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Ethanol exposure during gastrulation alters neuronal morphology and behavior in zebrafish

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ABSTRACT

Ethanol (EtOH) exposure during development has been shown to lead to deficits in fine and gross motor control. In this study we used zebrafish embryos to determine the effects of EtOH treatment during gastrulation. We treated embryos in the gastrulation stage (5.25 hours post fertilization (hpf) to 10.75 hpf) with 10 mM, 50 mM or 100 mM EtOH and examined the effects on general animal morphology, the c-start reflex behavior, Mauthner cell (M-cell) morphology and motor neuron morphology. EtOH treated fish exhibited a minor but significant increase in gross morphological deformities compared with untreated fish. Behavioral studies showed that EtOH treatment resulted in an increase in the peak speed of the tail during the escape response. Furthermore, there was a marked increase in abnormally directed c-starts, with treated fish showing greater incidences of c-starts in inappropriate directions. Immunolabeling of the M-cells, which are born during gastrulation, revealed that they were significantly smaller in fish treated with 100 mM EtOH compared with controls. Immunolabeling of primary motor neurons using anti-znp1, showed no significant effect on axonal branching, whereas secondary motor axons had a greater number of branches in ethanol treated fish compared with controls. Together these findings indicate that ethanol exposure during gastrulation can lead to alterations in behavior, neuronal morphology and possibly function.

1. Introduction

Embryonic organisms exposed to ethanol exhibit physical and mental deficits such as joint and cardiac abnormalities, motor and reflex developmental delays and mental retardation (Jones and Smith, 1973; Jones et al., 1973; Kalberg et al., 2006; Staisey and Fried, 1983). Here, we set out to determine if exposure to ethanol, particularly during gastrulation, has an effect on neurons involved in locomotion. Recent studies have explored the use of zebrafish as an animal model for embryonic alcohol exposure (Arenzana et al., 2006; Bilotta et al., 2004; Carvan et al., 2004; Dlugos and Rabin, 2007; Driscoll et al., 1990; Li et al., 2007) because they can offer certain advantages over mammalian models when studying embryonic ethanol exposure. For example, embryos develop outside the mother in a chorion or egg casing, thereby allowing one to accurately control the concentration and the time course of ethanol exposure compared with placental organisms. Additionally, semi-transparent zebrafish embryos can be used for whole preparation imaging. Drawbacks of the model include the absence of maternal–embryo interaction during gestation, and that relatively high doses of EtOH are typically required to induce defects that are similar to mammalian models (Cudd, 2005; Matsui et al., 2006). However the advantages offered by a zebrafish model for ethanol toxicity and teratogenicity are significant and allow for a wide range of studies that may be difficult to perform in other preparations.

M-cells are the largest neurons in the zebrafish CNS and are involved in the c-start escape reflex (Kimmel et al., 1981; Metcalfe et al., 1990). They are born at 8 hours post fertilization (hpf), during gastrulation, and are located in rhombomere 4 of the hindbrain (Mendelson, 1986). Under normal conditions they are tonically inhibited via glycinergic transmission (Ali et al., 2000b), and are activated via glutamatergic chemical transmission (Ali et al., 2000a). M-cells receive sensory information from a number of sources including the eighth cranial nerve, the trigeminal nerve and acousticovestibular inputs (Kimmel et al., 1990). Tactile stimuli to the head elicits a c-start escape response in 2 day old embryos (Foreman and Eaton, 1993; Liu and Fetcho, 1999; O'Malley et al., 1996), but as development proceeds, the M-cells become preferentially stimulated by acousticovestibular input after several days of development (Kohashi et al., 2012).

M-cells pioneer a descending pathway down the spinal cord and synapse with primary motor neurons as well as a number of interneurons (Fetcho and Faber, 1988). There are three primary motor neurons (sometimes 4) per spinal cord hemi-segment, one of which innervates the dorsal trunk musculature, whereas the other two innervate mid

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and ventral regions of the trunk (Eisen et al., 1986). Secondary motor neurons follow paths similar to those taken by primary motor neurons, although they do not branch as extensively and are present in greater numbers (Myers et al., 1986b). Zebrafish larvae possess two types of skeletal muscle fibers: slow, tonic (red) fibers and fast, twitch (white) fibers (Buckingham and Ali, 2004; Greer-Walker and Pull, 1975). White fibers are innervated by both primary and secondary motor neurons, while red fibers are only innervated by secondary motor neurons (de Graaf et al., 1990; Liu and Westerfield, 1988).

In previous studies we exposed embryos to relatively high concentrations of ethanol (0.5%–3%, which roughly corresponds to ~86 mM–~514 mM EtOH) between 8 hours post fertilization (hpf) and 24 hpf (Sylvain et al., 2010, 2011), and found that this exposure paradigm had a significant effect on motor neuron and muscle fiber morphology. In this study we sought to reduce the ethanol exposure by lowering the concentration of ethanol to (10 mM, 50 mM and 100 mM EtOH) values that more closely approximate blood alcohol concentrations (BAC) during alcohol consumption. For instance, on average, if a 120 lb female consumes 1–2 drinks in a single sitting, her BAC may be 0.033% (5.6 mM)–0.066% (11.3 mM), while a binge drinking episode may result in a BAC of 0.2% (34.4 mM) (Maier and West, 2001). In addition, we limited the exposure time to 5.5 hrs during the period of gastrulation, which occurs between 5.25 hpf and 10.75 hpf. We specifically wanted to determine if exposure during gastrulation had a detrimental effect on the startle response, and hypothesized that embryos exposed to alcohol during this brief but important developmental period would have altered locomotion, and abnormalities in M-cell and or motor neuron morphology. Our results indicate that the c-start escape behavior, and M-cell and secondary motor neuron morphology were adversely affected by embryonic exposure to alcohol during gastrulation, but there appeared to be little or no effect on primary motor neuron morphology.

2. Materials and methods

2.1. Animal care and ethanol exposure

The zebrafish used in this study were wild type zebrafish (*Danio rerio*) embryos of the AB strain that were maintained at the University of Alberta Aquatic Facility. For breeding, 3 to 5 adults, usually consisting of 3 females and 2 males, were placed in breeding tanks the evening before eggs were required. The following morning, fertilized eggs were collected from the breeding tanks, usually within 30 min of fertilization. Embryos and larvae were then housed in incubators on a 12 h light/dark cycle, and set at 28.5 °C. Embryos were exposed to ethanol (0 mM, 10 mM, 50 mM and 100 mM) during the period of gastrulation, which occurs between 5.25 hpf to 10.75 hpf. The exposure medium was then replaced at 10.75 hpf with 25 mL of fresh EM and incubated until behavioral recording at 48 hpf. For 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.

2.2. Immunohistochemistry staining

Embryos (48 dpf) were fixed in 2% paraformaldehyde for 1–2 h and washed with 0.1 M phosphate buffered saline (PBS) every 15 min for 2 h. The preparations were then permeabilized for 30 min in 4% Triton-X 100 containing 2% BSA and 10% goat serum. Tissues were incubated for 48 h at 4 °C in either mouse monoclonal anti-3A10 (Serafini et al., 1996) (Developmental Studies Hybridoma Bank (DHSB)), to identify M-cells, or mouse monoclonal anti-znp-1 which targets primary motor axons (Trevarrow et al., 1990), or mouse monoclonal anti-zn-8 (Trevarrow et al., 1990) (DHSB), which identifies secondary motor neurons. All primary antibodies were 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. The larvae were then washed for 7 h with PBS, and mounted in MOWIOL mounting media. All embryos were imaged on a Zeiss LSM confocal microscope and photographed under a 20× objective. Images were compiled using Zeiss LSM Image Browser software and are shown as z-stack compilations.

2.3. Behavior

Video observation of 2 day old zebrafish embryos was performed using an AOS video camera (AOS S-PRI 1995; 1250 FPS; shutter speed: 800 μs) mounted on a dissecting microscope (Roy et al., 2014). Embryos were immobilized in 2% low-melting point (26–30 °C) agarose (LMPA) dissolved in embryo medium (Sigma-Aldrich 2-Hydroxyethylagarose, type VII low gelling temperature). To observe embryo movements, the LMPA was cut away from the embryo's trunk and tails, leaving the heads embedded in the gel. Embryo media was added to the Petri dish to ensure that the larvae remained immersed in solution. Embryos were allowed to acclimate to their environment for 20 min prior to stimulus application. Borosilicate glass micropipettes (Sutter Instrument; O.D.: 1.2 mm, I.D.:094 mm, 10 cm length) were pulled using a Flaming/Brown Sutter Instrument pipette puller (model P-97). The pipettes were positioned as close as possible to the embryo's otolith without contacting the embryo. Embryo stimulation was performed using a 15 ms pulse of basic 2% phenol red (Sigma-Aldrich) dissolved in embryo media ejected from a Picospritzer II (General Valve Corporation). Behaviors were recorded for about 900 ms following the stimulus. This period of time was long enough to film the escape response and periods of swimming following the c-bend. The video-recordings were transferred and analyzed using a Motion Analysis Software, ProAnalyst® (Xcitex Inc., Cambridge, MA, USA).

2.4. Embryo imaging and morphological observations

Embryos were imaged at 2 dpf using a Lumenera Infinity2-1R color microscope camera mounted on a dissecting microscope. 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% MS222. Measurements of embryo length were done using a microscope eyepiece equipped with a micrometer.

2.5. Statistics

All values are reported as means ± SEM (standard error of the mean). Significance was determined using a one-way ANOVA followed by a Tukey post-hoc multiple comparisons test where appropriate ($p < 0.05$). When normality tests failed, the Dunn's method of comparison was performed followed by a Kruskal–Wallis one-way ANOVA on ranks. Statistical analysis was done using SigmaPlot 12.5 (SPSS) and Microsoft Office Excel 2007.

3. Results

3.1. Gross morphology

Embryos treated with ethanol exhibited minor morphological defects such as slightly curved tails, but overall, the embryos were not strikingly different from controls (Fig. 1). We found that there was a significant difference in the rate of hatching between treatments, such that embryos exposed to 50 and 100 mM ethanol experienced lower