are specific and not p53-mediated off-target defects (Liu et al, 2008). MOs were solubilized in water at a concentration of 0.1-1.0 mM before injection into one to two-cell stage embryos. Agrin MOs (0.1 pmol for subthreshold, 1 pmol for severe agrin morphant phenotypes) consistently produced reproducible phenotypes with an injection volume of 1 nl and robustly reduced agrin expression at 1 pmol injections. A general control MO purchased from Gene Tools to preclude MO toxicity, and used at the same concentration and volume, produces no detectable effects on zebrafish development (Kim et al, 2007).

For subthreshold agrin MO experiments, embryos were injected with 0.1 pmol LG2 MO, and at 6 hpf were then exposed to 0.5% or 1% ethanol until 24 hpf. For Shh mRNA rescue of agrin MO/ethanol exposure, embryos were co-injected with 0.1 pmol LG2 MO and 20 pg of capped ShhN 183 mRNA. N-terminal 183 amino acid zebrafish Shh (ShhN183) was synthesized by using 22 hpf zebrafish embryo mRNA as template and oligodT as primer, using Superscript reverse transcriptase. PCR primers were CGGAATTCATGCGGCTTTTGACGAGAGTG and

GCTCTAGATCAGCAATGAATGTGGGCTTTGG. The PCR product was subcloned into pCS2 vector and capped mRNA was synthesized with Ambion cap mRNA kit.

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). Digoxygenin-labeled riboprobes were transcribed from cDNAs encoding *agrin*, *Pax6a*, *Mbx1*, or *Shh*.

RESULTS

Ethanol-induced ocular defects in zebrafish resemble agrin morphant ocular phenotypes

Ocular defects are a common finding in FASD and include microphthalmia and optic nerve hypoplasia (Chan et al, 1991; Dangata and Kaufman 1997; Stromland 1985; Stromland and Pinazo-Duran 1994). Previous studies have shown that different strains of zebrafish exhibit differential susceptibility to ethanol-induced cyclopia (Bilotta et al, 2004; Arenzana et al, 2006), as well as ethanol-induced microphthalmia (Dlugos and Rabin 2007; Kashyap et al, 2007; Loucks et al, 2007; Reimers et al, 2004), impaired retinal lamination (Dlugos and Rabin 2007), and perturbed photoreceptor differentiation (Dlugos and Rabin, 2007; Matsui et al, 2006). In our studies using the AB strain of zebrafish, when embryos are exposed to ethanol from 6 hpf to 24 hpf pronounced microphthalmia is observed at both 24 hpf and 48 hpf (Figure 1 D,G,H). Microphthalmia is not evident following exposure to 0.5% ethanol (Figure 1 B, F), is most severe at 1.5% ethanol concentrations (Figure 1 D, H), and is observed in both 24 hpf and 48 hpf embryos (Figure 1 D,H). When eye diameter is quantified following ethanol exposure it can be seen that a statistically significant diminution in eye diameter occurs as a consequence of exposure to 1.0% and 1.5% ethanol, but not 0.5% ethanol (Figure 2).

Previously we have demonstrated that agrin MO-mediated knockdown of agrin expression leads to a number of phenotypes which include ocular defects characterized by microphthalmia, perturbed retinal lamination and photoreceptor differentiation, and optic nerve hypoplasia (Kim et al, 2007; Liu et al, 2008). As shown in Figure 1I, severe agrin morphants (injected with 1 pmol of MO) exhibit smaller optic vesicles than controls. Quantitation of eye size demonstrates a markedly significant decrease in eye diameter as a result of this agrin MO injection (Figure 2). This agrin MO ocular phenotype is remarkably similar to ethanol-induced microphthalmia, albeit more severe. Additionally, Figure 1 J-L illustrates that both ethanol exposure and agrin MO injection perturb retinal lamination in 72 hpf embryos. While a general developmental delay induced by ethanol may account for this effect on retinal lamination, we think this is unlikely since distinct retinal layers are present in 48 hpf retina, and the observed developmental delays as a result of ethanol on retinal development would not account for the loss of retinal lamination (Dlugos and Rabin, 2007). In addition, retinal lamination is perturbed with agrin knockdown as early as 48 hpf (Liu et al, 2008). Thus, a developmental delay of more than one day would be required to account for any phenotypes arising due to ethanol-induced developmental delay.

Agrin gene expression is reduced in response to embryonic ethanol exposure

As a first test of agrin's role in ethanol-induced abnormal eye development, agrin gene expression was examined using whole-mount in situ hybridization (ISH) in zebrafish embryos exposed to a range of ethanol concentrations. As shown in Figure 3A, agrin mRNA expression in the retina is observed in both its ganglion cell layer (RGC) and inner nuclear layer (INL). At the lowest concentrations of ethanol tested (0.5%), agrin expression appears normal in the retina (Figure 3B). However, in embryos exposed to 1.5% ethanol, ethanol-induced microphthalmia is readily observed, combined with marked reduction of agrin mRNA retinal expression (Figure 3C).

Since these ISH data suggest that agrin gene expression may be affected by embryonic ethanol exposure, and could account for observed FASD phenotypes such as microphthalmia, studies to examine whether agrin and ethanol may function via similar molecular/genetic pathways were conducted. In zebrafish this is readily accomplished using subthreshold levels of inhibition in a pathway; in this case combining subthreshold levels of agrin MO (that will produce only a minor eye phenotype) with subthreshold levels of ethanol (which do not produce the morphological phenotype). As shown in Figure 3B, we do not observe microphthalmia with acute exposure of embryos to 0.5% ethanol. When a subthreshold dose (0.1 pmol) of agrin MO is injected into one-cell zebrafish embryos, we observe modest microphthalmia, while agrin mRNA expression appears normal by ISH (Figure 3D). However, with a combination of subthreshold agrin MO and 0.5% ethanol or 1% ethanol, we observe marked microphthalmia as well as more dramatic reduction in agrin mRNA expression as compared to treatment with 1.5% ethanol alone (compare Figure 3C to Figure 3 E,F). Significantly smaller head size accompanied by brain hypoplasia is also observed with these treatments (Figure 3E,F). In addition, hematoxylin and eosin staining of eyes from agrin MO plus 0.5% ethanol treatment shows that retinal lamination at 72 hpf is perturbed, as was observed in severe agrin morphant embryos (Figure 1O).

While agrin gene expression in retina is perturbed by ethanol exposure, it appears that agrin mRNA expression levels, and pattern of expression, are largely unaffected in other CNS regions. For example, the pattern and level of agrin gene expression in hindbrain appears similar in control, 0.5% ethanol-exposed and 1.5% ethanol-exposed embryos (Figure 3G-I).

Agrin-dependent gene expression is perturbed by ethanol exposure

In previous studies (Liu et al, 2008) gene expression in zebrafish embryos was analyzed employing ISH in order to begin to understand the molecular mechanisms underlying the agrin morphant phenotypes resulting from loss of agrin function. For the analysis of ocular phenotypes *Pax6a* gene expression in retina was first examined, since it is well recognized that *Pax6* mutations produce microphthalmia (Glaser et al, 1992). In addition, *Pax6* has been shown to play a critical role in ethanol-induced microcephaly in *Xenopus* (Peng et al, 2004) and an altered expression pattern for *Pax6* in zebrafish eye has been observed following ethanol exposure (Kashyap et al, 2007; Loucks et al, 2007). As shown in Figure 4 A, B, we observe a marked reduction in expression of *Pax6a* in zebrafish retina as a consequence of reduced agrin protein expression that accompanies agrin MO injection. Similarly, *Pax6a* gene expression is disrupted in zebrafish eye following ethanol exposure (Figure 4 C-F). A

marked reduction in *Pax6a* expression is observed in 30 hpf embryos following 1.5% ethanol treatment, with reduced *Pax6a* mRNA expression also being detected in 48 hpf embryos, but to a lesser extent. Embryos treated with 0.5% ethanol exhibited *Pax6a* expression that was similar to untreated control embryos (Figure 4H). Thus, as shown by previous laboratories (Kashyap et al, 2007; Loucks et al, 2007), our data suggest that *Pax6* gene expression is affected by ethanol exposure, and may provide one possible mechanism for the effects of ethanol on eye development.

To establish that agrin loss-of-function and ethanol act via a shared molecular pathway to disrupt *Pax6a* expression, one-cell embryos were injected with a subthreshold dose of agrin LG2 MO, and then were exposed to 0.5% ethanol from 6-24 hpf. This combined agrin MO and ethanol treatment induces *Pax6a* mRNA expression patterns resembling either suprathreshold agrin MO injection alone or exposure to 1.5% ethanol alone (Figure 4G-J). *Pax6a* mRNA expression is markedly reduced in retina as a consequence of the combined subthreshold treatment with agrin MO and 0.5% ethanol (Figure 4J).

During our previous analysis of the effects of agrin loss-of-function on ocular development, we also examined the expression of the homeobox gene *Mbx*, which is required for eye and midbrain development (Kawahara et al, 2002). As shown in Figure 5 A,B and K,L, *Mbx* gene expression is markedly reduced in agrin morphant eyes when compared to control *Mbx* expression. We therefore extended these analyses to assess *Mbx* gene expression in zebrafish embryos exposed to ethanol. Interestingly, we observe a pronounced down-regulation of *Mbx* gene expression in zebrafish eye as a consequence of embryonic ethanol exposure, with the pattern of *Mbx* gene expression also being altered in the eyes of embryos exposed to 1.5% ethanol (Figure 5 E,F). In untreated and 0.5% ethanol treated embryos individual labeled cells are apparent in a distinctive pattern in retinas viewed from lateral and dorsal views (Figure 5A-D), while both the extent and pattern of *Mbx* mRNA expression is altered in 1.5% ethanol-exposed embryos.

To establish that agrin loss-of-function and ethanol act via a shared molecular pathway to disrupt *Mbx* expression, we again injected one-cell embryos with a subthreshold dose of agrin LG2 MO, followed by exposure of the embryos to 0.5% ethanol from 6-24 hpf. Combined treatment with subthreshold agrin MO and ethanol both induces marked down-regulation of *Mbx* gene expression in zebrafish eye and alters the pattern of *Mbx* expression (Figure 5G-J). Whereas *Mbx* mRNA is expressed in a well-defined pattern in zebrafish retina of untreated, 0.5% ethanol, or subthreshold LG2 MO embryos (Figure 5 A-D, G,H), *Mbx* mRNA expressed is markedly reduced and appears in a dispersed, undefined pattern in embryos exposed to 1.5% ethanol or combined subthreshold agrin MO and 0.5% ethanol (Figure 5 E,F,I,J). This is particularly evident for embryos exposed to both agrin LG2 MO and ethanol, with microphthalmia not being observed in either subthreshold agrin morphant or 0.5% ethanol embryos alone, but with microphthalmia observed in embryos treated with combined subthreshold agrin MO and 0.5% ethanol (compare Figure 5 C, G and I). Thus, these data provide strong support for the relationship between agrin loss-of-function and ethanol-induced morphological ocular phenotypes, as genes dependent on agrin function for normal developmental expression are also disrupted following ethanol exposure.

Since we observe ethanol- and agrin MO-dependent reduction in gene expression, as analyzed by ISH, for several developmental genes (i.e., *agrin*, *Pax6a*, *Mbx*), we wanted to demonstrate that a general transcriptional down-regulation is not induced by ethanol and therefore responsible for our observed reduction in agrin, *Pax6a* and *Mbx* gene transcription. Such a demonstration would provide support for the specificity of ethanol effects on agrin function, as well as the combined subthreshold treatment with agrin MO and ethanol on retinal gene expression. Arguing against a general effect of ethanol on gene transcription, recent studies in zebrafish using real-time PCR have shown that multiple developmentally-regulated genes, including *Shh*, are not affected by ethanol exposure prior to 4 dpf (Fan et al, 2010). Normal*Gli1* expression was also observed following acute exposure to 1.5% ethanol in zebrafish (Loucks et al, 2007). Since Shh signaling is considered a major target of ethanol-mediated FASD defects (Ahlgren et al, 2002; Aoto et al, 2008; Arenzana et al, 2006; Li et al, 2007; Loucks and Ahlgren 2009), we decided to analyze *Shh* mRNA expression by ISH in zebrafish embryos exposed to 1.5% ethanol from 6-24 hpf. As shown in Figure 6, we do not observe marked changes in *Shh* gene expression of 48 hpf zebrafish embryos following ethanol exposure. In particular, well-defined midline expression of *Shh* is detected in both control and 1.5% ethanol treated embryos when viewed dorsally (Figure 6 B,D). However, in lateral views it appears that the level and pattern of *Shh* mRNA expression is altered in ethanol treated embryos (Figure 6 A,C). These data suggest that ethanol-induced reductions in agrin, *Pax6a* and *Mbx* gene expression are not the result of a non-specific, general down-regulation of transcription as a consequence of embryonic ethanol exposure. Further support for specific, targeted effects of ethanol on gene expression is also suggested by agrin and *Mbx* gene expression patterns being apparently normal in brain, while being markedly reduced in eye (Figures 3 and 5).

Shh overexpression can rescue agrin-mediated ethanol-induced ocular phenotypes

Loss of Shh signaling has been shown to lead to eye defects that include microphthalmia (Chiang et al, 1996). Shh overexpression has also been shown to rescue ocular phenotypes (such as cyclopia) in zebrafish embryos treated with high doses of ethanol (Loucks and Ahlgren, 2009). Shh function in eye development is dependent on Fgf signaling, with Fgf8 signaling regulating Shh expression in eye (Vinothkumar et al, 2008). Our recent studies in zebrafish have shown that agrin loss-of-function, leading to microphthalmia and ocular defects, is mediated via disrupted Fgf signaling, with Fgf8 bead transplantation rescuing agrin morphant ocular defects (Liu et al, 2008). Therefore, our present results suggest a possible mechanism where agrin may be a critical regulator of Shh function during eye development, providing a possible molecular basis for the effects of ethanol exposure on agrin function. To obtain direct evidence that combinatorial treatment of zebrafish embryos with subthreshold doses of agrin MO and ethanol produces microphthalmia as a result of perturbed Shh signaling, we tested whether Shh mRNA overexpression can rescue the combined agrin MO/ethanol ocular phenotype. mRNA encoding the N-terminus of Shh (ShhN 183), which contains all of the biological activity of Shh protein and has been shown previously to rescue ethanol-induced ocular phenotypes in zebrafish (Loucks and Ahlgren, 2009), was overexpressed in zebrafish embryos to determine if Shh was capable of rescuing the microphthalmia observed in agrin morphant embryos exposed to ethanol. While injection of zebrafish embryos with subthreshold agrin MO, followed by 0.5% ethanol

treatment, produced microphthalmia in 59/85 (69.4%) embryos, co-injection of embryos with agrin MO and Shh mRNA, followed by 0.5% ethanol treatment, only produced microphthalmia in 16/89 (18.0%) embryos (Figure 7A). Thus, ethanol-mediated effects on agrin gene expression appear to result in perturbed Shh signaling in the developing zebrafish eye, producing ocular defects such as microphthalmia.

As shown in Figure 7B, embryos injected with Shh only, agrin MO only, or agrin MO plus Shh mRNA exhibit eye size that is similar to untreated embryos. This observation was confirmed by direct measurement of eye size in 20 embryos from each treatment group. Conversely, eye size in embryos treated with agrin MO in combination with 0.5% ethanol leads to reduced eye size (Figure 7B; 199.54 ± 20.50 μm vs. 253.70 ± 6.84 μm in untreated, $P<0.001$). Shh overexpression in embryos treated with agrin MO plus ethanol results in partial recovery of eye size (Figure 7B; 229.64 ± 15.38 μm). Importantly, quantitation of eye size in embryos from the Shh rescue experiments shows that eye size in agrin MO plus ethanol embryos is statistically different from all other treatment groups, including Shh rescued embryos ($P<0.001$).

To further confirm that combined agrin MO- and ethanol-mediated perturbation of normal eye development is a consequence of impaired Shh function, we analyzed *Mbx* gene expression in zebrafish retina. *Mbx* expression was analyzed by ISH in 72 hpf zebrafish embryos, with embryos subjected to both ethanol and agrin MO treatment either being injected at the one-cell stage with Shh mRNA, or being analyzed without Shh rescue. As described earlier, the characteristic pattern of *Mbx* gene expression was observed in untreated, 0.5% ethanol-exposed, and low dose agrin morphant embryos (Figure 8A-F). Shown in Figure 8 is a range of *Mbx* gene expression phenotype produced as a result of combined ethanol exposure and agrin MO injection. In all cases *Mbx* gene expression is clearly abnormal, ranging from marked down-regulation in expression to less pronounced decreases in expression that nonetheless are accompanied by an altered expression pattern in eye (Figure 8I-N). With Shh mRNA overexpression in the combined ethanol-exposed and agrin MO treated embryos, a clear rescue of the *Mbx* gene expression pattern was observed in the majority of Shh mRNA-injected embryos (Figure 8O,P). Thus, both morphological and quantitative analysis (eye size as shown in Figure 7) and *Mbx* gene expression analysis (Figure 8) demonstrate that combined agrin MO- and ethanol-mediated effects on eye development appear to share a common molecular pathway that involves Shh function.

DISCUSSION

While ethanol is known to have teratogenic effects on brain and craniofacial development, leading to the morphological and functional phenotypes of FASD in mammals, the underlying molecular mechanisms are not fully understood. The majority of information regarding FASD mechanisms has been obtained using rodent models for FASD, with the zebrafish model system yet to be extensively used for elucidating the molecular basis of FASD. 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). Other developmental effects of ethanol observed in zebrafish include increased cell death in hindbrain (Carvan et al, 2004; Loucks and Carvan 2004), otolith (otic vesicle) defects (Reimers et al, 2004) and impaired pharyngeal arch formation (Carvan et al, 2004).

Our recent observation that agrin loss-of-function in agrin morphant zebrafish embryos produced numerous ocular defects, that included microphthalmia, perturbed retinal lamination and photoreceptor differentiation, and optic nerve hypoplasia (Liu et al, 2008) provided a foundation for the studies described here. With the knowledge that all of these ocular defects are also induced by prenatal ethanol exposure in a variety of species including zebrafish (Chan et al, 1991; Dangata and Kaufman 1997; Stromland 1985; Stromland and Pinazo-Duran 1994; Bilotta et al, 2004; Reimers et al, 2004; Arenzana et al, 2006; Matsui et al, 2006; Dlugos and Rabin, 2007; Kashyap et al, 2007; Loucks et al, 2007), we decided to test the possibility that agrin function is a target of prenatal ethanol exposure. Our studies reported here support this hypothesis. We observe a marked reduction in agrin mRNA expression in zebrafish retina following ethanol exposure, as analyzed by ISH. Importantly, we demonstrate that subthreshold amounts of agrin MO that do not produce prominent ocular defects, combined with subthreshold 0.5% ethanol exposure that does not produce ocular defects or diminish agrin mRNA expression, produce prominent microphthalmia accompanied by pronounced reduction in agrin mRNA expression. These data strongly suggest that molecular pathways affected by agrin loss-of-function are also affected by embryonic ethanol exposure.

In our previous analyses of possible molecular mechanisms that may account for ocular defects resulting from agrin loss-of-function, we examined expression of genes encoding transcription factors known to be required for ocular development. *Pax6* is a paired-box gene that is a master regulator of eye development with its mutation resulting in microphthalmia (Glaser et al, 1992). *Mbx* is a paired-type homeobox gene that is also required for ocular development, with *Mbx* knockdown in zebrafish inducing perturbed *Pax6* expression (Kawahara et al, 2002). Our demonstration that *Mbx* gene expression is markedly reduced as a result of ethanol exposure is to our knowledge the first evidence that *Mbx* expression is sensitive to ethanol exposure during development. The sensitivity of *Mbx* gene expression to combined subthreshold treatment with agrin MO and 0.5% ethanol also indicates that its expression is regulated by agrin function via a molecular pathway that is a target of prenatal ethanol exposure. Interestingly, we also show that *Pax6* gene expression is disrupted by both agrin loss-of-function and ethanol treatment, as well as combined subthreshold treatment with agrin MO and ethanol. In light of the demonstration that *Mbx* function regulates *Pax6* expression, suggesting that *Mbx* acts upstream of *Pax6 (*Kawahara et al, 2002), this raises the interesting possibility that ethanol-induced diminution in *Pax6* expression is a consequence of perturbed *Mbx* expression in response to ethanol exposure. Previous studies have shown disparate effects of ethanol on *Pax6* expression, with *Pax6* reported to be unaffected by a 3 h ethanol exposure beginning at approximately 5 hpf, when

analyzed in 24 hpf zebrafish embryos (Blader and Strahle 1998). Conversely, 12 h exposure of Xenopus embryos to ethanol induces a marked decrease in *Pax6* expression resulting in microcephaly, which could be rescued by overexpression of *Pax6* (Peng et al, 2004). *Pax6* gene expression has also been shown to be altered in zebrafish retina following ethanol exposure (Kashyap et al, 2007; Loucks et al, 2007), including in 48 hpf zebrafish retina (Kashyap et al, 2007), a developmental age used in the present study where we demonstrate reduction of *Pax6a* expression as a result of ethanol exposure. We observed a more marked diminution in *Pax6a* mRNA expression in 48 hpf retina than reported previously (Kashyap et al, 2007), which may be attributable to differences in the developmental stages exposed to ethanol as we exposed embryos to ethanol from 6-24 hpf, whereas Kashyap et al (2007) exposed zebrafish embryos to ethanol from 24-48 hpf. Collectively, these studies strongly suggest that *Pax6* gene expression is a target of prenatal ethanol exposure, with the developmental timing of ethanol exposure playing a critical role in the sensitivity of *Pax6* gene expression to ethanol. Our results reported here also raise the question of whether ethanol-induced disruption of *Mbx* expression may be responsible for observed reductions in *Pax6* expression, in particular in ethanol-induced microcephaly (Peng et al, 2004).

Interestingly, recent studies using cultured myotubes to examine the role of ethanol in acetylcholine receptor aggregation demonstrate that agrin-induced receptor aggregation, but not laminin-induced receptor aggregation, is inhibited with ethanol exposure (Owen et al, 2010). Since acetylcholine receptor aggregation is dependent on the neuronal form of agrin and not muscle agrin (Smith and Hilgenberg 2002), these data suggest that either ethanol inhibits interaction of neuronal agrin with its receptor or other signaling components necessary for receptor aggregation, or that ethanol selectively inhibits components of the agrin-dependent pathway downstream of agrin binding to its receptor. Collectively, these data and our present studies suggest that agrin function may be a target of ethanol inhibition during several developmental processes, which include ocular development, branchial arch development (Turton and Cole, unpublished observations), and acetylcholine receptor aggregation in skeletal muscle (Owen et al, 2010).

Although an understanding of the effects of prenatal ethanol exposure on form and function has begun to be elucidated, the molecular basis of ethanol insult still remains poorly understood. Numerous studies provide strong support for interference with Shh signaling as a critical molecular event 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), characterized by reduced *Gli1* gene expression at high ethanol exposure (Loucks et al, 2007). Interestingly, Shh overexpression rescues cyclopia and skeletal defects associated with ethanol exposure in zebrafish embryos (Loucks and Ahlgren 2009), raising the possibility that our observed effects of ethanol on agrin function and ocular development may be linked to perturbed Shh function. Accordingly, Shh function is regulated by interactions with HSPGs such as glypicans and perlecan (Bornemann et al, 2004; Datta et al, 2006; Giros et al, 2007; Park et al, 2003; Takeo et al, 2005), and the establishment of functional Shh gradients is mediated by binding and transport of Shh following binding to HSPGs (Han et al, 2004a, 2004b). Overexpression of agrin in transgenic mice produces alterations in Shh expression in the optic stalk and ocular defects

similar to agrin loss-of-function in zebrafish (Fuerst et al, 2007). This study suggests that agrin expression must be tightly regulated for normal ocular development, with both elevated or reduced agrin expression producing similar ocular defects, possibly as a consequence of altered binding of agrin to heparin-binding growth factors such as Fgfs. The basement membrane HSPG perlecan, when mutated, results in diminished Shh signaling and brain morphological defects common to FASD (Giros et al, 2007). Collectively, these studies on HSPG function strongly implicate HSPGs as key regulators of Shh function.

Consistent with the demonstration of multiple roles for HSPGs in the regulation of Shh function, in the present study we have demonstrated that Shh mRNA overexpression can rescue microphthalmia that is produced by the combined partial knockdown of agrin expression, using an agrin MO, and low dose ethanol treatment. In addition, we demonstrate that Shh overexpression can rescue the combined agrin MO- and ethanol-mediated perturbation of *Mbx* gene expression in zebrafish retina. The ability to rescue the agrin/ethanol microphthalmia extends previous studies from our laboratory that showed that Fgf8-coated bead implantation in agrin morphant embryos rescued microphthalmia and retinal lamination phenotypes (Liu et al, 2008). Accordingly, as shown in the model in Figure 9, we propose a mechanism where agrin function acts upstream of Shh, via interactions with Fgf, to modulate ocular development, with ethanol perturbation of agrin function/expression impacting Fgf and Shh signaling. In preliminary experiments using solid-phase binding assays we have demonstrated binding of agrin to Shh protein (data not shown), raising the possibility that agrin regulation of Fgf function as well as direct binding to Shh protein could regulate Shh-dependent ocular development. In support of this model, previous studies have shown that Shh function in eye development is dependent on Fgf8 signaling (Vinothkumar et al, 2008), that loss of Shh signaling produces microphthalmia (Chiang et al, 1996), and that Shh overexpression rescues ocular phenotypes in zebrafish (Loucks and Ahlgren, 2009). Collectively, these observations by other groups indicate a molecular pathway whereby Fgf signaling is required for *Shh* expression and function, with loss of either Fgf or Shh function leading to disruptions in eye development that can manifest as microphthalmia. The data provided in the present studies, coupled with our previous demonstration that agrin loss-of-function in ocular development can be rescued by Fgf8 administration (Liu et al, 2008), strongly suggest that agrin may function upstream of Shh and Fgf signaling to modulate ocular development, with ethanol perturbation of agrin function/expression impacting downstream Fgf and Shh signaling, as well as expression of critical transcription factors such as *Pax6a* and *Mbx* (Figure 9). Our ability to rescue the ocular defects induced by combined ethanol exposure and agrin MO injections are consistent with this hypothesis. However, further experiments, testing whether Fgf overexpression can rescue agrin/ethanol phenotypes, or whether combined Fgf/agrin knockdown phenocopies ethanol/agrin phenotypes, will be required to confirm this model.

In summary, the present study suggests that ethanol-induced ocular defects are at least in part the result of perturbed agrin expression and/or function, with a molecular pathway involving ethanol-mediated disruption of *Pax6a* and *Mbx* gene expression also likely contributing to ethanol-induced ocular defects. Other developmental defects that are observed with ethanol exposure, such as defects in branchial arch formation (Carvan et al,

2004) and otic vesicle development (Reimers et al, 2004), are also observed following agrin loss-of function, raising the interesting possibility that agrin loss-of-function may be play a role in other ethanol-mediated developmental defects.

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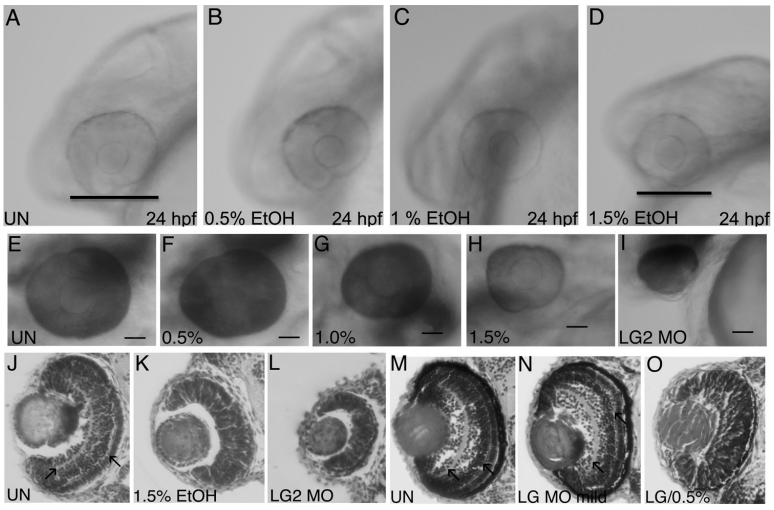

Figure 1. Acute ethanol exposure and agrin knockdown induce microphthalmia in zebrafish embryos

Zebrafish embryos were treated with ethanol beginning at 6 hpf, with microphthalmia and brain hypoplasia observed at 24 (A-D) and 48 hpf (E-I) when embryos are exposed to 1% or 1.5% ethanol. UN, untreated. The bars in A and D are indicative of eye diameter and illustrate differences in eye size in different treatments at 24 hpf. I, agrin MO (1 pmol) injection that induces severe agrin morphant phenotypes produces microphthalmia when compared to untreated embryos (E) at 48 hpf. J-O, assessment of retinal lamination by hematoxylin and eosin staining shows that retinal lamination observed in 72 hpf control embryos (J, M) is perturbed in embryos exposed to 1.5% ethanol (K) or injected with 1 pmol agrin MO (L). Retinal lamination appears normal in embryos injected with a low dose of agrin LG2 MO (0.1 pmol, N), and in embryos treated with 0.5% ethanol (data not shown). Retinal lamination is perturbed in 10/15 embryos injected with the subthreshold dose of agrin MO (0.1 pmol) combined with 0.5% ethanol exposure (O). Arrows in J, M and N indicate the inner and outer plexiform layers, which are not readily detectable in ethanol and agrin MO treated embryos. At least 25 embryos were analyzed for morphological analyses in A-I. At least 15 embryos were analyzed for morphological analysis of retinal lamination using H & E staining. Calibration bars in E-I are 50 μm.

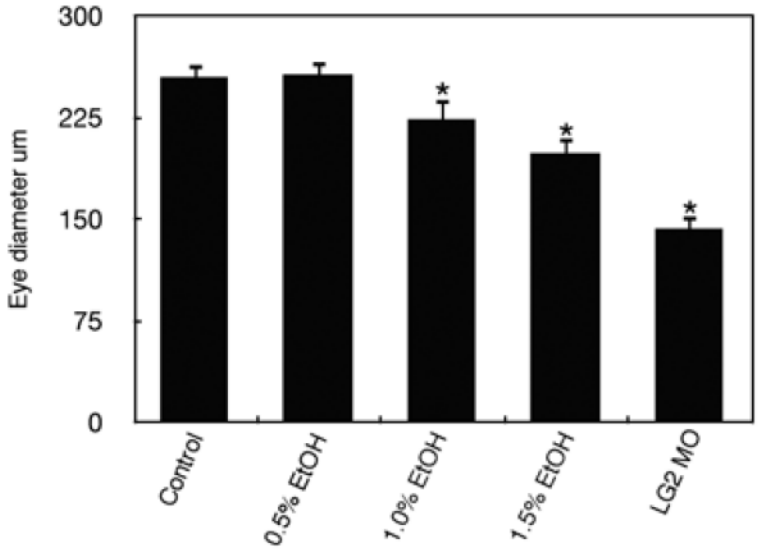

Figure 2. Eye diameter is reduced in ethanol exposed and agrin morphant embryos

Zebrafish embryos treated with ethanol or agrin MO as described in Figure 1, but in the absence of PTU, were analyzed at 48 hpf. Eye diameter was measured for 20 embryos for each treatment. Mean ± SEM is shown. * statistically significant compared to control (untreated) embryos at $P < 0.001$ using ANOVA.

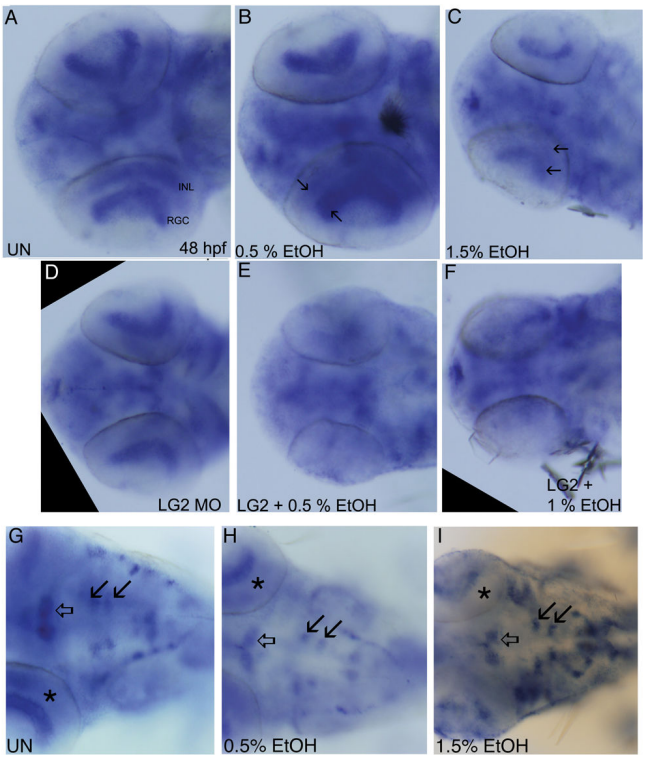

Figure 3. Agrin mRNA expression is diminished in retina of ethanol exposed zebrafish embryos

Agrin mRNA expression in 48 hpf zebrafish embryos was assessed by whole-mount ISH. Normal agrin mRNA expression is observed in embryos exposed to 0.5% ethanol (n= 17/17; arrows denote INL and GCL), when compared to untreated embryos (n=16/16). Pronounced microphthalmia combined with reduced agrin mRNA expression in INL and RGC layers of retina (arrows) is observed when zebrafish embryos are exposed to 1.5% ethanol from 6-24 hpf (C; n= 2/25 normal expression pattern). D-F, agrin mRNA expression in zebrafish embryos treated with subthreshold agrin MO (0.1 pmol) and ethanol. Subthreshold agrin MO, which produces a mild eye phenotype (D, n= 17/18 normal expression pattern), displays synergistic effects with low ethanol exposure (0.5%) to produce a severe microphthalmia and brain hypoplasia in zebrafish embryos (E). In addition, agrin gene expression is markedly reduced when subthreshold agrin MO and ethanol are combined and used to treat zebrafish embryos (E; n=1/17 normal expression pattern). F, subthreshold agrin MO plus 1% ethanol exposure (N=1/20 normal expression pattern). G-I, agrin gene expression patterns and levels do not appear to be as sensitive to ethanol exposure in hindbrain. The open arrows and solid arrows denote hindbrain nuclei that do not exhibit down-regulation of agrin mRNA expression in response to ethanol exposure. * denotes eyes, which display diminished agrin gene expression in embryos exposed to 1.5% ethanol.

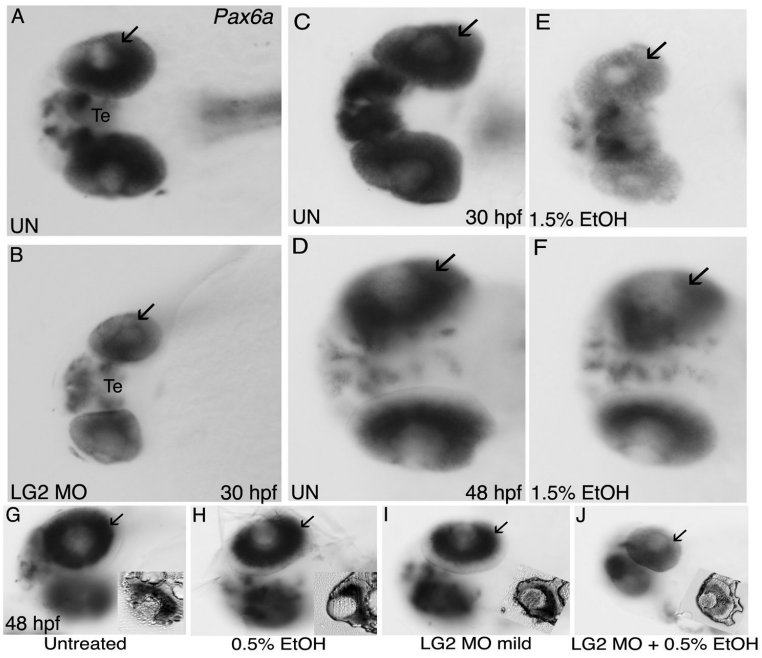

Figure 4. *Pax6a* mRNA expression is diminished in retina of agrin morphants and ethanol-exposed zebrafish embryos

A,B, *Pax6a* gene expression is markedly reduced in 30 hpf zebrafish retina following agrin MO treatment (1 pmol) that produces severe agrin morphant phenotypes (A, n=9/9; B, n=0/10)). Microphthalmia is also observed in these embryos. C-F, Zebrafish exposed to 1.5% ethanol from 6-24 hpf, and then allowed to recover until 30 or 48 hpf, exhibit reduced *Pax6a* gene expression (E, n=1/10; F, n=2/22) when compared to untreated embryos (C, n=9/9; D, n= 13/13). Te, optic tectum. G-J**,** *Pax6a* mRNA expression in zebrafish embryos treated with subthreshold agrin MO (0.1 pmol) and ethanol. Subthreshold agrin MO, which produces a mild eye phenotype, displays synergistic effects with subthreshold ethanol exposure (0.5%) to disrupt *Pax6a* mRNA expression as analyzed by whole-mount ISH in 48 hpf embryos and in cryostat sections of 48 hpf zebrafish eyes (insets). G, untreated, n=13/13; H, 0.5% ethanol treatment only from 6-24 hpf, n=15/15; I, subthreshold agrin MO producing a mild ocular phenotype, n=16/17; J, subthreshold agrin MO plus 0.5% ethanol exposure; n=4/21 normal expression pattern. Both whole-mount embryos (arrows denote eye) and cryostat sections (inset) demonstrate markedly less *Pax6a* expression in retina as a result of combined agrin MO and ethanol exposure.

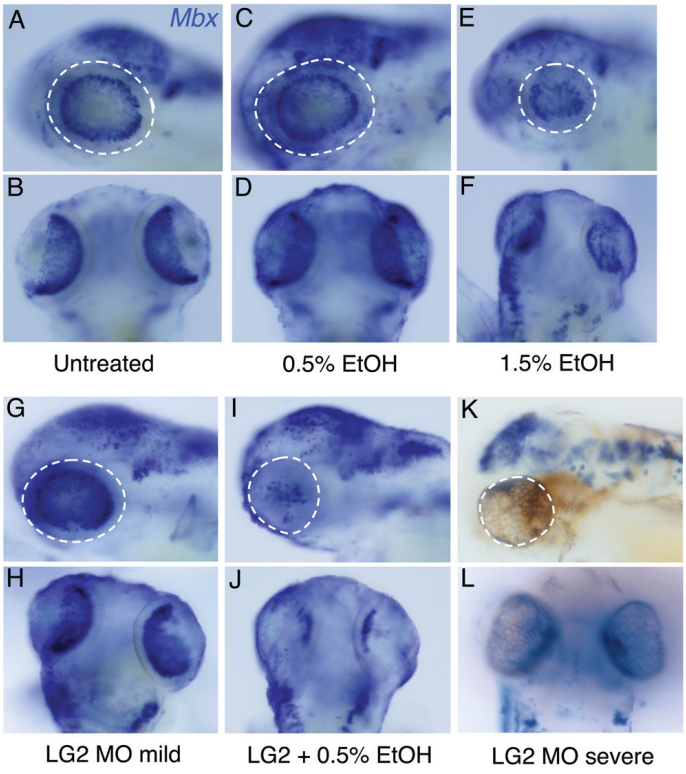

Figure 5. *Mbx* mRNA expression in zebrafish embryos treated with subthreshold agrin MO and ethanol

Subthreshold agrin MO, which produces a mild eye phenotype, displays synergistic effects with subthreshold ethanol exposure (0.5%) to perturb *Mbx* mRNA expression as analyzed by ISH in 72 hpf embryos. A,C,E and G,I,K show lateral views of *Mbx* expression in eye. B,D,F and H,J,L show dorsal views of *Mbx* gene expression in eye. The dashed lines illustrate the boundaries of eyes, associated microphthalmia, and pattern of *Mbx* expression in lateral views of embryos. The extent of *Mbx* expression in dorsal view, which appears in a crescent pattern, is clearly perturbed in embryos treated with both agrin MO and ethanol. A,B, untreated, n=16/16; C,D, 0.5% ethanol from 6-24 hpf, n=16/16; E,F, 1.5% ethanol from 6-24 hpf, n=3/24 normal expression pattern; G,H, subthreshold (0.1 pmol) agrin MO, n=19/21 normal expression pattern; I,J, subthreshold agrin MO plus 0.5% ethanol, n=2/31 normal expression pattern; K, L, For severe agrin MO phenotype, agrin MO (1.0 pmol) that produces pronounced ocular defects was injected in one-stage embryos (n= 0/9 normal expression pattern for severe morphants). It can be seen that subthreshold agrin MO combined with 0.5% ethanol produces ocular defects and perturbed *Mbx* gene expression that is observed with both 1.5% ethanol and severe agrin MO phenotypes.

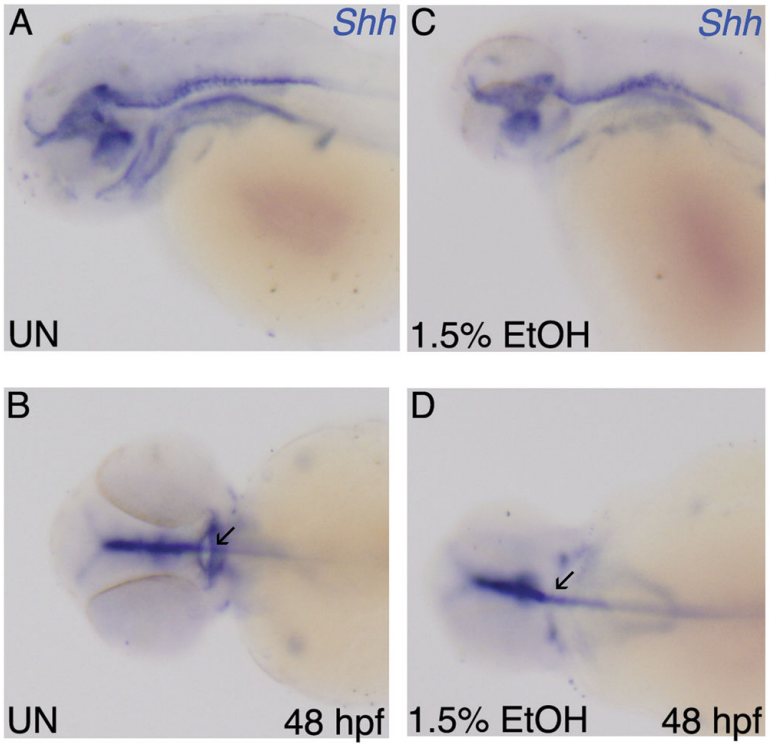

Figure 6. *Shh* mRNA expression in response to ethanol exposure in zebrafish embryos

48 hpf embryos were analyzed for *Shh* expression using ISH. A,B, untreated; C,D, 1.5% ethanol exposed embryos. It can be seen that the pattern and level of ventral midline expression of *Shh* appears largely normal as a result of ethanol treatment that produces ocular defects and brain hypoplasia, except as seen in lateral views (compare A and C) the level of Shh expression appears to be reduced and altered in its pattern. The arrows in C and D indicate *Shh* expression that appears disrupted in the region of the midbrain-hindbrain boundary in ethanol-exposed embryos.

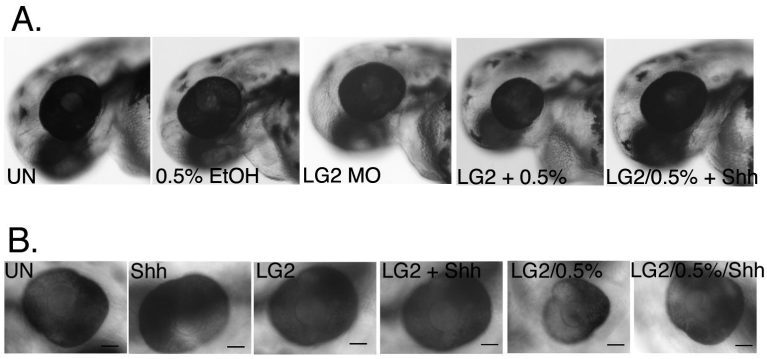

Figure 7. Role of Shh signaling in agrin- and ethanol-induced ocular defects

A, Subthreshold (0.1 pmol) agrin MO, which produces a mild eye phenotype, displays synergistic effects with subthreshold ethanol exposure (0.5%) to produce microphthalmia in 59/85 (69.4%) embryos at 48 hpf, that is typical of the ocular phenotype produced by 1.5% ethanol treatment. Co-injection of subthreshold agrin MO with 20 pg Shh mRNA, followed by 0.5% ethanol treatment from 6-24 hpf, only produces microphthalmia in 16/89 (18.0%) embryos when analyzed at 48 hpf. B, Effects of Shh, agrin MO and ethanol treatment on ocular development, specifically eye diameter. It can be seen that only combined agrin MO and ethanol exposure reduce eye diameter, with this phenotype rescued by Shh overexpression. Note that embryos depicted in A and B were not treated with PTU in these morphological analyses, but similar phenotypes are observed when compared to embryos treated with ethanol and agrin MO in the presence of PTU.

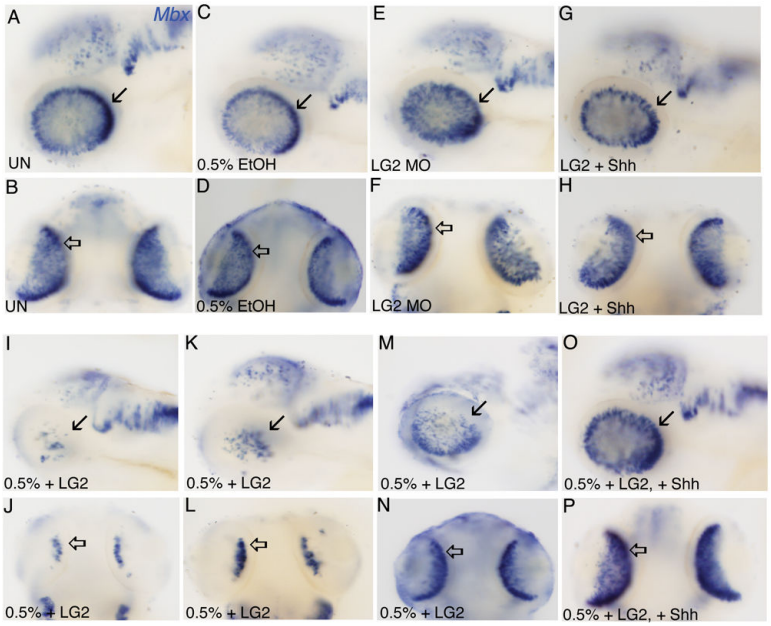

Figure 8. *Mbx* mRNA expression in zebrafish embryos treated with subthreshold agrin MO and ethanol and rescued with *Shh* overexpression

Zebrafish embryos were analyzed for *Mbx* gene expression as described in Figure 5. Embryos subjected to combinatorial agrin MO and ethanol treatments were allowed to develop to 72 hpf with or without Shh mRNA overexpression following Shh mRNA injection of one-cell stage embryos. A-F, *Mbx* gene expression in retina appears normal in low dose ethanol (0.5%, n= 10/10 normal expression pattern) or subthreshold (0.1 pmol) agrin MO embryos (n=14/18 normal expression pattern) in both lateral (arrows) and dorsal (open arrows) views. G,H, retinal *Mbx* gene expression after injection with subthreshold (0.1 pmol) agrin MO plus Shh mRNA (n=14/19). I-N, Combined 0.5% ethanol exposure and subthreshold agrin MO injection produces a range of *Mbx* expression in eyes, which is clearly altered when compared to untreated embryos (n=3/17 normal expression pattern). O,P, Shh mRNA overexpression rescues the perturbed retinal *Mbx* expression pattern in ethanol/agrin MO-treated embryos (n= 16/22 rescued expression pattern). The effect of Shh overexpression alone on *Mbx* gene expression was also analyzed, with *Mbx* expression appearing normal in embryos injected with Shh mRNA only (data not shown).

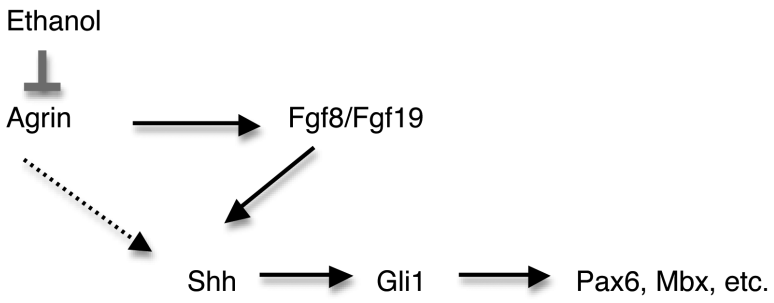

Figure 9. Model for proposed role of agrin function, and ethanol exposure, in ocular development

Schematic model depicting possible mechanism for the effect of ethanol on agrin expression and ocular development. Previous studies from our laboratory have shown that Fgf8 bead implantation rescues agrin loss-of-function eye defects (Liu et al, 2008), while other studies indicate Fgf signaling acts upstream of Shh in ocular development (Vinothkumar et al, 2008) and Shh overexpression rescues ocular phenotypes in zebrafish (Loucks and Ahlgren 2009). Ethanol exposure may therefore lead to perturbed agrin gene expression and hence agrin function in zebrafish eye, leading to diminished Fgf and Shh signaling, perturbed expression of Shh-dependent genes, ultimately resulting in ocular defects (i.e., microphthalmia). The dashed line from agrin to Shh suggests agrin may directly regulate Shh function as a result of binding to Shh.