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Retinoic acid prevents synaptic deficiencies induced by alcohol exposure during gastrulation in zebrafish embryos

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ABSTRACT

In this study, we examined the effects of alcohol exposure during gastrulation on zebrafish embryos, specifically focusing on excitatory synaptic activity associated with neurons (Mauthner cells) that are born during gastrulation. Furthermore, we determined whether co-treatment of alcohol and retinoic acid (RA) could prevent the effects of alcohol exposure during gastrulation. We exposed zebrafish embryos to ethanol (150 mM), RA (1 nM), or a combination of RA (1 nM) plus ethanol (150 mM) for 5.5 h from 5.25 h post fertilization (hpf) to 10.75 hpf (gastrulation). Ethanol treatment resulted in altered hatching rates, survivability and body lengths. Immunohistochemical analysis of Mauthner cells (M-cells) suggested that ethanol treatment resulted in smaller M-cell bodies and thinner axons, while electrophysiological recordings of AMPA miniature excitatory postsynaptic currents (mEPSCs) associated with M-cells showed that ethanol treated animals had a significantly reduced mEPSC frequency. Other mEPSC parameters such as amplitude, rise times and decay kinetics were not altered by exposure to alcohol. Locomotor studies showed that ethanol treatment resulted in altered C-bend escape responses. For instance, the C-bends of alcohol-treated fish were larger than control embryos. Thus, ethanol treatment during gastrulation altered a range of features in embryonic zebrafish. Importantly, co-treatment with RA prevented all of the effects of ethanol including survivability, body length, M-cell morphology, AMPA mEPSC frequency and escape response movements. Together these findings show that ethanol exposure during the brief period of gastrulation has a significant effect on neuronal morphology and activity, and that this can be prevented with RA co-treatment.

1. Introduction

Fetal alcohol spectrum disorder (FASD) is a birth disorder that results from prenatal ethanol exposure (Jones, 1975; Sokol et al., 2003). The prevalence of FASD ranges from 0.3 to 5% within most populations, reaching as high as 8.9% in low socioeconomic populations (May et al., 2009). Ethanol-induced defects include craniofacial, cardiac, central nervous system, learning, motor, sensory, and ocular defects. Several animal models have been used to study the biological mechanisms by which embryonic exposure to ethanol alters development (Sulik, 2005). Although each model system has its own advantages and disadvantages, recent studies have explored zebrafish (*Danio rerio*) as an animal model to study ethanol-mediated developmental toxicity (Arenzana et al., 2006; Bradfield et al., 2006; Carvan et al., 2004; Dlugos and Rabin, 2007; Marrs et al., 2010; Shan et al., 2015; Sylvain et al., 2010, 2011). Zebrafish offer key advantages for alcohol toxicity studies, even though there is not a maternal-fetal interaction. For instance, the fertilized eggs can be exposed to alcohol at precise time points and the embryos can be closely followed throughout development. In our studies, we exposed the embryos during gastrulation, when a key command neuron known as the M-cell is born (Kimmel et al., 1990). The embryonic zebrafish is one of the rare preparations in which we can study how cells born during a brief episode of alcohol exposure, develop in response to that exposure. We examined the M-cell approximately 2 days after ethanol exposure, to study its morphology, physiology, synaptic activity and overall function.

Retinoic acid (RA) is a Vitamin A derivative and a morphogen at low concentrations, but a teratogen at high doses (Collins and Mao, 1999). It is known to play a role in organogenesis, organ homeostasis, cell and neuronal growth and differentiation, CNS

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development and limb morphogenesis (Duester, 1991; Durston et al., 1989; Maden et al., 1990) (Kumar et al., 2010; West et al., 1994). Vitamin A, acquired through nutrition is subsequently converted to RA by alcohol dehydrogenases (ADHs) or retinol dehydrogenases (RDHs), and then aldehydye dehydrogenase (ALDHs) enzymes (Rhinn and Dolle, 2012). RA binds to nuclear Retinoic Acid Receptors (RARs) which form heterodimeric complexes with Retinoid X Receptors (RXRs). The RAR-RXR heterodimers bind to RA response elements (RAREs), located in the enhancer regions of RA target genes and transcription is initiated (Cunningham and Duester, 2015; Dilworth et al., 2000; Duester, 2008; Niederreither and Dolle, 2008; Rhinn and Dolle, 2012). A link between FASD and RA signaling has been clearly established and it is thought that many of the defects induced by ethanol exposure occur via an alteration of RA biosynthesis and signaling (Duester, 1991; Keir, 1991; Leo and Lieber, 1999; McCaffery et al., 2004; Pullarkat, 1991; Sulik et al., 1981). Some of the developmental genes modulated by RA signaling include Cdx1, Cyp26a1, Drd2, Fgf8, Hnf1b, homeobox genes (Hox), Pax6, and Wnt8a (Cunningham et al., 2015).

Vitamin A levels are depleted upon consumption of alcohol, thereby producing complex developmental phenotypes that resemble FASD-associated defects. The detoxification of EtOH requires the function of ADHs and ALDHs, which are also used for RA biosynthesis. Therefore EtOH may act as a competitive inhibitor of RA biosynthesis (Deltour et al., 1996; Duester, 1991). Moreover, retinol accumulates in tissues such as the kidneys and lungs when it is not oxidized to RA, and high levels of retinol may induce morphological defects. Additionally, ethanol exposure alters the expression of RAR and RXR (Kumar et al., 2010). Thus, EtOH exposure alters RA signaling on multiple levels.

When zebrafish embryos exposed to ethanol for 21 h between 3 hph and 24 hpf, are supplemented with low levels of retinoic acid (10^{-9} M), some of the morphological defects caused by alcohol exposure are prevented (Marrs et al., 2010). In our study, we tested whether a high, but brief exposure to ethanol (150 mM) during gastrulation could alter zebrafish development and synaptic function, and whether co-treatment with RA could prevent the deleterious effects of ethanol. Low doses of RA have been shown to ameliorate the effects of alcohol, but higher doses (plasma concentrations of around 0.1 μg/ml in rats) can result in observable malformations (Collins et al., 1995, 1994; Tembe et al., 1996). We were careful to choose a dose of RA (10^{-9} M) in the normal physiological range that produces mild to no teratogenic effects (Herrmann, 1995; Marrs et al., 2010; Sarmah and Marrs, 2013b). Our findings suggest that even a brief exposure to ethanol during gastrulation may alter embryonic development and synaptic activity. Importantly, this can be prevented by supplementation with RA.

2. Materials and methods

2.1. Animal care, ethanol and retinoic acid exposure

Wild type zebrafish (*Danio rerio*) embryos were collected from the University of Alberta Aquatic Facility. For breeding, 5 adults, consisting of 3 females and 2 males, were placed in breeding tanks the evening before eggs were required. The following morning, eggs were collected from the breeding tanks, usually within 30 mins of release by the females. A 12 h light/dark cycle and 28.5 °C temperature was set for housing the embryos and larvae in incubators.

Embryos were exposed to ethanol (150 mM), 10^{-9} M RA (Sigma, catalog number R2625, also known as Tretinoin, all-*trans*-Retinoic acid), or 10^{-9} M RA plus 150 mM ethanol during the period of gastrulation, which occurs between 5.25 h post fertilization (hpf) to 10.75 hpf by adding them to the Embryo Media (EM). The exposure medium was then replaced at the end of each exposure period with 25 ml of fresh EM, washed several times in fresh EM and incubated until behavioral recording at 2 days post fertilization (dpf). In immunohistochemical studies, pigment formation was blocked by adding 0.003% phenylthiourea (PTU) dissolved in egg water at 24 hpf. All protocols were carried out in compliance with guidelines described by the Canadian Council for Animal Care (CCAC) and the University of Alberta.

Embryo Media contained 13.7 mM NaCl (BDH), 4.1 mM $NaHCO_3$ (BDH), 1.0 mM $MgSO_4$ (BDH), 0.98 mM CaCl2 (Fisher Scientific), 0.54 mM KCl (BDH), 0.044 mM KH_2PO_4 (Sigma-Aldrich), and 0.025 mM Na_2HPO_4 (BDH). Penicillin-Streptomycin and the antifungal agent Methylene Blue (Sigma-Aldrich) were added to the EM solution. RA (10 mM) stock solution (Sigma-Aldrich) was prepared by dissolving 50 mg RA into 16.64 ml of DMSO. 10^{-9} M RA was chosen as optimal concentration for treatments that minimized its toxic effect and displayed maximum rescue phenotypes as previously described (Collins and Mao, 1999; Marrs et al., 2010; Sarmah and Marrs, 2013b). The final concentration of DMSO that embryos were exposed to was 0.00001%.

2.2. Imaging and morphology

Embryos were imaged at 2 dpf using a Lumenera Infinity2-1R colour microscope camera mounted on a Leica stereomicroscope. All immunohistochemical imaging were done on a Zeiss LSM confocal microscope and photographed under a 20 x objective. Images were compiled using Zeiss LSM Image Browser software and are shown as z-stack compilations of the trunk unless specified.

The number of fish still alive and the number of fish that had hatched out of the chorion were recorded on each day until 5 dpf. Gross deformities were observed at 2 dpf where body length of the fish, the number of larvae exhibiting pericardial edema and axial malformations was counted for each treatment. Measurements of embryo length were done using a microscope eyepiece equipped with a micrometer. Morphological observations were performed using a dissecting microscope; embryos were placed in a 16-well plate with one embryo per well and anesthetized in 0.02% MS-222.

2.3. Immunohistochemistry

Embryos (2 dpf) were fixed in 2% paraformaldehyde for 1–2 h and washed with 0.1 M PBS-T (phosphate buffered saline (PBS) and 0.5% Tween-20 (Hanington et al., 2008) every 15 min for 2 h. PBS consisted of (in mM): 150 NaCl, 8 NaH_2HPO_4, 2 $NaH_2PO_4{\cdot}2H_2O$, and had a pH of 7.2. The preparations were then permeabilized for 30 min in 4% Triton-X 100 containing 2% BSA (bovine serum albumin) and 10% goat serum. Tissues were incubated for 48 h at 4 °C in mouse monoclonal anti-3A10 (Serafini et al., 1996) (Developmental Studies Hybridoma Bank (DSHB)), to identify Mauthner neurons. The primary antibody was diluted at 1:250 in PBS. Tissues were washed in PBS twice every 15 min for 2–3 h and then incubated for 4 h at room temperature in the secondary antibody, Alexa Fluor® 488 goat anti-mouse IgG, (Molecular Probes, Life Technologies), at a dilution of 1:1000. Preparations were then washed for 7 h in PBS, followed by mounting in MOWIOL mounting media for viewing.

2.4. Electrophysiology

Zebrafish embryos at 2 days post-fertilization (dpf) were dissected and prepared for electrophysiology as described previously (Drapeau et al., 1999; Roy and Ali, 2013). In brief, the embryos were anesthetized in 0.02% tricaine (MS-222) (Sigma-