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Effects of Ethanol on Photoreceptors and Visual Function in Developing Zebrafish

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Abstract

Purpose—Children born to mothers who have consumed alcohol during pregnancy have an array of retinal abnormalities and visual dysfunctions. In the past, rodent systems have been used to study the teratogenic effects of ethanol on vertebrate embryonic development. The exact developmental windows in which ethanol causes specific developmental defects have been difficult to determine because rodents and other mammals develop in utero. In this study, we characterized how ethanol affects the function and development of the visual system in an ex utero embryonic system, the zebrafish.

Methods—Zebrafish embryos were raised in fish water containing various concentrations of ethanol from 2 to 5 days after fertilization. The effects of ethanol on retinal morphology were assessed by histologic and immunohistochemical analyses and those on retinal function were analyzed by optokinetic response (OKR) and electroretinography (ERG).

Results—Zebrafish embryos exposed to moderate and high levels of ethanol during early embryonic development had morphological abnormalities of the eye characterized by hypoplasia of the optic nerve and inhibition of photoreceptor outer segment growth. Ethanol treatment also caused an increased visual threshold as measured by the OKR. Analysis with the ERG indicated that there was a severe reduction of both the a- and b-waves, suggesting that ethanol affects the function of the photoreceptors. Indeed, low levels of ethanol that did not cause obvious morphologic changes in either the body or retina did affect both the OKR visual threshold and the a- and b-wave amplitudes.

Conclusions—Ethanol affects photoreceptor function at low concentrations that do not disturb retinal morphology. Higher levels of ethanol inhibit photoreceptor development and cause hypoplasia of the optic nerve.

Some children born to mothers who have consumed alcohol during pregnancy have a number of morphologic, sensory, and cognitive abnormalities, including vision deficits, collectively known as fetal alcohol syndrome (FAS). It was originally thought that FAS was the result of alcohol abuse; however, smaller doses or shorter durations of prenatal alcohol consumption also produce harmful, though more subtle, effects referred to as alcohol-related birth defects (ARBDs) or alcohol-related neurodevelopment disorder (ARND).¹ Even though FAS was described several decades ago,² little is known about the mechanistic underpinnings of ethanol teratogenicity.³

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The retina is one of the organs affected by ethanol during embryogenesis. As many as 90% of children in whom FAS is diagnosed have some type of ocular problem, ranging from microphthalmia and retinal dysmorphologies to reduced visual function.^{4,5} In rats, ethanol exposure during embryogenesis has been linked to optic nerve hypoplasia.^{6,7} In trying to understand the effects of alcohol on visual development, Katz and Fox⁸ analyzed the visual function of rat pups born to mothers exposed to ethanol during pregnancy. The rat pups exhibited deficiencies in both photopic and scotopic vision and had lower rhodopsin levels than non-ethanol-treated rat pups. These results suggested that ethanol's effect on the development of visual function in vertebrates could alter the expression of genes regulating the development of the photo-receptors.

One of the challenges of analyzing ethanol's teratogenicity in vertebrates using rodents as model systems is that mammals develop in utero. Therefore, ethanol concentrations and exposure times that result in a specific phenotype are difficult to determine because the metabolic function of the mother must be considered. Other vertebrates, such as zebrafish and *Xenopus laevis*, develop ex utero, so specific concentrations of ethanol over specific developmental periods are easily achieved. Treating zebrafish and *Xenopus* embryos with ethanol results in phenotypes comparable to those described for children with FAS, suggesting that the same molecular mechanisms are disturbed by ethanol treatment in vertebrates.^{9–11} Moreover, unlike mouse, zebrafish contain abundant cone photoreceptors that differentiate relatively early, making it a better system for the study of color vision in vertebrates.^{12,13}

The goal of this study was to obtain a detailed analysis of the effect of ethanol in zebrafish retinal development and function during the period of photoreceptor differentiation. We demonstrate that treating zebrafish embryos with ethanol causes the retinal abnormalities described in rodent models with FAS. Furthermore, ethanol compromises photoreceptor function at levels that do not affect photoreceptor development or morphology.

Materials and Methods

Breeding Fish and Treating Zebrafish Embryos with Ethanol

Ekkwill and AB strain zebrafish were maintained as an inbred stock at the Harvard zebrafish facility and were bred as previously described.¹⁴ We limited our studies to two strains of zebrafish because ethanol may affect the development of various strains differently.^{15,16}

Staged zebrafish embryos¹⁷ were raised until 48 hours postfertilization (hpf), when they were transferred to 6-well dishes containing 10 mL fish water and varying concentrations of United States Pharmacopoeia (USP) grade ethanol (1% to 2% by volume; Pharmco Products, Brookfield, CT) or methanol (1% to 2% by volume; Sigma, St. Louis, MO). The fish water and the appropriate alcohol were changed on a daily basis. To determine optokinetic response (OKR) and to conduct electroretinogram (ERG) analysis at 5 days postfertilization (dpf), ethanol-treated animals were removed from the alcohol-supplemented water and placed into alcohol-free water 4 hours before the behavioral or electrophysiology experiments were conducted (chronic treatment). Some zebrafish were raised in alcohol-free water until 5 dpf and then were placed in alcohol-supplemented water for 4 hours before the behavioral or electrophysiology experiments were conducted (acute treatment). Other zebrafish were acutely treated with ethanol, but after the alcohol treatment, the alcohol-supplemented water was replaced with fresh fish water for 4 hours before the behavioral and electrophysiology experiments were conducted.

The Harvard University Institutional Animal Care and Use Committee approved all experimental protocols, which conformed to National Institutes of Health guidelines on animal

use. In addition, the experiments were conducted in adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Histology

Embryos were fixed, embedded in plastic resin, and cut in transverse sections 1 to 5 μm in width, as previously described.¹⁸ For electron microscopy, larval eyes were fixed and embedded as previously described.¹⁰ Transverse 60- to 80-nm sections were mounted and stained with lead citrate and uranyl acetate. All transverse sections for light microscopy and transmission electron microscopy (TEM) were cut through the central retina and contained the optic nerve.¹⁰ A transmission electron microscope (JEOL USA, Peabody, MA) was used to view and photograph the specimens. Negatives were scanned using commercial software (Adobe Photoshop; Adobe Systems, Inc., San Jose, CA) and a scanner (UMAX Power Look 3000; UMAX Technologies, Inc., Dallas, TX).

The width of the optic nerve and the length of the outer segments were measured from TEM negatives (Image J; National Institutes of Health, Bethesda, MD). For each category (control, 1.5% ethanol, and 1.75% ethanol) measurements from either the optic nerve or the different photoreceptors (central cones, peripheral cones, and rods in the ventral patch) were taken from 4 to 7 retinas of 4 to 7 fish. Measurements were not corrected for shrinkage, but the mean outer segment length was similar to previously reported findings.¹⁹

Immunohistochemistry

Fixed larvae were washed in PBS and stored in 100% methanol at 4°C. Embryos were rehydrated in 50% and 30% methanol. Embryos were then permeabilized in acetone for 7 minutes at -20°C, followed by Proteinase K treatment (20 mg/mL; Sigma) for 90 minutes at room temperature. Embryos were refixed in 4% paraformaldehyde for 30 minutes and were then immersed in a blocking buffer (PBS containing 1% BSA, 1% dimethyl sulfoxide [DMSO], 2% normal goat serum, 0.25% Triton X-100, and 0.25% Tween-20) for 1 hour at room temperature and then incubated in primary antibody overnight at 4°C. The primary antibody solution contained the blocking solution and either rabbit anti-rhodopsin (1:250), rabbit anti-red opsin (1:250),²⁰ or Zpr-1 (1:20).¹⁸ Embryos were washed with PBS and then incubated overnight at 4°C with an alkaline phosphatase-conjugated secondary antibody (1:125; Sigma). The embryos were then rinsed with PBS, stained with a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution (Sigma), embedded in resin; 1- to 5- μm transverse serial sections through the optic nerve were obtained.

Visual Behavior and Electrophysiology on Isolated Larval Eye

To examine visual behavioral responses, the optokinetic response (OKR) assay was performed as previously described.^{18,21} All OKR assays were conducted between the hours of 10 AM and 6 PM in a darkened room. For testing, two to three zebrafish embryos were transferred to small Petri dishes containing 5% methyl cellulose and placed within a drum lined with vertical black and white stripes, 1 cm in width. The drum was illuminated with a tungsten light source, $5.6 \times 10^{-2} \mu\text{W}/\text{cm}^2$, attenuated by 3.5 log units, and the drum was rotated at 8.1 rpm.

Isolated whole-eye electroretinograms (ERGs) were obtained using published methods.^{18,22} The isolated eye was bathed in Mangel's ringers solution²² throughout the course of a recording session, which lasted between 30 to 75 minutes. ERGs were recorded at 24°–25°C. A two-channel optical bench with separate 100-W tungsten light sources for the stimulus and the background light was used. For light-adapted ERGs, the 1409 W/cm² background light was attenuated by a -1.6 log unit neutral-density (ND) filter. For recordings obtained under scotopic conditions, isolated eyes were dark-adapted for 30 minutes before testing and the interstimulus interval was gradually increased from 10 seconds at log I = -6, to 60–90 seconds

at log I = 0.¹⁸ The stimulus was produced by a tungsten halogen light, 9503 $\mu\text{W}/\text{cm}^2$ unattenuated intensity and was adjusted with ND filters. Recordings were bandpass-filter (0.1 to 100 Hz) amplified (Dagan Cornerstone amplifier; total gain approximately 10K; Dagan, Minneapolis, MN) and were collected using a personal computer and commercial software (PCLAMP; Axon Instruments, Burlingame, CA). The duration of the stimulus was 800 to 1000 ms, while the interstimulus time was 15 seconds. Data were either single responses or averages of three to seven responses, depending on signal-to-noise ratios. Amplitudes of the a-waves were measured from the resting potential to the bottom of the a-wave. The b-waves were measured from the bottom of the a-wave to the peak of the b-wave. A-waves were isolated by bathing the eye in 150 μM L(+)-2-amino-4-phosphonobutyric acid (L-AP4; Tocris, Ellisville, MO) and 15 μM DL-threo- β -benzyloxyaspartate (TBOA; Tocris). A manifold was used to switch the superfusion between control and the drug solutions. After switching to the a-wave cocktail, we waited until the effect of the new solution had stabilized before data were collected.

Statistical Analysis

Statistical analyses were performed using unpaired two-sample *t*-test assuming unequal variances (Excel; Microsoft Corporation, Redmond, WA) or one-way analysis of variance (ANOVA; Statistica, StatSoft Inc., Tulsa, OK). Post hoc comparisons, when appropriate, were made with the use of the Tukey Kramer or the Sidák multiple comparisons test.

Results

Ethanol Treatment Causes Morphological Problems throughout the Body and in the Eye

Photoreceptor differentiation commences around 2 days post-fertilization (dpf), and the differentiation of the photoreceptor layer is easily observable by light microscopy at 5 dpf.^{9, 10} Zebrafish embryos were treated with various concentrations of ethanol (1%–2% ethanol by volume) from 2 to 5 dpf. Embryos exposed to 1% ethanol swam normally around the Petri dish, but were more active than untreated controls. These fish appeared morphologically normal (Figs. 1A, 1B). Embryos treated with 1.25% to 1.5% ethanol had a phenotype different from that of wild-type fish, with slightly flatter forebrains, swollen hearts, swollen guts, and abnormal craniofacial development (Figs. 1C, 1D). These larvae could swim, but were not as active as the controls or 1% ethanol-treated fish. Embryos treated with 1.75% and 2% ethanol had numerous morphological problems, including a dorsally curved body, swollen heart with blood sometimes pooling in the chambers, rounded forebrain, irregular jaw, and smaller eyes (Figs. 1E, 1F). Embryos treated with these higher concentrations of ethanol were also listless; they swam little but had normal touch responses.

Transverse sections of the retina through the optic nerve show the retinal morphology of wild-type (Fig. 1G) and ethanol-treated animals (Figs. 1H–L). By 5 dpf, the five principal laminae in the retina are easily identified and the photoreceptors in untreated embryos are differentiated with well-defined inner and outer segments (Fig. 1G).

All retinas from the ethanol-treated embryos maintained proper lamination and had normally differentiated lenses (Figs. 1H–L), but they were noticeably smaller than those of untreated controls, and retinal alterations, especially of the photoreceptors, were observed. The severity of the phenotype was positively correlated with the amount of ethanol to which the embryos were exposed. Animals treated with 1%–1.25% ethanol had normal photoreceptor outer segments (Figs. 1H, 1I), whereas fewer outer segments were observed in embryos treated with 1.5%–1.75% ethanol (Figs. 1J, 1K). Embryos treated with 2% ethanol had few outer segments (Fig. 1L). Embryos treated with medium to high concentrations of ethanol (1.5%–2%) also had thinner GCLs; some exhibited periocular swelling around the eye, and all had smaller ciliary marginal zones (CMZs) (Fig 1I–1L), the area of proliferating cells that mediates the

continuous growth of the eye in cold-blooded vertebrates,^{23,24} and populates the retina with all its neuronal cell types.²⁵ Because of the poor health of the 2% ethanol-treated animals, we did not include them in further studies.

To determine whether the effects were ethanol specific,²⁶ zebrafish embryos were treated with 1%–2% methanol by volume from 2 to 5 dpf. Methanol-treated larvae could not be distinguished from untreated controls because they swam normally and did not exhibit any observable dysmorphology (data not shown). When retinal sections were analyzed by light microscopy, all the methanol-treated eyes had proper retinal lamination and no obvious morphological differences. No degeneration was observed in any of the laminae in 2% methanol-treated animals.

Closer examination of the morphology of the retinas was needed to determine whether the ethanol caused any abnormalities that could not be identified at the light microscopic level. Several studies of children with FAS as well as studies of ethanol teratogenesis in rats have shown that hypoplasia of the optic nerve is a consequence of ethanol exposure.^{3,6,7,27,28} Transmission electron micrographs of the optic nerve were obtained from untreated and ethanol-treated zebrafish embryos. In control animals, the optic nerve fibers were compact and homogenous (Fig. 2A). Analysis of the optic nerve from ethanol-exposed embryos showed that the optic nerve was particularly sensitive to this treatment. Numerous pyknotic profiles and prominent intercellular spaces between the fibers were always observed in the optic nerves of the 1.5% (Fig. 2B) and the 1.75% (Fig. 2C) ethanol-treated animals. Despite the morphological changes, there was no change in the width of the optic nerve. The mean width of the optic nerve of controls was $5.8 \mu\text{m} \pm 0.8$, whereas it was $5.6 \mu\text{m} \pm 0.33$ in 1.5% ethanol-treated embryos and $5.2 \mu\text{m} \pm 0.34$ in 1.75% ethanol-treated embryos ($P > 0.5$; $n = 4$ retinas from 4 embryos per condition).

Retinal ganglion cells were also examined in control (Fig. 2D) and in 1.5% and 1.75% ethanol-treated embryos (Figs. 2E, 2F). Both concentrations of ethanol affected the development of the ganglion cell layer and resulted in acellular holes and nuclei that had condensed chromatin (4 retinas from 4 embryos per condition).

Photoreceptors begin to differentiate in zebrafish at approximately 43 hpf, shortly after they become postmitotic.^{10,29} Morphological differentiation of these cells progresses quickly thereafter, with outer segments first becoming visible by 60 hpf.¹⁰ Close examination of the cone and the rod photoreceptors (Fig. 3) using electron microscopy indicated that both types of photoreceptors formed after treatment with 1.5% and 1.75% ethanol. The overall structure of the photoreceptors, except for the outer segments, appeared to be largely intact in ethanol-treated embryos. Similarly, the mitochondria in the photoreceptor inner segments also appeared normal in ethanol-treated embryos (data not shown).

Photoreceptor outer segments were most significantly affected by ethanol treatment. Some outer segment degeneration was observed in the ethanol-treated animals in the periphery of the retina (Fig. 3F), which might have contributed to the thinner appearance of the ONL. Many vacuoles and holes were found between the outer segments, and there were areas in which the RPE had withdrawn (Fig. 3F, arrowheads). Some of the inner segments of the photoreceptors contained visible, darkly staining cellular debris (data not shown). Although the outer segment membranous disks of the cone photoreceptors from the ethanol-treated embryos appeared to be properly stacked and organized, when examined at higher magnification, the length of cone outer segments in the cones found close to the optic nerve and in the periphery were significantly decreased compared with those of untreated controls ($P < 0.001$; Figs. 3J, 3K). The mean outer segment length for cones in the central retina was $3.76 \mu\text{m} \pm 0.86 \mu\text{m}$ for the controls ($n = 5$ to 6 photoreceptors per retina; 4 retinas from 4 animals). Animals treated with

1.5% ethanol had a mean outer segment length of $2.19 \mu\text{m} \pm 0.7 \mu\text{m}$ ($n = 4$ to 5 photoreceptors per retina; 7 retinas from 7 animals), whereas animals treated with 1.75% ethanol had a mean outer segment length of $1.34 \mu\text{m} \pm 0.58$ ($n = 4$ to 7 photoreceptors per retina; 5 retinas from 5 animals).

Rod photoreceptors are easily identifiable by 5 dpf in the ventral patch of the retina, where the developing rods are highly concentrated (Fig. 3G). Treatment with 1.5% ethanol (Fig. 3H) and 1.75% ethanol (Fig. 3I) caused a significant reduction in the size of rod outer segments in the ventral patch compared with untreated controls ($P < 0.001$; Fig. 3L). The mean rod outer segment in a control animal was $5.81 \mu\text{m} \pm 1.3 \mu\text{m}$ ($n = 4$ to 5 photoreceptors per retina; 6 retinas from 6 animals), but the mean rod outer segment in a 1.5%-treated animal was $4.29 \mu\text{m} \pm 1.13 \mu\text{m}$ ($n = 4$ to 5 photoreceptors per retina; 7 retinas from 7 animals), and in the 1.75%-treated animal it was $2.03 \mu\text{m} \pm 0.54 \mu\text{m}$ ($n = 4$ photoreceptors per retina; 4 retinas from 4 animals), indicating that the decrease in rod outer segment length was dose dependent.

The analysis of photoreceptor morphology revealed that ethanol disrupts the proper maturation of the photoreceptor outer segments. Opsin expression begins at approximately 50 hpf.⁹ To test whether the reduction in outer segment growth was correlated with an inhibition of opsin expression, ethanol-treated embryos were stained with antibodies directed against rhodopsin (Figs. 4A–4C), red opsin (Figs. 4D–4F), and the red-green double cones (data not shown). Rhodopsin, red opsin, and green opsin were expressed in the outer segments of fish treated with ethanol.

Ethanol Affects Vision in Acute- and Chronic-Treated Animals

Visual Behavior—To determine whether the chronic ethanol-treated animals had visual behavioral deficits, 5 dpf untreated and ethanol-treated larvae were tested using the OKR assay. To reduce the likelihood that the chronically treated embryos (animals treated with ethanol from 2 dpf through 5 dpf) were intoxicated and thus their visual function affected, all embryos were removed from the ethanol-supplemented water and placed into fresh fish water 4 hours before testing. The dimmest light ($\log I = -3.5$) was used to illuminate the black-and-white stripes during the first trial, and then the intensity level was raised. Each trial lasted 30 seconds. Embryos chronically treated with 1.5% and 1.75% ethanol (black bars) had significantly higher visual thresholds than those of untreated controls ($P < 0.001$; white bar).

Similarly, to determine whether vision in the zebrafish was altered by acute ethanol exposure, fish were raised in ethanol-free water for 5 days and then were treated with ethanol for 4 hours before being tested with the OKR assay (Fig. 5). Acute ethanol treatment (hatched bars) resulted in a significant increase in the average visual threshold compared with untreated controls ($P < 0.001$; white bars). The increase in threshold, on the other hand, was less than that observed in chronically treated larvae (black bars). There was a significant improvement in the performance of fish that were allowed to swim in fresh fish water for 4 hours after an acute 4-hour ethanol treatment ($P < 0.01$; hatched bars). Nevertheless, animals raised in ethanol from 2 to 5 dpf had higher visual thresholds than did fish that were acutely treated with ethanol and then allowed to swim in fresh water before testing ($P < 0.001$), illustrating that prolonged exposure to ethanol has a significant effect on embryonic visual function.

Electroretinography—An increased visual threshold in the OKR test indicated a deficit in visual function but could also indicate potential defects in the optic tectum or the muscle cells that control eye movements. In addition, some periorbital swelling around the eye was observed in ethanol-treated animals (Fig. 1) that could have prevented the fish from rotating their eyes, thus indirectly increasing the visual threshold observed using the OKR. To differentiate between these possibilities, ERGs were recorded from 5 dpf larvae to analyze outer retinal function. Representative ERGs from control (Fig. 6A) and 1% ethanol-treated embryos (Fig.

6B) are shown. The responses illustrated were elicited under photopic conditions with a light stimulus 800 to 1000 ms in duration. Most wild-type and some of the ethanol-treated animals responded to the dimmest flash ($\log I = -3$), but the b-wave amplitudes elicited from ethanol-treated animals were reduced at all light intensities (Fig. 6C). The b-wave amplitudes were significantly smaller with the 1% ethanol-treated embryos and embryos treated with greater concentrations of ethanol at the -1 and 0 log intensity (I) levels compared with untreated controls ($P < 0.01$). Although d-wave amplitudes were present in ethanol-treated animals, they were severely reduced at the -2 and -1 log I levels (data not shown).

Treating embryonic rats with ethanol resulted in changes in both photopic and scotopic vision.⁸ To determine whether scotopic vision was also compromised, ERGs were obtained from dark-adapted retinas after a 30-minute exposure to total darkness. The b-wave forms for control and ethanol-treated embryos were similar. The magnitude of the b-wave responses from the ethanol-treated embryos was considerably smaller than wild-type amplitudes in response to the brightest stimuli used ($\log I = 0$) (Fig. 6D). A measurable b-wave was obtained using a stimulus at -4 log intensity, whereas under photopic conditions the smallest measurable response was obtained at -3 log intensity. The d-wave in both the control and ethanol-treated embryos was not present in the dark-adapted ERG waveform (data not shown).

To test whether the decrease in outer retina function was mediated by a decrease in photoreceptor function, the a-wave was isolated by superfusing zebrafish eyes with Mangel's ringer solution containing $150 \mu\text{M}$ L(+)-2-amino-4-phosphonobutyric acid (L-AP4) and $15 \mu\text{M}$ DL-threo- β -benzyloxyaspartate (TBOA) (Fig. 6E). L-AP4, also known as APB, is a group III metabotropic glutamate receptor (mGluR) agonist that blocks the light response of rod-driven ON bipolar cells and eliminates the ERG b-wave in many animals by inactivating metabotropic glutamate receptor type 6 (mGluR6).²² Previous studies have shown that L-AP4 removes most, but not all, b-waves in larval zebrafish ERGs.^{18,22,30,31} In teleosts, excitatory amino acid transporters (EAATs) are linked to Cl^- channels and mediate the light-evoked response of ON bipolar cells receiving input from cones, and account for the remaining b-wave.³² Most of the b-wave is abolished when isolated larval zebrafish eyes were treated with L-AP4 and the EAAT inhibitor TBOA.^{18,22,33} The remaining response consisted of a sustained, negative-going potential—a reflection of the photoreceptor response—and the positive d-wave. The b-wave returned after a 20-minute wash in Mangel's ringer solution (data not shown). The average a-wave amplitude was drastically reduced in ethanol-treated animals in a dose-dependent manner when compared with untreated larvae (Fig. 6F). Moreover, the a-wave amplitudes were significantly smaller in the 1% and 1.25% ethanol-treated embryos and embryos treated with greater concentrations of ethanol at the -1 and 0 log intensity levels when compared with untreated controls ($P < 0.01$). These data, in conjunction with the reduced b-wave and OKR response data, indicate that ethanol at concentrations as low as 1% compromises the photoreceptor response.

Finally, to determine whether methanol treatment resulted in deficits in outer retinal function, OKRs and ERGs were also recorded from age-matched untreated controls and 1.75% methanol-treated animals. All methanol-treated animals had OKRs similar to those of untreated controls ($P > 0.5$). No significant differences in amplitudes were observed in either the b-wave or the d-wave at all light intensities tested in the methanol-treated fish ($P > 0.5$).

Discussion

Zebrafish treated with moderate to high levels of ethanol during the developmental period when photoreceptors differentiate had morphological abnormalities in the eye, as assessed by both light microscopy and transmission electron microscopy. Ethanol treatment also caused an increased visual threshold, as measured by the OKR. Analysis of the ERG indicated that there

was a severe reduction of a- and b-waves, indicating that the outer retina was significantly affected by ethanol treatment. Interestingly, low-concentrations of ethanol caused a reduction in the a- and b-wave amplitudes, though the embryos looked normal and their retinas morphologically resembled those of controls.

Ethanol Affects Photoreceptor Differentiation and Visual Function in a Dose-Dependent Manner

The defects in eye development and visual function observed in children exposed to alcohol during fetal development range from severe retinal dysmorphologies to abnormal visual function.³ The range of phenotypes characterized in children with FAS is consistent with alcohol disruption of several stages in fetal visual development, presumably correlated with the mother's drinking patterns. To understand the full spectrum of the effects that ethanol has on fetal eye development, it is necessary to characterize the mechanisms underlying ethanol's teratogenesis at each stage of visual development.

Studies examining the effects of ethanol exposure on retinal development using rodents have revealed two major targets: the optic nerve^{6,7} and the photoreceptor layer.⁸ We tested whether treating zebrafish embryos with ethanol would recapitulate the phenotype observed in rodents.

Ethanol exposure disrupts the development of the zebrafish optic nerve in a manner similar to that observed in rats.^{6,7} Ethanol treatment also inhibits the growth of the outer segment of photoreceptors in zebrafish embryos. Both rods and cones are affected by ethanol exposure, consistent with the photopic and scotopic effects that were observed in rats exposed to ethanol during embryogenesis.⁸

The inhibition of photoreceptor outer segment differentiation was dose dependent in zebrafish embryos; outer segments were shorter in embryos exposed to 1.75% ethanol than in those exposed to 1.5% ethanol. In addition, a temporal dependency with ethanol exposure was observed. Cones found in the central retina—those that differentiated first during development—were less affected than the more peripheral cones. These levels of ethanol exposure did not appear to cause degeneration or cell death in the outer retina. Instead, the photoreceptor cells looked morphologically intact, and the structures of the laminae in the outer segment were normal when analyzed by transmission electron microscopy, suggesting that ethanol has a specific effect on the inhibition of outer segment growth.

The decrease in outer segment length in the retinas of zebrafish treated with ethanol may correlate with a total inhibition of opsin expression. Opsins are one of the most abundant proteins in the outer segment, so it is conceivable that a complete inhibition of opsin expression would inhibit outer segment maturation. As in rats, treatment of zebrafish embryos with ethanol did not result in a total inhibition of opsin expression. Furthermore, our results suggest that the decrease in rhodopsin expression observed in ethanol-treated rat pups⁸ could be simply a result of shorter outer segments.

Treating embryonic rats with ethanol resulted in changes in both scotopic and photopic vision.⁸ Ethanol treatment also affected photopic visual behavior in zebrafish embryos when the embryos were treated before any cell type in the retina had differentiated.¹² In the present study, chronic ethanol exposure disrupted visual function in a dose-dependent manner, similar to the effect of ethanol on outer segment growth. The OKR analysis indicated that acute ethanol exposure affected visual function in zebrafish embryos, consistent with the changes in color vision that are observed in humans after an acute exposure to alcohol.^{34,35} Normal light-adapted visual function recovered when the animals were allowed to swim in an ethanol-free environment for several hours.

Because cones and rods present a similar outer segment phenotype, a decrease in the function of rods could occur in zebrafish as in rats.⁸ In the present study, measurable ERG recordings were obtained from both light- and full-field dark-adapted zebrafish embryos at 5 dpf (Fig. 6). Thirty minutes was sufficient to dark adapt the retinas and the b-wave responses became more sensitive by at least 1 log unit than ERGs obtained under photopic conditions. All the dark-adapted ERG recordings had no obvious d-wave. We found that there was a significant reduction in visual function in ethanol-treated dark-adapted zebrafish. The reduction in the d-wave form and the shift in b-wave sensitivity are consistent with recordings obtained from the adult zebrafish.³⁶

Anatomic and immunohistochemical studies indicate that rods are formed in the zebrafish retina as early as 50 to 60 hpf,^{9,10,37} and that substantial rod visual function does not occur until 2 weeks postfertilization.³⁸ Nevertheless, we consistently observed responses at low light levels in dark-adapted 5 dpf larvae, implying that there may be some degree of rod function early on. Behavioral data using the OKR assay indicates that some rod function is observed in 5–6 dpf dark-adapted wild-type zebrafish and in the no optokinetic response f(w21) (*nof*) mutant zebrafish (Johanna Lampert, personal communication, 2006). The *nof* mutant does not have any functioning cones³⁹ so any observed behavioral responses must come from functioning rod photoreceptors. Therefore, our data imply that the rod photoreceptors in ethanol-treated zebrafish embryos are not functioning properly based on the reduced ERG responses.

OKR analysis cannot differentiate between the effects on the retina and those in the optic tectum and muscle cells mediating the saccade, but ERGs provide a clear analysis of the visual function parameters within the retina. As with the OKR, ERGs revealed that ethanol had a profound dose-dependent effect on retinal visual function. When the a-wave was isolated, its decreased size suggested that the observed disruption in visual function in ethanol-treated fish could be accounted for by a defect in photoreceptor function.

It has been proposed that ethanol may disrupt retinoic acid (RA) metabolism in the developing vertebrate embryo.^{40–43} The organs that are affected in children with FAS are the same organs that are affected in animals that are exposed to RA during their embryonic development.⁴³ RA exposure can rescue the effects of ethanol exposure in vertebrates,⁴⁴ so ethanol may lower the levels of RA in the developing vertebrate embryo by competing with retinol for the activity of alcohol dehydrogenases. This would cause a reduction in rhodopsin and a corresponding shift of the voltage-intensity (V-I) curve. Nevertheless, there was no shift in the V-I curve, but rather a flattening of the V-I curve (reduction in the a- and b-wave amplitudes), indicating that the mechanism of how ethanol exerts its effects on the photoreceptors is still unclear.

Zebrafish as a Model System for the Study of FAS

Although FAS was described almost four decades ago,² very little is known about the mechanisms that underlie the teratogenic effects of ethanol in vertebrates. Most of the work aimed at analyzing the effects and mechanisms of ethanol teratogenesis have used rodents as the animal model.³ An obvious disadvantage of using systems in which the embryos develops in utero is that it is difficult to assess the role of maternal metabolism in the process; thus, it is difficult to establish the direct effect of ethanol on vertebrate development. Therefore, studying FAS using mammals as model systems can be complemented by studying the effects of ethanol on the development of vertebrate embryos that develop ex utero, such as zebrafish.

Allowing zebrafish embryos to grow in water containing the desired amount of ethanol results in a phenotype that recapitulates the FAS phenotype, and includes abnormalities in heart and craniofacial development.^{12,15,45–47} The levels of ethanol to which zebrafish embryos were exposed to obtain a retinal phenotype were similar to those reported by others.^{12,16, 45–47}

Low levels of ethanol did not cause obvious gross morphologic changes in either the body or the retina but it did affect both the OKR visual threshold and the a- and b-wave amplitudes, indicating that there are physiological defects even when morphology and rod development appear normal.

Interestingly, the ethanol levels necessary to recapitulate the FAS phenotype in zebrafish are an order of magnitude higher than the blood alcohol levels considered lethal in humans. It has been proposed that the levels of ethanol that human FAS fetuses are exposed to are unknown and may be much higher than the blood alcohol level of the mother because the vertebrate fetus does not produce alcohol dehydrogenase until the liver begins to differentiate.⁴⁷ An alternative hypothesis is that, as with oxygen and some nutrients, cold-blooded vertebrate embryos absorb ethanol through their skin; hence, the levels of alcohol that reach the embryo's bloodstream may be within the levels to which mammalian embryos are exposed to during their development. In fact, the amount of ethanol that is toxic to the zebrafish is an order of magnitude lower after animals have developed gills (Christian Lawrence, personal communication, 2002).

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Figure 1.

Ethanol treatment affects zebrafish development. Zebrafish embryos were raised in fish water (A, G) or fish water supplemented with 1% ethanol (B, H), 1.25% ethanol (C, I), 1.5% ethanol (D, J), 1.75% ethanol (E, K), or 2% ethanol by volume (F, L) from 2 days postfertilization (dpf) through 5 dpf. (A–B) Treatment with low concentrations of ethanol (1%) resulted in no observable morphological differences compared with untreated controls. (B–F) Increasing the concentration of ethanol treatment resulted in smaller retinas, swollen hearts (*arrow*), swollen guts (*white asterisks*), and inhibited head development (*arrowhead*), similar to the phenotype observed in children with FAS. (G–L) Transverse retinal sections are oriented dorsal (up) to ventral (down). Eye size varied with ethanol concentration, but lamination and cellular

components were similar. Substantial periocular swelling (*black asterisks*) was observed in some embryos treated with concentrations greater than 1.25%. No outer segments were observed in retinas treated with 2% ethanol, and abnormal outer segments were observed in retinas treated with 1.5% and 1.75% ethanol. Labeled are the lens (Lens), optic nerve (ON), retinal pigment epithelia (RPE), outer nuclear layer (ONL), inner nuclear layer (INL), inner plexiform layer (IPL), dorsal marginal zone (dMZ), ventral marginal zone (vMZ), and ganglion cell layer (GCL). Scale bar: (A–F) 10 μm ; (G–L) 100 μm .

Figure 2.

Ethanol treatment affects the development of the optic nerve and the retinal ganglion cell layer. Zebrafish embryos were raised in untreated fish water (**A, D**) or water treated with 1.5% ethanol (**B, E**) or 1.75% ethanol (**C, F**) from 2 to 5 dpf. Transmission electron micrographs were taken from the optic nerve (**A–C**) and from the ganglion cell layer (**E–F**). There was an observable increase in the incidence of hypoplasia of the optic nerves in the retinas of the ethanol-treated embryos. $n = 10$ to 12 retinas from 10 to 12 animals per condition. Scale bar, $1\ \mu\text{m}$.

Figure 3.

Ethanol treatment affects the development of the photoreceptor outer segments. Embryos were untreated (A, D, G) or received 1.5% ethanol (B, E, H) or 1.75% ethanol (C, F, I) from 2 to 5 dpf. Transmission electron micrographs were taken from the central retina near the optic nerve (A–C), the peripheral retina (D–F), and the ventral patch (G–I). Cone and rod outer segments (OS) were significantly shorter and more greatly degenerated in the ethanol-treated animals, particularly in the periphery, than in the untreated animals (J–L). At the higher ethanol concentrations, the retinal pigment epithelium (RPE) did not extend as far into the cone outer segments as it did in untreated animals (arrowheads). $n = 4$ to 7 retinas from 4 to 7 animals per condition. Error bars, $\pm$ SD. ** $P < 0.001$. Scale bar, 1 μ m.

Figure 4.

Ethanol treatment did not inhibit opsin and rhodopsin expression in cones and rods, respectively. Embryos were untreated (**A, D**) or were treated with either 1.5% (**B, E**) or 1.75% ethanol (**C, F**) from 2 to 5 dpf. Embryos were then fixed and processed for immunohistochemistry using antibodies directed against either rhodopsin (**A–C**) or red opsin (**D–F**). The fish were embedded in plastic, and serial sections were obtained. Rhodopsin (*arrowhead*) and red opsin (*arrow*) were expressed in the outer segment of rods and cones. Scale bar, 20 μm . $n = 5$ retinas per treatment condition from 3 treatments.

Figure 5.

Ethanol affects visual behavior in acute- and chronic-treated animals. Animals raised in ethanol from 2 to 5 dpf and placed in fresh water 4 hours before testing (chronic treatment, *black bars*) had higher visual thresholds than untreated controls as measured by OKR. When ethanol was added to the fish water for 4 hours (acute treatment, *hatched bars*), the result was an increase in the average visual threshold compared with untreated controls (*white bar*). The acute affect was rescued by allowing the larvae to swim in fresh fish water for 4 hours ($P > 0.05$; *gray bars*), indicating that chronic exposure to ethanol affects the function of the visual system permanently. $n = 18$ to 25 embryos per condition. Error bars, $\pm$ SD. * $P < 0.01$; ** $P < 0.001$.

Figure 6.

Chronic ethanol treatment reduces the amplitude of both a- and b-waves. Representative ERG recordings from (A) one wild-type fish and (B) one fish treated with 1.0% ethanol under constant background illumination to stimuli range in intensity over a range of 4 log units. In wild-type and ethanol-treated recordings, the waveform was dominated by the b- and d-waves. ERG recordings were made from isolated eyes.^{18,22} Each trace was an average of 3 to 7 consecutive responses. (C) The graph is a comparison of b-wave amplitudes from light-adapted wild-type and ethanol-treated fish ($n = 10$ to 14 embryos per condition) as a function of stimulus intensity. Ethanol treatment resulted in reduced b-wave amplitude in a dose-dependent manner. (D) The graph is a comparison of b-wave amplitudes from dark-adapted wild-type and ethanol-

treated fish ($n = 10$ to 14 embryos per condition) as a function of stimulus intensity. Ethanol treatment resulted in reduced b-wave amplitudes in a dose-dependent manner. **(E)** Some larval eyes were treated with a cocktail consisting of L-AP4 and TBOA, which resulted in pharmacologically isolating the a-wave, by abolishing most of the b-wave. Shown is a trace from a wild-type animal with the a-wave cocktail. **(F)** Ethanol treatment in a dose-dependent manner resulted in a reduced a-wave amplitude ($n = 10$ to 14 embryos per condition). Error bars, $\pm$ SD. $*P < 0.01$.