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Moderate developmental alcohol exposure reduces repetitive alternation in a zebrafish model of fetal alcohol spectrum disorders

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ABSTRACT

The damaging effects of alcohol on a developing fetus are well known and cause a range of conditions known as fetal alcohol spectrum disorder (FASD). High levels of alcohol exposure lead to physical deformity and severe cognitive deficits, but more moderate exposure leads to a range of subtle cognitive effects such as reduced social behavior, higher propensity to develop addictions, and reduced spatial working memory. Previous studies have demonstrated that following exposure to relatively low levels of ethanol during early brain development (equivalent in humans to moderate exposure) zebrafish display a range of social and behavioral differences. Here, our aim was to test the hypothesis that moderate developmental ethanol exposure would affect aspects of learning and memory in zebrafish. In order to do this, we exposed zebrafish embryos to 20 mM [0.12% v/v] ethanol from 2 to 9 dpf to model the effects of moderate prenatal ethanol (MPE) exposure. At 3 months old, adult fish were tested for appetitive and aversive learning, and for spatial alternation in a novel unconditioned y-maze protocol. We found that MPE did not affect appetitive or aversive learning, but exposed-fish showed a robust reduction in repetitive alternations in the y-maze when compared to age matched controls. This study confirms that moderate levels of ethanol exposure to developing embryos have subtle effects on spatial working memory in adulthood. Our data thus suggest that zebrafish may be a promising model system for studying the effects of alcohol on learning and decision-making, but also for developing treatments and interventions to reduce the negative effects of prenatal alcohol.

1. Introduction

Consumption of alcohol by women during pregnancy can result in a range of physical and behavioral abnormalities in the fetus, symptoms which are collectively known as fetal alcohol spectrum disorders (FASDs) (Dörrie et al., 2014; van Wieringen et al., 2010). The most severe and easily diagnosed disorder is fetal alcohol syndrome (FAS), which is characterized by craniofacial malformations, central nervous system dysfunction, growth retardation and reduced intellectual abilities (Archibaldma et al., 2001; Mattson, 1998). Although FAS is an extreme case caused by high levels of chronic alcohol abuse, lower levels of alcohol intake have also been shown to cause a range of milder, less obvious symptoms including deficits in social behavior (Fernandes and Gerlai, 2009), decision-making and planning (Sood et al., 2001; Berman and Hannigan, 2000) and an increased susceptibility to substance abuse in later life, even following adoption (i.e., controlling for environmental effects (Cadoret et al., 1995)).

Though heavy chronic abuse of alcohol by a pregnant woman leads to obvious symptoms in the child, behavioral symptoms of milder cases of FASD are rarely accompanied by physical deformities and are thus problematic to diagnose (Astley and Clarren, 2000). As a result, the number of children affected by milder forms of FASDs is likely to be much higher than those reported (Stoler and Holmes, 1999; May et al., 2014). In the absence of physical symptoms, details of any alcohol consumption must derive from self-report and is open to response biases (Jacobson and Jacobson, 1994). Thus, to better understand the effects of amount, frequency and timing of exposure of the embryo to alcohol, animal models have been used to bridge the gap (Patten et al., 2014). Traditionally most animal models of FASDs have been carried out in rodents. However, recently zebrafish have come to light as an alternative model for neurobehavioral research, striking a balance between similarities with human and rodent models, complex behavioral interactions, ease of genetic manipulation, low cost of maintenance and high throughput (Kalueff et al., 2014; Kalueff, 2017; Kalueff and Cachat, 2011).

In rodents, the effects of moderate prenatal ethanol exposure on learning have been mixed and unclear, with some conflicting reports of effects on some aspects of learning (Patten et al., 2014; Abel, 1979;

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Carvan et al., 2004). This lack of consistency may be due to the complexities associated with rodent models of prenatal exposure, such as dosing regime (injection vs gavage vs voluntary drinking), maternal effects (i.e. during gestation) and effects of rearing (e.g., cross-fostering vs maternal rearing) (Marquardt and Brigman, 2016; Valenzuela et al., 2012). It is critical, therefore, to get a more developed understanding on the effects of moderate exposure to ethanol during early brain development, and zebrafish may offer a useful complementary model organism in which to achieve this.

Since the pioneering work from Fernandes and Gerlai (2009) and Carvan et al. (2004), zebrafish have been proving to be excellent models for examining the effects of low-to-moderate concentrations of ethanol exposure on the developing embryo on behavioral endpoints. For example, we have shown that zebrafish exposed as larvae to moderate amounts of alcohol display alterations (in adulthood) in social behavior and anxiety, an increased propensity to develop habits, and corresponding changes in mRNA expression in genes typically associated with the reward pathway, including dopamine, serotonin, u-opioid and nicotinic acetylcholine receptors (Parker et al., 2016; Parker et al., 2014). Despite some evidence that exposed embryos show reductions in ability to learn a spatial two-choice guessing task (Carvan et al., 2004) no studies have carried out a full assessment of the effects of moderate exposure to ethanol during early brain development on different aspects of learning and memory. The aim of this study was therefore to characterize aspects of learning and memory in adult zebrafish that have been exposed to moderate levels of ethanol during early brain development. We approached our aim by examining appetitive and aversive learning, and repetitive alternation in a novel unconditioned search protocol using a y-maze. Y-maze tests are widely used to measure exploratory behavior in rodents (Hughes, 2004; Dember and Fowler, 1959; Roberts et al., 1962) and spatial memory in zebrafish (Cognato et al., 2012).

2. Methods

2.1. Subjects and ethanol treatment

Embryos (AB wild-type strain) were collected from multiple individual pairings, sorted and cleaned, and placed at random (fish from each individual pairing mixed into final groups) in groups of ~40/petri dish in a translucent incubator (28 °C) on a 14/10-hour light/dark cycle. The concentration of ethanol used was based on our previous research, and the ethanol treatment protocol was as previously described (Parker et al., 2014; Parker et al., 2016). Briefly, at 48 h post-fertilization, embryos were visually inspected and sorted to ensure all were at the same developmental stage (long-pec phase), then transferred into multiple replicates (5/concentration) of either 20 mM (0.12 percent [v/v] ethanol in aquarium water (ethanol-treatment), or to fresh aquarium water with no alcohol (control). Our previous work, and that of others, has shown that 20 mM ethanol gives a final alcohol concentration of ~0.04 g/dl blood alcohol [BAC] (Fernandes et al., 2014; Parker et al., 2014). The reason for choosing 48 h to start treatment, is that by this stage all embryos have emerged from the chorion, so we can be sure the concentration of ethanol getting into the embryos is uniform. In addition, 48 hpf represents the long-pec phase of development, when the main catecholaminergic neural development takes place (Guo et al., 1999). At 5-days-post-fertilization, embryos were transferred, still in their treatment medium, into larger containers (10 × 10 × 20 cm [depth × width × length]) containing 500 ml solution (ethanol or aquarium water), and remained in the incubator. During treatment, water/ethanol media were changed daily. Fish remained in the treatment solution for 7 days, until 9-days post-fertilization, after which all fish were transferred within their initial treatment groups into fresh aquarium water and placed on our re-circulating system, initially in groups of 40 in 1.4 L tanks (Aquaneering Inc., San Diego, CA, USA). Juvenile zebrafish (at 1-month of age) were moved to groups of ~20 in 2.8 L tanks on the re-circulating system, on a 14/10-hour light/dark cycle, at ~28.5 °C(± 1 °C). Fish were tested on behavioral procedures at 3 months of age. Fish were fed a mixture of live brine shrimp and flake food 3 times/day (once/day at weekend). No fish was used for multiple protocols, and following the experiment, all remaining ethanol-exposed fish were euthanized (Aqua-Sed™, Vetark, Winchester, UK). We used a mixture of male and female fish for all behavioral testing. Previous research with zebrafish has not revealed sex effects for developmental alcohol exposure, and sex was not evaluated as a variable in this study. Finally, there were no differences in mortality or in gross morphology in any of the groups, although specific data are not reported here.

2.2. Ethical statement

All experiments were carried out following scrutiny by the University of Portsmouth Animal Welfare and Ethical Review Board, and under license from the UK Home Office (Animals (Scientific Procedures) Act, 1986) [PPL: P9D87106F].

2.3. Randomization and blinding

All experiments were carried out and reported under the ARRIVE guidelines (Kilkenny et al., 2010). First, all embryos were randomly allocated to treatment from multiple pair breedings. During treatment, experimental staff carried out ethanol treatment (see above) but technical staff were not aware of treatment allocation (blinded). This was achieved by putting the name of the experiment on treatment dishes and an individual dish identifier, but not indicating which level of treatment on the dish. This protocol was continued throughout development (i.e. when fish were on the housing rack). When testing was carried out, it was done so double-blinded; i.e., when fish were individually housed (for the appetitive learning protocol), each tank was numbered, but the treatment group was not known by either the experimenter or the technical staff. This was achieved by creating an excel sheet with identities in a hidden column. Identity was not revealed until data had been examined for outliers and analyses were carried out. For protocols where the fish came from a group (aversive learning and y-maze), fish were taken from numbered housing tanks, but the treatment level was not revealed until after data had been examined for outliers and was ready for final analysis. Finally, sample sizes for all experiments were determined by initial pilot studies or previous research (details in sections below).

2.4. Materials

Behavioral testing of adults was carried out using the Zantiks (Zantiks Ltd., Cambridge, UK) AD system (https://www.zantiks.com/products/zantiks-ad), a commercially available, fully integrated behavioral testing environment for adult zebrafish (Brock et al., 2017, and see Fig. 1). All tank inserts were acrylic, with opaque sides and inserts and a transparent base. The test tanks were placed into the Zantiks AD system (see Fig. 1A and B) one tank/time. Each Zantiks AD system was fully controlled via any mobile/web enabled device. Fig. 1B and C display the tanks used to measure appetitive learning. This Zantiks AD unit is designed to carry out multiple learning protocols in zebrafish but in the present experiment, fish were trained to swim into an initiator zone (Fig. 1C, red area) in order to receive a food reinforcer (ZM200 zebrafish food) in the food delivery zone (Fig. 1C, orange area). Tanks were filled with 3 L water during testing. The total length of the testing environment was 200 mm, and 140 mm width.

Fig. 2 displays the tanks equipment used for Pavlovian fear conditioning. The stimuli used were based on previous work (Brock et al., 2017; Valente et al., 2012) and comprised either a checker board design ('check') (black/white alternating squares) or a dark grey ('grey') background. Each tank comprised four lanes