# COMPARISON OF MOLECULAR MARKER EXPRESSION IN EARLY ZEBRAFISH BRAIN DEVELOPMENT FOLLOWING CHRONIC ETHANOL OR MORPHOLINO TREATMENT

**Chengjin Zhang**[1], **Oswald Boa-Amponsem**[1,2], and **Gregory J. Cole**[1,3,*]

[1]Julius L. Chambers Biomedical/Biotechnology Research Institute, North Carolina Central University, Durham, NC 27707

[2]Integrated Biosciences Program, North Carolina Central University, Durham, NC 27707

[3]Department of Biological and Biomedical Sciences, North Carolina Central University, Durham, NC 27707

## Abstract

This study was undertaken to ascertain whether defined markers of early zebrafish brain development are affected by chronic ethanol exposure or morpholino knockdown of agrin, sonic hedgehog, retinoic acid and fibroblast growth factors, four signaling molecules that are suggested to be ethanol sensitive. Zebrafish embryos were exposed to 2% ethanol from 6–24 hpf or injected with *agrin*, *shha*, *aldh1a3* or *fgf8a* morpholinos. *In situ* hybridization was employed to analyze *otx2*, *pax6a*, *epha4a*, *krx20*, *pax2a*, *fgf8a*, *wnt1* and *eng2b* expression during early brain development. Our results showed that *pax6a* mRNA expression was decreased in eye, forebrain and hindbrain of both chronic ethanol exposed and select MO treatments. *Epha4a* expression in rhombomere R1 boundary was decreased in chronic ethanol exposure and *aldh1a3* morphants, lost in *fgf8a* morphants, but largely unaffected in *agrin* and *shha* morphants. Ectopic *pax6a* and *epha4a* expression in midbrain was only found in *fgf8a* morphants. These results suggest that while chronic ethanol induces obvious morphological change in brain architecture, many molecular markers of these brain structures are relatively unaffected by ethanol exposure.

### Keywords

zebrafish; fgf; sonic hedgehog; ethanol; retinoic acid; FASD

## Introduction

Alcohol is a teratogen affecting central nervous system (CNS) development and results in congenital abnormalities of the eyes (Chan et al., 1991; Dangata et al., 1997; Stromland, 1985; Stromland et al., 1994), forebrain (Clarren et al., 1978; Mattson et al., 1996) and

*To whom correspondence should be addressed: Dr. Gregory J. Cole, Department of Biological and Biomedical Sciences, North Carolina Central University, Durham, NC 27707, T: 919.530.6034, F: 919.530.7773, gcole@nccu.edu.

hindbrain (Cragg et al., 1985; Goodlett et al., 1990). Understanding the molecular mechanisms that lead to fetal alcohol spectrum disorders (FASD) is a critical emphasis of FASD research. A wealth of evidence indicates that at least some of the effects of ethanol on CNS development may occur via disruption of key extracellular signaling proteins, in particular pathways involving sonic hedgehog (Shh) and fibroblast growth factors (fgfs). Both fgf2 and fgf8 function is affected by ethanol exposure in prenatal mice (Aoto et al., 2008; Hard et al., 2005; Rubert et al., 2006) and in zebrafish fgf signaling is disrupted by ethanol (Zhang et al., 2013). Shh signaling is another critical target of fetal ethanol exposure (Ahlgren et al., 2002; Aoto et al., 2008; Arenzana et al., 2006; Li et al., 2007; Loucks et al., 2009; Zhang et al., 2013), and *shh* mRNA overexpression in zebrafish rescues many phenotypes induced by ethanol exposure (Loucks et al., 2009; Zhang et al., 2013; 2011).

Agrin is a cell surface and extracellular matrix (ECM) heparan sulfate proteoglycan (HSPG) (Tsen et al., 1995) that regulates zebrafish ocular development via a fgf-dependent mechanism (Liu et al., 2008). We have shown previously that ethanol-mediated disruption of ocular development results from perturbed agrin function (Zhang et al., 2011), and ethanol-mediated disruption of forebrain and cerebellar GABAergic neuron development occurs via a molecular pathway involving Shh and fgfs, which is modulated by agrin (Zhang et al., 2013).

An additional molecular target of prenatal ethanol exposure is retinoic acid (RA) (Duester et al., 1991; Kot-Leibovich et al., 2009; Kumar et al., 2010; Leo et al., 1999; Pillarkat et al., 1991; Sulik et al., 1981; Yelin et al., 2007; 2005; Zachman et al., 1998). Morpholino (MO) knockdown of *aldh1a3*, an RA biosynthetic enzyme, leads to ocular phenotypes that are similar to ethanol exposure and fgf or shh loss of function (Yahyavi et al., 2013). Our recent work has also shown that *aldh1a3* knockdown induces disruption of zebrafish midbrain-hindbrain boundary (MHB) development as observed with ethanol exposure (Zhang et al., 2015).

A complex interplay among multiple extracellular signaling molecules, that are disrupted by prenatal ethanol exposure, likely underlies the hallmark morphological features of FASD (Zhang et al., 2015; 2013; 2011). Our studies have demonstrated synergistic effects of ethanol exposure and *agrin*, *shh*, or *aldh1a3* MO knockdown. While there are no obvious phenotypes observed in low ethanol exposure or low MO alone, more severe phenotypes occur with combined ethanol and *agrin*, *shh*, or *aldh1a3* MO knockdown. These data strongly suggest that ethanol exposure may disrupt *agrin*, *shh*, or *aldh1a3* signaling.

While pronounced alterations in brain architecture result from prenatal ethanol exposure, the molecules responsible for the manifestation of these morphological alterations in brain structure are poorly understood. To further investigate the molecular mechanisms underlying these key features of FASD, we decided to compare the effects of MO treatment to ethanol exposure, as assessed by brain morphology and expression of known markers of zebrafish brain architecture. Employing either chronic ethanol exposure or MOs to *agrin*, *Shha*, *aldh1a3* or *fgf8a* (signaling molecules that have been shown previously by numerous laboratories to be ethanol sensitive; Aoto et al, 2008; Loucks and Ahlgren 2009; Zhang et al, 2011; Zhang et al, 2015) we analyzed the expression of well characterized markers of

zebrafish eye, forebrain, midbrain, MHB or hindbrain development. Our goal was to ascertain whether ethanol and these targeted MOs would produce common or different molecular changes, thereby providing insight into molecular processes sensitive to ethanol that may lead to the dysmorphogenesis observed in FASD. Our studies showed that *fgf8a* MO knockdown had the most marked effect on expression of these markers, which ranged from ectopic expression (*pax6a*, *epha4a*), to loss of expression in specific brain regions (*wnt1*, *eng2b*, *pax2a*). Chronic ethanol exposure surprisingly did not alter the expression of most of these CNS markers, with only down-regulated *pax6a* expression in eye, forebrain, hindbrain and decreased *epha4a* expression in R1 boundary being altered by ethanol. While *agrin* and *shh* MOs primarily altered expression of *pax6a*, *aldh1a3* MO produced more marked alterations in the expression of these markers, with decreased MHB *pax2a*, *fgf8a* and *wnt1*, decreased *pax6a*, and decreased R1 *epha4a*. Importantly, although both chronic ethanol exposure and specific MOs resulted in morphological disruption of the MHB, the MHB expression of these markers was largely unaffected by many of the MO and ethanol treatments. These data suggest that the morphological development of the MHB is independent of the expression of many known functional markers of this critical brain patterning structure, since ethanol and MO treatments that morphologically disrupt the MHB do not alter expression of well characterized MHB markers.

## Materials and methods

### Animals

Zebrafish (AB strain) were obtained from Zebrafish International Resource Center and housed in automated 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 2% ethanol in fish water from 6 to 24 hour post-fertilization (hpf). This duration and concentration of ethanol is not lethal to embryos with a majority of embryos surviving past the larval stage (Zhang et al., 2013), and even 48 h exposure to 2% ethanol in zebrafish embryos has been reported by other laboratories (Zamora et al., 2013). We have previously shown that 2% ethanol results in approximately 65 mM ethanol in zebrafish embryos (Zhang et al, 2013), with this chronic ethanol concentration required to induce FAS phenotypes in a majority of zebrafish embryos. Embryos were incubated in 100 mm plates with the freshly diluted 2% ethanol from 6 hpf, with 10 to 30 embryos per plate. At 24 hpf ethanol was removed and embryos were washed once with fresh fish water. Embryos were then collected and fixed in 4% paraformaldehyde in PBS.

### Antisense morpholino oligonucleotides (MOs) injection

Antisense MOs (Gene Tools, Philomath, OR) were designed against exon/intron splice sites for agrin (LG2:5′-CCTCTCCTTTAC GCTGTGAAGACAA-3′) (Kim et al., 2007; Zhang et al., 2011), shha (5′-CAGCACTCTCGTCAAAAGCCGCATT-3′) (Nasevicius et al., 2000; Zhang et al., 2013) and fgf8a (fgf8E2I2:5′-TAGGATGCTCTTACCATGAACGTCG-3′)

(Draper et al., 2001). MO to aldh1a3 was designed to target the start codon (TATAGTCCCGTTCTGTGCCATAGC) (Bill et al., 2009; Yahyavi et al., 2013). MOs were solubilized in water at a concentration of 0.5–1.5 mM. 1 nl of *agrin-LG2* MO, *shha* MO, *aldh1a3* MO or *fgf8a* MO was injected into one to two-cell stage embryos. Control MO at the same concentration and volume as that of *agrin* MO produced no detectable effects (Kim et al., 2007). *Shh* morphants display phenotypes characteristic of a *shh* mutation (Nasevicius et al., 2000). Control MO for *aldh1a3* does not produce any obvious defects and only wild-type human mRNA for *ALDH1A3*, but not mutant human mRNA for *ALDH1A3*/ p.Lys190*, rescue the morphant phenotype (Yahyavi et al., 2013). *Fgf8a* MO injected embryos phenocopy *fgf8* mutants (Draper et al., 2001). All of the MOs used have been well characterized and validated in previous publications with regard to specificity and absence of off-target effects (Nasevicius et al., 2000; Draper et al., 2001; Kim et al., 2007; Yahyavi et al., 2013).

### Midbrain hindbrain boundary (MHB)

Malformation of the MHB was assessed visually based on absence of the defined border between the midbrain and hindbrain at 24 hpf. The presence of the MHB was defined as the presence of 3 or 4 ridges (tectal and cerebellar boundaries) perpendicular to the anterior-posterior axis of the CNS at the midbrain–hindbrain junction. Absence of this defined border was scored as a disruption of MHB development.

### 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). Plasmids to synthesize anti-sense digoxygenin-labeled riboprobes of *otx2*, *epha4a*, *pax2a*, *fgf8a*, *wnt1*, *eng2b*, and *krx20* were generous gifts from Yun-Jin Jiang. A plasmid that synthesizes *pax6a* anti-sense RNA probe was a generous gift from Ju-ahng Lee. Embryos were cleared in 50% glycerol and then viewed using an Olympus MVX-10 stereomicroscope.

### Statistical analysis

Each treatment has been repeated at least three times, and combined number of embryos from the three biological replicates was used to calculate ratios of embryos showing effects in response to ethanol exposure and MO injection. Differences in percent of embryos displaying changed marker expression as a result of treatments were analyzed for statistical significance using one-way Anova.

## Results

### Effects of ethanol and perturbation of agrin, Shh, RA and fgf signaling on forebrain, midbrain and eye development

O*tx2* was used as a forebrain and midbrain marker for analysis of mRNA expression. It marks the caudal limit of the developing mesencephalon (Millet et al., 1996) with expression ending at the caudal end of the mesencephalon (Figure 1A–B). We detected no obvious change in *otx2* expression in all MO treated embryos, as well as ethanol exposed embryos

(Figure 1C–J), an exception being caudally expanded expression in 39/48 of *fgf8a* morphants (Figure 1K–L; Table 1).

We used *pax6a* expression as a marker of eye and forebrain development, and observed decreased expression in 37/45 of embryos exposed to chronic 6–24 hpf 2% ethanol (Figure 2B; Table 1, Table S1), 42/53 of *agrin* MO-injected embryos (Figure 2C; Table 1, Table S1), 32/48 of *shh* MO-injected embryos (Figure 2D; Table 1, Table S1), 41/46 of *aldh1a3* MO-injected embryos (Figure 2E; Table 1, Table S1) and 51/56 of *fgf8a* MO-injected embryos (Figure 2F; Table 1, Table S1). Interestingly, ectopic *pax6a* expression in midbrain was found only in *fgf8a* morphants (51/56 embryos) (Figure 2F; Table 1, Table S1).

*Epha4a* was another forebrain and midbrain marker employed, with no obvious change in forebrain and anterior midbrain expression observed in all treatments (Figure 3C–L). Expanded *epha4a* expression in posterior ventral midbrain was only detected in 36/42 *fgf8a* morphants (Table 1, Table S1), with this expanded expression extending to the R1 boundary region of hindbrain (Figure 3K–L).

### Effects of ethanol and perturbation of agrin, Shh, RA and fgf signaling on hindbrain development

We were interested in determining whether ethanol exposure or perturbation of agrin, Shh, RA and fgf signaling would affect expression of hindbrain markers. *Pax6a* is normally expressed in the entire hindbrain including R1-6 (Figure 2A),and *pax6a* expression levels were decreased hindbrain of all treatments (Figure 2B–F; Table 1). *Epha4a* was expressed in rhombomere boundaries R1, R3 and R5 in WT embryos (Figure 3A–B). However, decreased expression in R1 boundary was detected in 57/71 of chronic ethanol exposed embryos (Figure 3C–D; Table 1, Table S1) and 27/45 of *aldh1a3* morphants (Figure 3I–J; Table 1, Table S1). Most *agrin* and *shh* morphants displayed relatively normal R1 expression (Figure 3E–H; Table 1), with only 4/51 and 6/42 of embryos, respectively, showing noticeably decreased R1 boundary expression. The R1 boundary expression was also lost in 36/42 *fgf8a* morphants (Figure 3K–L; Table 1, Table S1). *Krx20* serves as a hindbrain marker, being normally expressed in R3 and R5 (Figure 4A). We observed no obvious change of the boundary expression of *krx20* in R3 and R5 in all treatment groups (Figure 4B–4F; Table 1). However, it can be seen that the pattern of *krx20* R3 and R5 expression in all treatment groups was altered from control embryos, likely a result of delayed development resulting from the treatments, that produced a change in R3 and R5 morphology.

### Analysis of MHB morphological development

We have shown previously that chronic 6–24 hpf exposure of embryos to 2% ethanol (Zhang et al., 2015) and *agrin* MO (Kim et al., 2007; Liu et al., 2008) disrupted MHB formation. As shown here, *Shh* MO (Figure 5B) and *fgf8a* MO treatment perturbed MHB formation, both at the ratio of 30/30 (Figure 5C). To determine if molecular markers of the MHB were also affected by ethanol or MO treatments, we selected several MHB markers including *pax2a* (anterior MHB), *fgf8a*, *wnt1* (dorsal median midbrain, MHB and dorsal lateral hindbrain), and *eng2b* (MHB and posterior midbrain) (Thisse et al., 2001; 2004; 2005). Interestingly we observed that although these treated embryos had a similar high ratio of a morphologically

disrupted MHB, the molecular changes associated with this altered MHB were different. We detected no obvious change in MHB *pax2a* expression in chronic ethanol exposed (Figure 5E), *agrin* (Figure 5F) or *shh* morphants (Figure 5G). We identified two groups of *aldh1a3* morphants based on *pax2a* expression, Group I showed relatively normal MHB expression (Figure 5H) and Group II (20/57; Table 1) had almost complete loss of MHB *pax2a* expression (Figure 5I). All *fgf8a* morphants showed almost complete loss of MHB *pax2a* expression (Figure 5J; Table 1).

*Fgf8* expression in MHB was relatively normal in ethanol exposed embryos (Figure 6B), *agrin* morphants (Figure 6C) and *shh* morphants (Figure 6D). *Aldh1a3* morphants could again be divided into two groups based in this case on variable *fgf8a* expression: Group I had relatively normal MHB expression of *fgf8a* (Figure 6E) and Group II (19/68 embryos) had markedly decreased MHB *fgf8a* expression (Figure 6F; Table 1). We observed a similar pattern of marker expression when using *wnt1* as a MHB marker with normal dorsal median midbrain, MHB and dorsal lateral hindbrain expression in ethanol exposed embryos (Figure 7C–D), *agrin* morphants (Figure 7E–F) and *shh* morphants (Figure 7G–H). While *Wnt1* expression in dorsal median midbrain and dorsal lateral hindbrain was similar in *aldh1a3*, *fgf8a* morphants and WT embryos (Figure 7I–N), its MHB expression was almost completely lost in Group II *aldh1a3* morphants (Figure 7K–L; Table 1; 10/53) and lost in all *fgf8a* morphants (Figure 7M–N; Table 1). Using *eng2b* as marker for MHB and posterior midbrain, only *fgf8a* morphants (45/45 embryos) showed loss of expression in both MHB and posterior midbrain (Figure 8K–L; Table 1).

## Discussion

Zebrafish embryos exposed to ethanol display the hallmark craniofacial defects of FASD (McCarthy et al., 2013; Swartz et al., 2014), including microphthalmia, which are dependent on the dosage and timing of ethanol exposure (Ali et al., 2011; Zhang et al., 2014). Many of the hallmark phenotypes of FASD are also produced with disruption of gene function in zebrafish (Eberhart et al., 2016). Relevant to these studies, our previous work showed *agrin* morphants displayed similar ocular defects to those induced by ethanol exposure, suggesting agrin as a target of prenatal ethanol exposure (Zhang et al., 2011). Disruption of Shh, fgf and RA signaling also produced FASD phenotypes such as microphthalmia and abnormal MHB (Duester et al., 1991; Kot-Leibovich et al., 2009; Kumar et al., 2010; Leo et al., 1999; Marrs et al., 2010; Pillarkat et al., 1991; Sulik et al., 1981; Yelin et al., 2007; 2005; Zachman et al., 1998; Zhang et al., 2011). The ability to rescue FASD phenotypes with *shh* mRNA overexpression (Loucks et al., 2009; Zhang et al., 2011; 2013) or RA treatment (Muralidharan et al., 2015; Peng et al., 2004; Zhang et al., 2015) also provided support for these signaling pathways serving as molecular targets of ethanol. There also appears to be crosstalk between multiple molecular signaling pathways that are impacted by ethanol, since Shh, fgf and RA rescued ethanol induced phenotypes in zebrafish, and even overexpression of Shh or fgf8/fgf19 rescued FASD phenotypes induced by combined ethanol and agrin treatment (Zhang et al., 2011; 2013).

To further understand possible molecular targets of ethanol exposure that manifest in FASD, we chose to analyze several markers expressed in brain regions known to be affected by

ethanol exposure: eye, forebrain, midbrain, MHB (isthmus in rodent) and hindbrain. The genes selected were well-characterized markers of zebrafish brain development, and the main objective of the present study was to compare the expression of these established markers as a result of ethanol exposure to gene knockdown, using MOs to genes that have been suggested to be ethanol sensitive (*agrin*, *Shha*, *aldh1a3* and *fgf8a*) and thus potentially responsible for the brain dysmorphologies observed in FASD. Surprisingly, chronic ethanol exposure that induced FAS phenotypes in 100% of embryos only had modest effects on expression of these CNS markers. Most of the markers analyzed exhibited no change in expression following chronic ethanol exposure, and included well-characterized genes such as *fgf8*, *krox20*, *wnt1*, *eng2b*, and *otx2*. Ethanol exposure decreased ocular expression of *pax6a* as previously reported (Zhang et al., 2011; 2015), but also decreased *pax6a* expression in forebrain and hindbrain. These data are consistent with studies in Xenopus and mouse, that showed reduced *pax6* in these regions (Aronne et al., 2008; Peng et al., 2004). Ethanol also decreased *epha4a* expression in rhombomere R1 boundary, but not in other brain regions. As R1 gives rise to the cerebellum (Moens and Prince, 2002), which is known to be sensitive to prenatal ethanol exposure (Cragg et al., 1985; Goodlett et al., 1990; Zhang et al., 2013), this finding is of interest as it provides possible new insight into the mechanisms underlying ethanol disruption of cerebellar development. Interestingly, our laboratory has shown that chronic ethanol exposure disrupted zebrafish cerebellar development at early stages by a mechanism involving Shh and fgf signaling (Zhang et al., 2013), yet *shh* MO did not disrupt R1 boundary expression of *epha4a* while ethanol had this effect.

Although chronic ethanol exposure disrupted the morphological development of the MHB, well known markers of the MHB, such as *pax2a* and *fgf8a*, did not display altered expression levels. With regard to *pax2a*, our data confirm earlier studies that showed this MHB marker was not affected by early ethanol exposure (Coffey et al, 2013; Joya et al, 2014), although Coffey et al (2013) showed decreased *pax2*a expression following later exposure to ethanol. *Pax2a* MHB expression was only lost in a small subset of *aldh1a3* morphants and in *fgf8a* morphants in the present study. Similarly, *fgf8a* MHB expression was only markedly decreased in some *aldh1a3* morphants, but not in chronic ethanol embryos or *agrin* or *shh* morphants. These data are in contrast to mouse studies that found ethanol exposure to cause decreased *fgf8* expression in telencephalon (Aoto et al., 2008), although our analysis focused on MHB *fgf8a* expression. Not surprisingly, we found loss of *wnt1* MHB expression in all *fgf8a* morphants. The loss of MHB gene expression in *fgf8a* morphants was consistent with studies using the *fgf8 ace* mutant, where the initial molecular identity of the isthmic domain (MHB) was not maintained (Jászai et al., 2003). However, our current results clearly showed that the perturbation of morphological MHB formation did not disrupt expression of genes typically associated with the MHB. These data suggest that even in the absence of a defined MHB, genes that may be critical to midbrain and hindbrain patterning are still expressed, indicating that the cellular and functional integrity of the MHB was intact even in the absence of a well-defined morphological MHB. Adding to the complexity of this picture is our recent work showing that RA treatment rescued a morphologically abnormal MHB induced by combined *aldh1a3* MO and ethanol treatment, or *Shh* MO combined with ethanol treatment, but *Shh* mRNA overexpression could not rescue MHB phenotypes in *aldh1a3* MO combined with ethanol treatment (Zhang et al.,

2015). Taking into account the current analysis of expression of molecular markers of the MHB, these data suggest that RA signaling may act upstream of *pax2a* and *fgf8a*, and that ethanol may be acting downstream of these genes, thus accounting for an absence of an effect on their expression.

Numerous studies have shown that the MHB, or the isthmus organizer in rodent, is a critical patterning center in the CNS (Joyner et al., 2000; Pera et al., 2002), with isthmic fgf8 required for both tectum and cerebellum development (Chi et al., 2003). Both the strength and duration of fgf8 MHB expression are crucial for patterning different tectal-isthmo-cerebellar structures (Sato et al., 2009). It therefore was not surprising that perturbed MHB *fgf8a* expression resulted in loss of expression of *pax2a/wnt1/eng2* in MHB and ectopic expression of *pax6a/epha4a* in midbrain. We detected relatively normal *fgf8a* expression in MHB in all chronic ethanol exposed embryos, *agrin* and *shh* morphants and most *aldh1a3* morphants, despite the absence of a morphologically normal MHB. This was despite our previous studies that showed either *fgf8* or *fgf19* mRNA overexpression was capable of rescuing decreased *GAD1* and *Atonal1a* expression phenotypes in combined ethanol and *agrin* or *shh* MO treated embryos (Zhang et al., 2013). We also detected decreased *fgf19* expression in forebrain following chronic ethanol exposure and in *agrin* and *shh* morphants (Zhang et al., 2013). These data therefore suggest redundancy in *fgf* function, with *fgf19* having a more critical role in ethanol pathology, and manifestation of some features of FASD, than *fgf8*. Our data also suggest that morphological disruption of the MHB occurs while expression of MHB molecular markers is maintained in ethanol exposed embryos and *agrin* or *shh* morphants. The observation that the early markers of neural domains and MHB are not affected in ethanol-exposed embryos suggests that the morphologically disrupted MHB is a downstream effect that likely is not due to defects in the patterning of the neural primordium. However, the observed changes in *pax6a* and *epha4a* gene expression as a consequence of ethanol exposure suggest these genes may be important mediators of the effects of ethanol in the manifestation of FASD. When considered in the context of our previous studies that have suggested a role for *agrin*, *shh*, *fgf8/19* and RA in ethanol teratogenicity (Zhang et al, 2013; 2015), and our demonstration that genes such as *Gli1* and *fgf19* were reduced in expression, this body of work begins to shed more light on possible molecular pathways that are disrupted by ethanol exposure during embryogenesis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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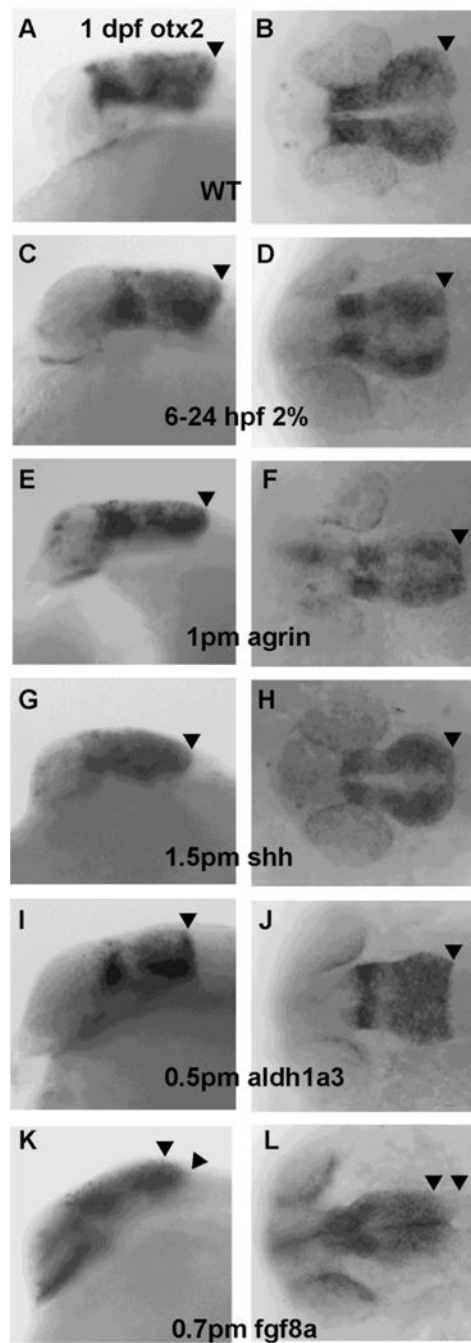
**Figure 1. Caudally expanded expression of *otx2* mRNA only occurs in *fgf8a* morphants**
A–B, wild-type embryos (WT); C–D, embryos exposed to 2% ethanol from 6–24 hpf; E–F, embryos injected at the one to two- cell stage with 1 pmol *agrin* MO; G–H, embryos injected with 1.5 pmol *shh* MO; I–J, embryos injected with 0.5 pmol *aldh1a3* MO; K–L, embryos injected with 0.7 pmol *fgf8a* MO. A,C,E,G,I, and K embryos are lateral views; B,D,F,H,J, and L embryos are dorsal views. 39/48 of *fgf8a* morphants have caudally expanded *otx2* expression which was not observed with the other treatments. The arrowhead (A–J) points to

the caudal expression limit of *otx2* at the MHB. The two arrowheads (K–L) mark the caudally expanded territory expression of *otx2* in *fgf8a* morphants.
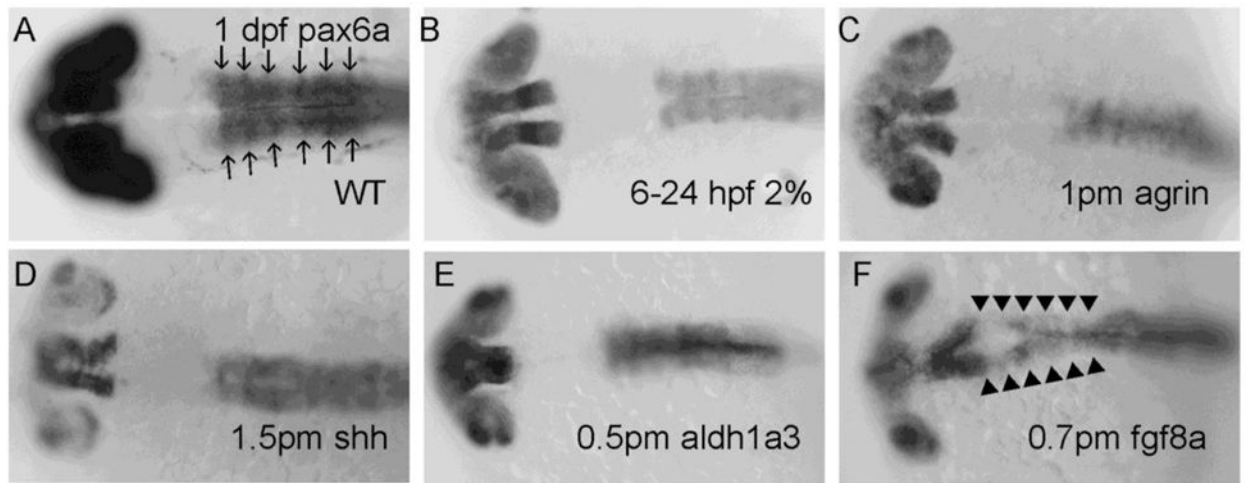
**Figure 2. Decreased *pax6a* mRNA expression in 1 dpf eye, forebrain and hindbrain following chronic ethanol exposure and different MOs treatments**

A, WT; B, embryo exposed to 2% ethanol from 6–24 hpf; C, embryo injected with 1 pmol *agrin* MO; D, embryo injected with 1.5 pmol *shh* MO; E, embryo injected with 0.5 pmol *aldh1a3* MO; F, embryo injected with 0.7 pmol *fgf8a* MO. All embryos are dorsal views. Noted that decreased *pax6a* expression was observed in B–F and ectopic expression in midbrain (indicated to by arrowheads) was only observed in *fgf8a* morphants (F). Arrows in (A) denote rhombomeres R1–R6.
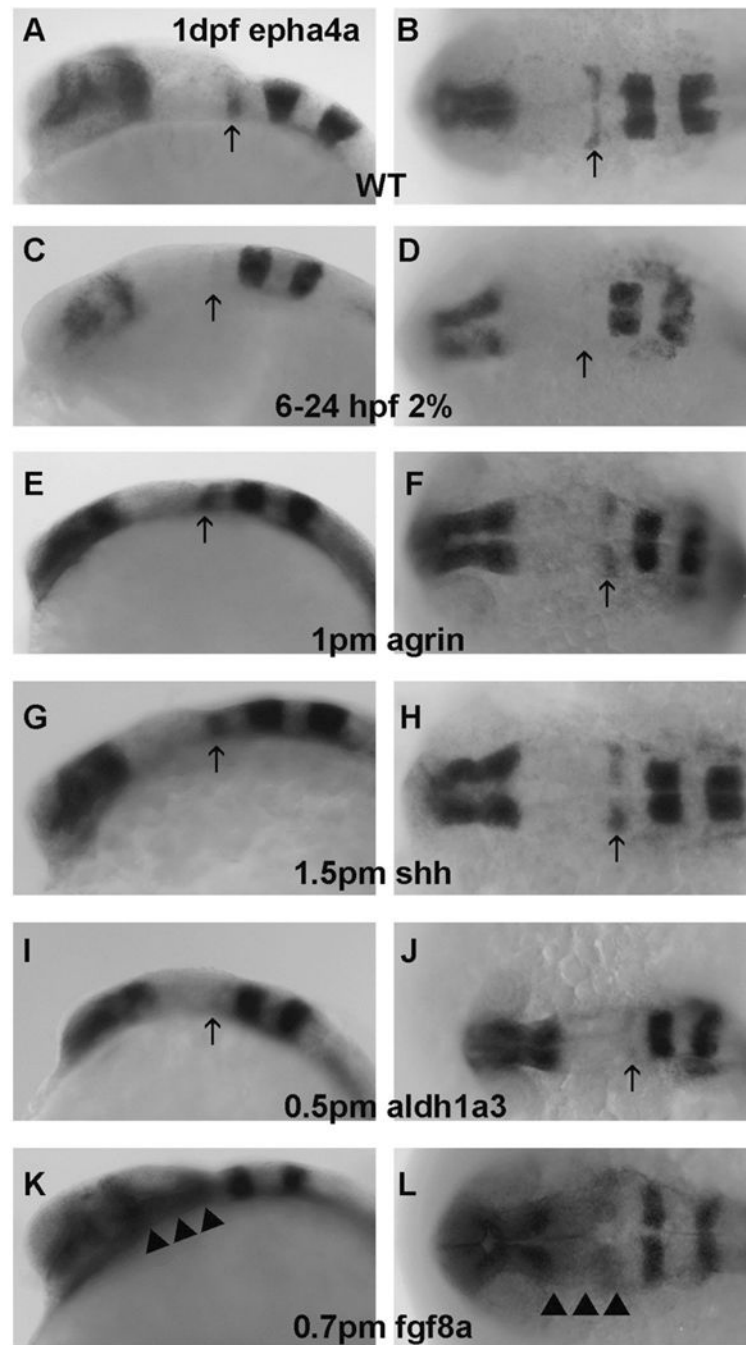
**Figure 3. *EphA4a* mRNA expression in R1 boundary is selectively decreased in embryos exposed to chronic ethanol treatment or *aldh1a3* MO**
A–B, WT; C–D, embryos exposed to 2% ethanol from 6–24 hpf; E–F, embryos injected with 1 pmol *agrin* MO; G–H, embryos injected with 1.5 pmol *shh* MO; I–J, embryos injected with 0.5 pmol *aldh1a3* MO; K–L, embryos injected with 0.7 pmol *fgf8a* MO. A,C,E,G,I, and K embryos are lateral views; B,D,F,H,J, and L embryos are dorsal views. Arrows indicate R1 boundary expression. Note that *ephA4a* expression in R1 decreased with ethanol exposure (C–D), *aldh1a3* MO(I–J) or *fgf8a* MO (K,L). Ectopic *epha4a* expression in posterior midbrain (indicated by arrowheads) was only found in *fgf8a* morphants (K–L).
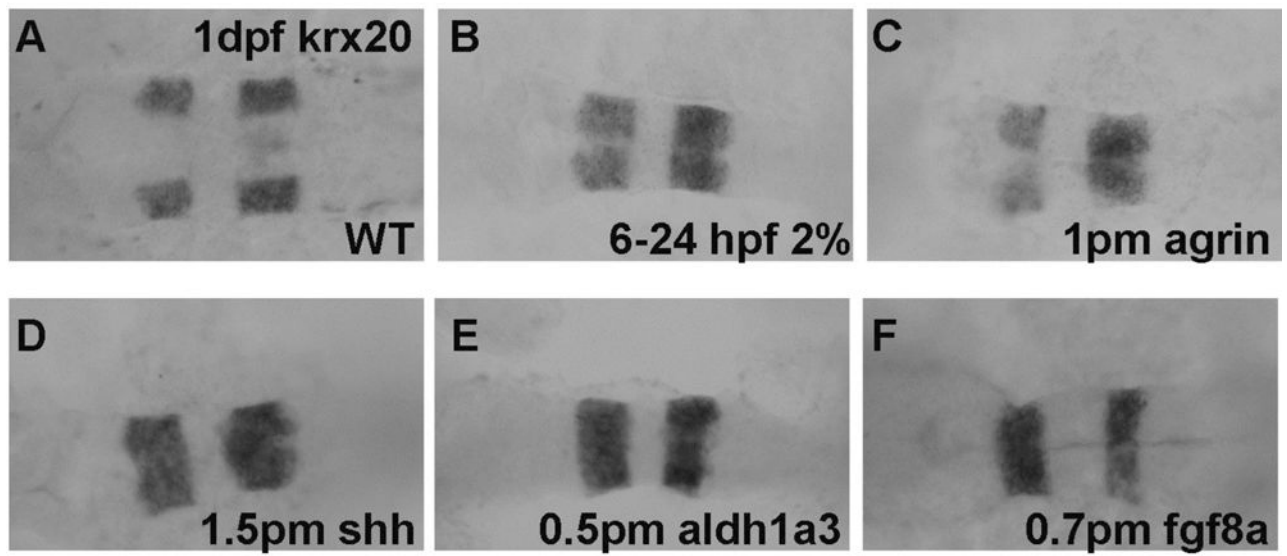
**Figure 4.** *Krx20* **mRNA expression is unchanged in R3 and R5 boundaries following ethanol or MO treatments**

A, WT; B, embryo exposed to 2% ethanol from 6–24 hpf; C, embryo injected with 1 pmol *agrin* MO; D, embryo injected with 1.5 pmol *shh* MO; E, embryo injected with 0.5 pmol *aldh1a3* MO F, embryo injected with 0.7 pmol *fgf8a* MO. All embryos are dorsal views.
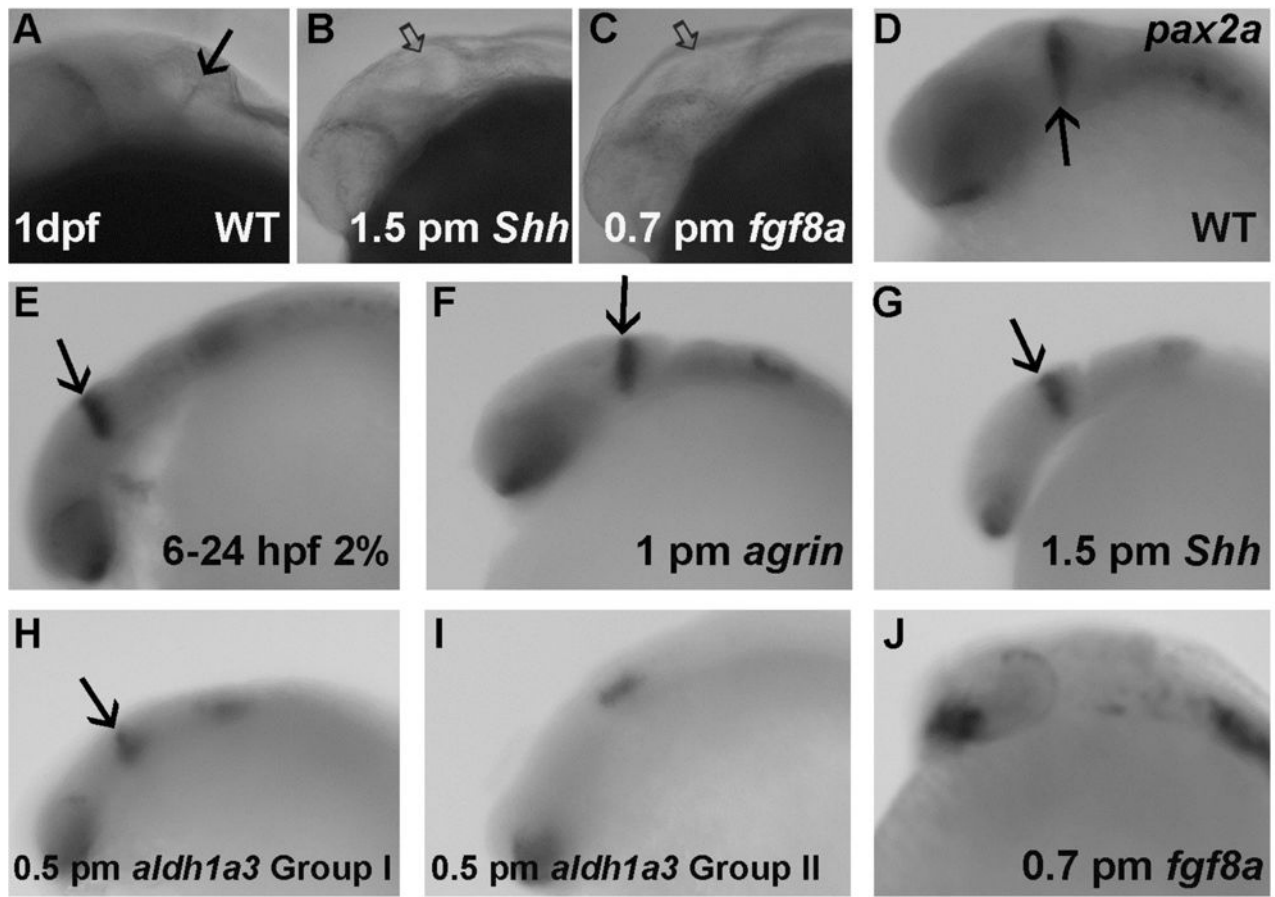
**Figure 5. Analysis of *pax2a* gene expression in ethanol and MO treated embryos**
A–C, MHB morphology following *shh* and *fgf8a* MO treatment; D–J, WISH of *pax2a* expression. D, WT; E, embryo exposed to 2% ethanol from 6–24 hpf; F, embryo exposed to 1 pmol *agrin* MO; G, embryo injected with 1.5 pmol *shh* MO; H–I, embryos injected with 0.5 pmol *aldh1a3* MO, either showing normal *pax2a* expression (H) or loss of MHB *pax2a* expression (I); J, embryo injected with 0.7 pmol *fgf8a* MO; All embryos are lateral views. Solid arrows indicate the morphologically normal MHB or *pax2a* expression in MHB. The open arrows indicate a morphologically abnormal MHB. Note that MHB morphology was disrupted in B–C and *pax2a* expression in MHB was lost in I and J.
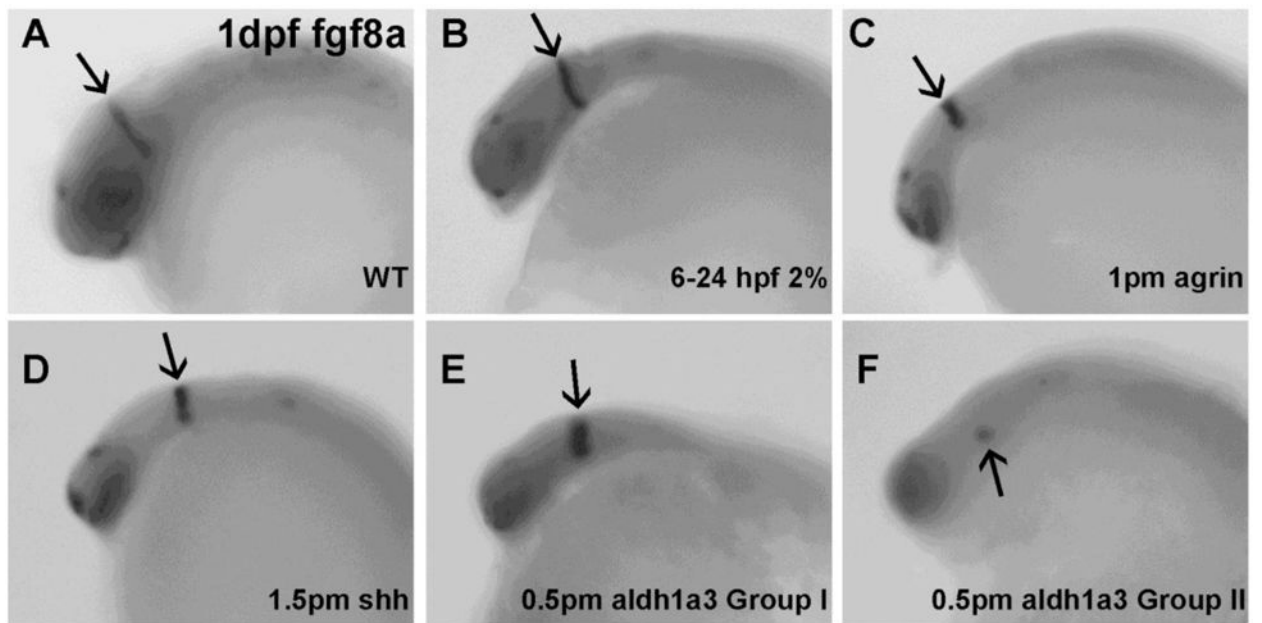
**Figure 6.**
**Decreased *fgf8a* mRNA expression in MHB is observed in *aldh1a3* morphant embryos.**
A, WT; B, embryo exposed to 2% ethanol from 6–24 hpf; C, embryo injected with 1 pmol *agrin* MO; D, embryo injected with 1.5 pmol *shh* MO; E–F, embryos injected with 0.5 pmol *aldh1a3* MO. All embryos are lateral views. Arrows indicate *fgf8a* expression in MHB. Noted that *fgf8a* expression in MHB was noticeably decreased in a subset of embryos injected with *aldh1a3* MO (F).
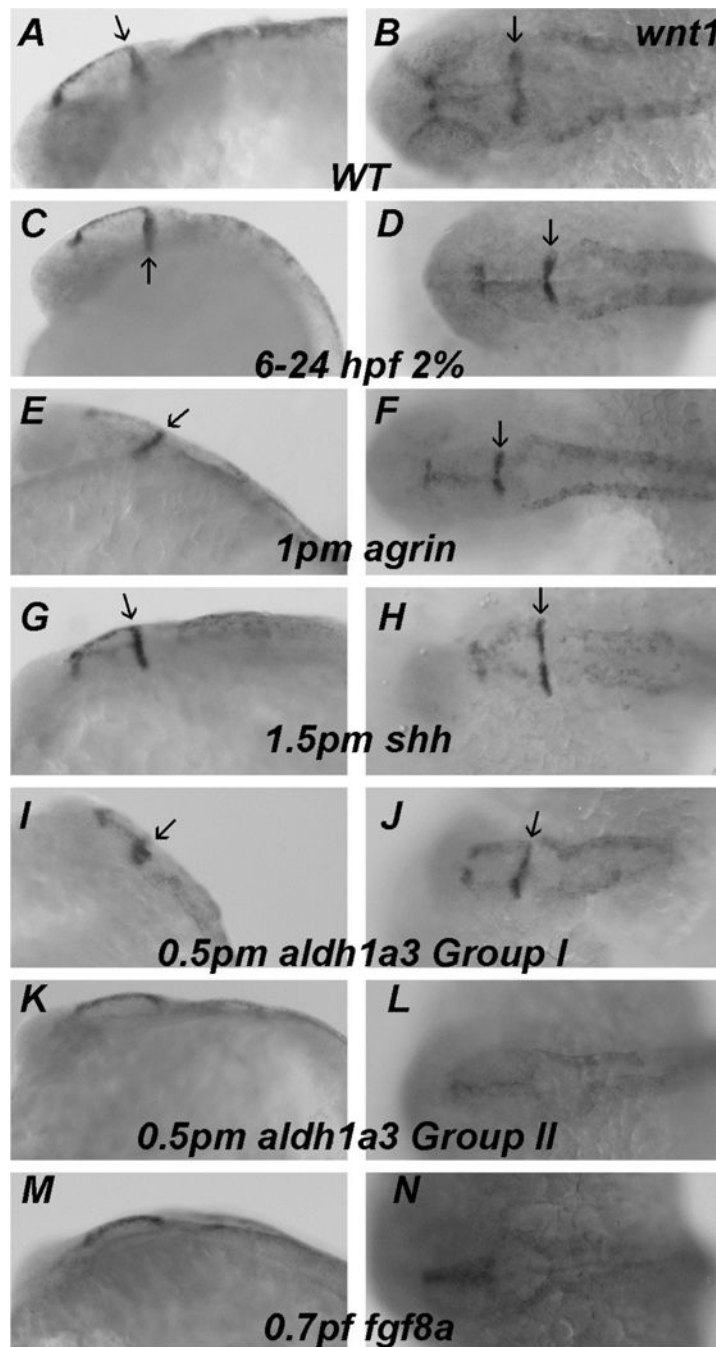
**Figure 7. Loss of *wnt1* expression in MHB occurs selectively in *aldh1a3* and *fgf8a* morphants**
A–B, WT; C–D, embryos exposed to 2% ethanol from 6–24 hpf; E–F, embryos injected with 1 pmol *agrin* MO; G–H, embryos injected with 1.5 pmol *shh* MO; I–J, embryos injected with 0.5 pmol *aldh1a3* MO that had relatively normal MHB expression; K-L, embryos injected with 0.5 pmol *aldh1a3* MO that lost MHB expression; M–N, embryos injected = with 0.7 pmol *fgf8a* MO. A,C,E,G,I,K and M embryos are lateral views; B,D,F,H,J,L and N embryos are dorsal views. Arrows indicate MHB expression of *wnt1*. Note that *wnt1*

expression in MHB is lost in a subset of embryos injected with *aldh1a3* MO (K,L) and in all embryos injected with *fgf8a* MO (M,N).
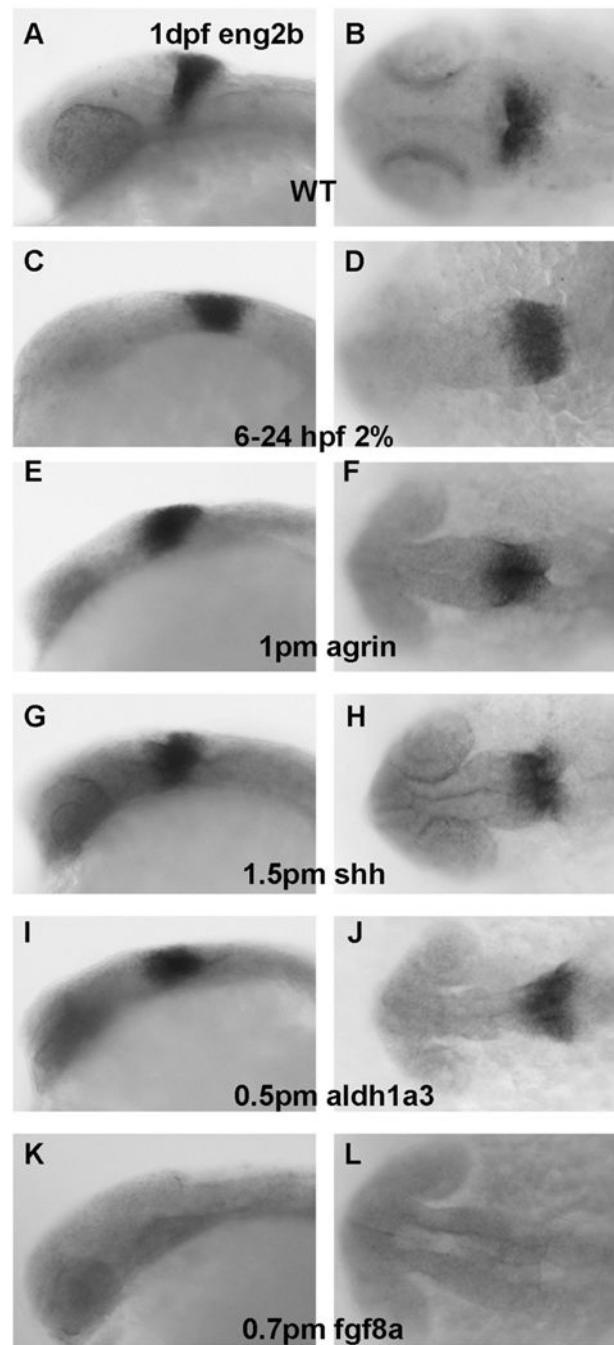
**Figure 8. E*ng2b* expression is unchanged with ethanol exposure and is selectively lost in MHB and posterior midbrain in *fgf8a* morphants**

A–B, WT; C–D, embryos exposed to 2% ethanol from 6–24 hpf; E–F, embryos injected e with 1 pmol *agrin* MO; G–H, embryos injected with 1.5 pmol *shh* MO; I–J, embryos injected with 0.5 pmol *aldh1a3* MO; K–L, embryos injected with 0.7 pmol *fgf8a* MO. A,C,E,G,I, and K embryos are lateral views; B,D,F,H,J, and L embryos are dorsal views. Note that *eng2b* expression in MHB and posterior midbrain is only lost with *fgf8a* MO treatment (K,L).

**Table 1**

Ratio of embryos that displayed altered marker expression in different treatments.

| Treatments | Ratio of embryos showing altered marker expression | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | *otx2* | *pax6a* | *epha4a* | *krx20* | *pax2a* | *fgf8a* | *wnt1* | *eng2b* |
| WT | 0/45 0±0% | 0/45 0±0% | 0/45 0±0% | 0/45 0±0% | 0/45 0±0% | 0/45 0±0% | 0/45 0±0% | 0/45 0±0% |
| 6–24 hpf 2% | 0/45 0±0% | 37/45 * 82±14% | 57/71 * 80±4% | 0/45 0±0% | 0/45 0±0% | 0/45 0±0% | 0/45 0±0% | 0/45 0±0% |
| 1 pm *agrin* | 0/45 0±0% | 42/53 * 79±1% | 4/51 7±6% | 0/45 0±0% | 0/45 0±0% | 0/45 0±0% | 0/45 0±0% | 0/45 0±0% |
| 1.5 pm *shh* | 0/45 0±0% | 32/48 * 67±10% | 6/42 * 14±7% | 0/45 0±0% | 0/45 0±0% | 0/45 0±0% | 0/45 0±0% | 0/45 0±0% |
| 0.5 pm *aldh1a3* | 0/45 0±0% | 41/46 * 89±10% | 27/45 * 60±12% | 0/45 0±0% | 20/57 * 36±9% | 19/68 * 26±12% | 10/53 * 18±5% | 0/45 0±0% |
| 0.7 pm *fgf8a* | 39/48 * 82±11% | 51/56 * 91±7% | 36/42 * 86±7% | 0/45 0±0% | 45/45 * 100±0% | N.A | 45/45 * 100±0% | 45/45 * 100±0% |

Each treatment was repeated three times, and the combined number of embryos was used to calculate ratios of embryos showing effects in response to ethanol exposure and MO injection.

*
indicates P<0.05 comparing the treated embryos to WT embryos.