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Long-term behavioral impairment following acute embryonic ethanol exposure in zebrafish

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ABSTRACT

Background: Developmental exposure to ethanol has long been known to cause persisting neurobehavioral impairment. However, the neural and behavioral mechanisms underlying these deficits and the importance of exposure timing are not well-characterized. Given the importance of timing and sequence in neurodevelopment it would be expected that alcohol intoxication at different developmental periods would result in distinct neurobehavioral consequences.
Methods: Zebrafish embryos were exposed to ethanol (0%, 1%, 3%) at either 8–10 or 24–27 h post-fertilization (hpf) then reared to adolescence and evaluated on several behavioral endpoints. Habituation to a repeated environmental stimulus and overall sensorimotor function were assessed using a tap startle test; measurements of anxiety and exploration behavior were made following introduction to a novel tank; and spatial discrimination learning was assessed using aversive control in a three-chambered apparatus. Overt signs of dysmorphogenesis were also scored (i.e. craniofacial malformations, including eye diameter and midbrain-hindbrain boundary morphology).
Results: Ethanol treated fish were more active both at baseline and following a tap stimulus compared to the control fish and were hyperactive when placed in a novel tank. These effects were more prominent following exposure at 24–27 hpf than with the earlier exposure window, for both dose groups. Increases in physical malformation were only present in the 3% ethanol group; all malformed fish were excluded from behavioral testing.
Discussion: These results suggest specific domains of behavior are affected following ethanol exposure, with some but not all of the tests revealing significant impairment. The behavioral phenotypes following distinct exposure windows described here can be used to help link cellular and molecular mechanisms of developmental ethanol exposure to functional neurobehavioral effects.

1. Introduction

The widespread effects of ethanol exposure on the fetal central nervous system (CNS) are well known to culminate in a constellation of behavioral impairments, a syndrome seen in a wide range of species (see reviews Cole et al., 2012; Gerlai et al., 2000; Ungerer et al., 2013). Moreover, it appears that the timing of ethanol exposure can be an important determinant of the CNS structures that are primarily affected and therefore presumably determines the nature of functional impairment (Hamre and West, 1993). In humans, the cognitive and behavioral effects associated with developmental ethanol exposure are classified clinically as fetal alcohol syndrome (FAS), or more broadly, fetal alcohol spectrum disorder (FASD). Characteristic signs and symptoms of FAS are growth retardation, craniofacial malformations, and neurodevelopmental abnormalities including intellectual delay (Jones and Smith, 1973; Warren and Foudin, 2001). While many of the overt characteristics (e.g. facial malformations and CNS structure) of FAS can be recapitulated with early embryonic ethanol exposure in rodents (see Sulik et al., 1981; Sulik, 2005) some of the subtle cognitive and behavioral characteristics have not been well characterized following developmentally distinct time points of ethanol exposure. However, zebrafish have been used to model some behavioral aspects of fetal alcohol exposure (Bilotta, 2003; Loucks and Ahlgren, 2012) and it has been shown that moderate concentrations of ethanol exposure from 24 to 27 hpf impairs social affiliation in adult zebrafish (Fernandes and Gerlai, 2009) and 0–6 dpf of exposure to ethanol decreases accuracy on a spatial discrimination task (Carvan et al., 2004).

Despite the emerging emphasis on exposure timing and the importance of episodic binge drinking as a risk during pregnancy, the general

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practice in animal models of fetal alcohol effects (FAE) is to expose the fetus or larvae chronically to ethanol, which often corresponds to an entire trimester of human gestation (or more) and cannot sustain high-dose exposures due to lethality (see Zhang et al., 2011, 2013). This model fails to reflect the binge nature of ethanol consumption by humans, in which relatively high doses of ethanol are consumed during a short span of time. Therefore, in addition to characterizing the functional impact of ethanol during distinct developmental windows, a more valid model of human ethanol consumption is achieved by mimicking episodes of maternal binge drinking.

The small freshwater zebrafish (*Danio rerio*) has emerged as a powerful tool for uncovering neural mechanisms of numerous syndromes and diseases because of the relative ease of using genetic and molecular tools in this species, coupled with highly conserved neural architecture and the capacity for complex behavior. The primary goal of this study was to characterize the behavioral effects of early (gastrulation) and late (organogenesis) developmental exposure to moderate-to-high doses of ethanol in zebrafish. Such data should facilitate further characterization of cellular and behavioral mechanisms that underlie FAS. To this end, the present design utilized a zebrafish model to investigate the persistent neurobehavioral deficits that result from short-term ethanol exposure during early development.

2. Methods

2.1. Animals

Zebrafish (*D. rerio*) from the AB strain (progenitors obtained from the Zebrafish International Resource Center) were bred in-house and generated all embryos used in the present study. All fish were housed in automatic fish housing systems at 28.5 °C while at North Carolina Central University (NCCU) (Aquaneering, San Diego, CA, USA) and while at Duke University (Aquatic Habitats, Apopka, FL, USA). Fish were group housed with <20 fish per 3 L tank on the multi-tank flow-through system and maintained on a 14 hr light,10 hr dark cycle 7 days per week. Ethanol exposure was carried out at NCCU and fish were subsequently transferred to Duke University for behavioral assessment, approximately 20 fish per ethanol exposure condition were reared for behavioral testing, fish with physical malformations (described below) were excluded from behavioral analyses. All behavioral testing was conducted between the hours of 12:00 and 5:00 pm, which was during the light phase. All fish were fed twice daily, artemia (Brine Shrimp Direct, Ogden, Utah) in the morning and dry flake fish food (TetraMin®, Tropical Flakes, Melle, Germany) in the evening; evening feeding was always withheld until the completion of behavioral testing. Each behavioral test is described in detail below, tests were conducted within subject on separate days with each fish experiencing each test only once, although the tap startle and spatial discrimination procedures are each made up of 10 intra-session trials. The protocols used were approved by the Institutional Animal Care and Use Committees at Duke University and North Carolina Central University in accordance with NIH and United States regulations.

2.2. Ethanol treatment of zebrafish embryos

All breeding, embryo harvesting and ethanol exposures were conducted at North Carolina Central University, Durham, NC, USA, in the Cole laboratory. Zebrafish embryos in fish water containing a 1:500 dilution of 0.1% methylene blue (to prevent fungal infection) were exposed to 1.0% and 3.0% ethanol from 8 to 10 hpf or 24 to 27 hpf. Ethanol was diluted with fish water to its final concentration, and at the selected developmental stage for ethanol treatment embryos were placed in fresh fish water containing ethanol. At the end of the exposure period, fish water containing ethanol was removed, embryos were washed once with fresh fish water and then transferred to fresh fish water and placed on an aquarium rack system for rearing. At approximately 2 months of age (which corresponds to the late fry juvenile stage of development) all fish were transferred to the Levin laboratory at Duke University, Durham, NC, USA for approximately one-week acclimation prior to behavioral testing.

2.3. Brain and facial malformation scoring

Eye size was measured at 2 dpf as previously described (Zhang et al., 2011, 2014), and involved measuring the longest axis along the eye, and calculated against a standard 50 μm ruler under the same magnification. For 2 dpf eye measurements, we designated a diameter of less than 240 μm as the "small eye" phenotype, since all untreated eyes were at least 250 μm in diameter. Malformation of the midbrain-hindbrain boundary (MHB) was assessed visually based on absence of the defined border between the midbrain and hindbrain. The presence of the MHB was defined as the presence of 3 or 4 ridges (tectal and cerebellar boundaries) perpendicular to the anterior-posterior (AP) axis of the CNS at the midbrain-hindbrain junction. Absence of this defined border was scored as a disruption of MHB development and has been previously described (see Zhang et al., 2014).

2.4. Behavioral testing

2.4.1. Novel tank dive task

The novel tank paradigm capitalizes on a species-specific pattern of responding to a novel environment in which zebrafish initially dwell on the tank floor before gradually exploring, occasioning the water surface after several minutes undisturbed in a novel environment (Bencan and Levin, 2008; Bencan et al., 2009; Levin, 2011; Levin et al., 2007). This initial "dive response" is an effective strategy for predatory avoidance inasmuch as it removes the possibility of being consumed from below; the later exploration is presumably food-seeking/foraging behavior as zebrafish feed along the water column, including from the water's surface. Therefore, any deviation from this dive/explore response pattern is considered maladaptive.

Zebrafish were individually tested in 1.5-liter plastic tanks filled with 1350 ml of tank water, the surface of the water was approximately 10 cm from the tank floor. The trapezoid shaped tanks were 22.9 cm along the bottom and 27.9 cm along the top. The diagonal side of the tank was 15.9 cm and the opposite vertical side was 15.2 cm, the same design as used previously (see Levin et al., 2007). Behavior was tracked in real-time using EthoVision tracking software (Noldus Information and Technology, Wageningen, The Netherlands) which calculated distance from the tank floor and total distance traveled. Each trial was five-minutes in duration and commenced immediately after a fish was placed in the novel tank. The video signal was transmitted through a Samsung camcorder that was positioned approximately 88 cm away from the testing tanks.

2.4.2. Tap startle and habituation test

24 h after completion of the dive test, animals were tested for sensorimotor function and habituation learning using a tap startle (Levin et al., 2007; Sledge et al., 2011). The startle test apparatus consisted of flat white 20.4 cm × 38.1 cm surface with white 12.7 cm × 15.2 cm frontal and rear blocking barriers attached. On the flat surface there were arranged eight identical 5.1 cm × 7.6 cm clear cylindrical arenas made of Plexiglas in a 2 × 4 array. Each of the eight arenas was filled with 30 ml of tank water. The apparatus was positioned between two white opaque barriers, which faced each other and projected a bare white screen. Mechanical solenoids were positioned beneath each arena. A Samsung camcorder was located approximately 71 cm above the apparatus. The solenoids were used to administer taps to the bottom of the cylindrical arena under control of the computer. Zebrafish were taken from the holding tanks and each one was placed into one of eight cylindrical arenas. Zebrafish were allowed to acclimate in the arena for 10 min prior to testing then