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Brief communication

Short exposure to low concentrations of alcohol during embryonic development has only subtle and strain- dependent effect on the levels of five amino acid neurotransmitters in zebrafish

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ABSTRACT

The zebrafish has been successfully employed to model and study the effects of embryonic alcohol exposure. Short exposure to low alcohol concentrations during embryonic development has been shown to significantly disrupt social behavior as well as the dopaminergic and serotoninergic systems in zebrafish. However, analysis of potential effects of embryonic alcohol exposure on other amino acid neurotransmitter systems has not been performed. Here we analyzed neurochemicals obtained from adult AB and TU strain zebrafish that were immersed in 0.00% (control), 0.25%, 0.50%, 0.75% or 1.00% alcohol solution (vol/vol%) at 24 h post-fertilization for 2 h. From whole brain extracts, we quantified glutamate, aspartate, glycine, taurine and GABA levels using high performance liquid chromatography (HPLC). We found embryonic alcohol exposure not to have any significant effect on the levels of glutamate, aspartate, glycine and GABA in both AB and TU zebrafish. AB zebrafish showed a significant elevation of taurine levels, but only in the highest alcohol dose group compared to control. These results, albeit mainly negative, together with prior findings suggest that behavioral abnormalities resulting from embryonic alcohol exposure described before for AB zebrafish may primarily be due to altered dopaminergic and serotoninergic mechanisms. Furthermore, a Principal Component Analysis conducted with all neurochemicals tested in this and in our prior study, found a strain-dependent correlation structure response to embryonic alcohol treatment, confirming that embryonic alcohol effects may be genotype dependent.

1. Introduction

The zebrafish is a tropical freshwater species, gaining popularity in behavioral brain research partly due to the large number of genetic tools developed for it (Driever et al., 1994). In addition to these tools, zebrafish offer several practical advantages, including ease of maintenance and their prolific nature. Zebrafish have easily identifiable and measurable behaviors, including shoaling, a form of group forming social behavior (Miller and Gerlai, 2007). Zebrafish embryos develop quickly, reaching free-swimming stage in 5 days, and are transparent for this entire period, allowing observation of morphological development. Importantly, zebrafish fertilize externally, enabling manipulation of the embryo without potential confounding maternal factors. Zebrafish genes have high enough nucleotide sequence homology to human genes, and the function of proteins encoded by these genes has often been found evolutionarily conserved across these species warranting translational relevance (Stewart et al., 2014). The zebrafish is also excellent as a high-throughput screening tool. For example, randomly induced mutations affecting numerous target phenotypes, and eventually the genes carrying these mutations, can be identified with phenotypical screens (Amsterdam and Hopkins, 2006). Phenotypic screens also make the zebrafish a good model for identifying efficacious compounds (Macrae and Peterson, 2015).

The zebrafish is a social species, and exhibits shoaling, a phenomenon observed both in nature and in the laboratory, and one which may be induced and quantified in several ways (Al-Imari and Gerlai, 2008; Buske and Gerlai, 2011; Miller and Gerlai, 2007). For example, the sight of conspecifics has been shown to be rewarding for zebrafish, and to activate the dopaminergic system (Al-Imari and Gerlai, 2008; Engeszer et al., 2007; Saif et al., 2013). Shoaling has been shown to strengthen as the fish mature between their age of 7 and 100 days post-fertilization, and the levels of neurotransmitters dopamine, serotonin and their metabolites, DOPAC and 5HIAA, have been shown to increase during this maturation period in a manner that correlates with shoaling (Buske and Gerlai, 2012; Mahabir et al., 2013).

In the context of embryonic alcohol exposure, the zebrafish was first

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used for modeling Fetal Alcohol Syndrome, a severe form of Fetal Alcohol Spectrum Disorders (Carvan III et al., 2004). Subsequently, the zebrafish was also utilized to model the milder and more prevalent form of Fetal Alcohol Spectrum Disorders (Fernandes and Gerlai, 2009). In this latter model, alcohol administered at low concentration for a short period of time during embryonic development was shown to disrupt zebrafish shoaling as well as the dopaminergic and serotoninergic neurotransmitter systems (Buske and Gerlai, 2011; Fernandes and Gerlai, 2009; Mahabir et al., 2014). This suggested that dopamine, serotonin and their metabolites may mediate the effects of embryonic alcohol exposure induced changes in shoaling in zebrafish. However, the results were only correlative, and did not exclude the possibility that other neurotransmitter systems may also be involved.

Mahabir et al. (2014) investigated the effect of embryonic alcohol exposure on the development of functioning of the dopaminergic and serotoninergic neurotransmitter systems. These neurotransmitter systems have previously been found to be significantly disrupted by early embryonic ethanol treatment in zebrafish (Buske and Gerlai, 2011). Mahabir et al. (2014) quantified the level of dopamine, serotonin and the metabolites of these neurotransmitters in the brain of zebrafish using HPLC, and found that AB zebrafish exhibited a significant dose-dependent embryonic ethanol exposure induced reduction of dopamine, DOPAC, serotonin and 5HIAA. Importantly, embryonic ethanol induced impairments were found to become increasingly robust with age, a between subject (cross sectional) developmental analysis. The question remained, however, how specific the embryonic ethanol exposure effect was on these neurotransmitter systems. That is, the question of whether embryonic ethanol exposure had a global effect on brain development leading to retardation of the development of several neurotransmitter systems, not just the dopaminergic or the serotoninergic, was not addressed. The current study was conducted to investigate this possibility.

Here, using the same brain tissue samples from which dopamine, serotonin and their metabolites were previously quantified (Mahabir et al., 2014), we explore potential embryonic alcohol exposure induced changes in the levels of five other amino acid neurotransmitters in zebrafish. It is notable that in the Mahabir et al. (2014) study two strains of zebrafish were employed, AB and TU, and the ethanol effect was only found in AB. In the current study, we also analyze the effects of embryonic ethanol treatment on these two genetically distinct (Guryev, 2006; Meyer et al., 2013) strains of zebrafish. The rationale for using two genetically distinct strains of zebrafish is several fold. For example, identified genetic differences between strains in embryonic ethanol exposure induced neurochemical changes may be utilized for uncovering molecular mechanisms underlying alcohol effects. Furthermore, knowing about such strain (genetic) differences may also be useful for selecting the appropriate mapping and host strains in mutagenesis studies, and also for drug screening studies aimed at identifying small molecules that alter the effects induced by embryonic ethanol treatment.

2. Materials and methods

2.1. Housing and alcohol treatment

Adult zebrafish (*Danio rerio*) of AB and TU strains were maintained and bred in our vivarium (University of Toronto Mississauga, Mississauga, ON, Canada) to obtain fertilized eggs. The progenitors of AB and TU fish were obtained from the Zebrafish International Resource Center (Eugene, Oregon, USA). Experiments in this study were approved by the University of Toronto Animal Care Committee. Obtained eggs were placed in system water (deionized and sterilized water supplemented with 60 mg/l Instant Ocean Sea Salt (Big Al's Pet Store, Mississauga, ON, Canada)) until 24 h post-fertilization (hpf). To precisely time fertilization and limit variation in the developmental stage at which the embryos were, we placed females and males in a breeding tank separated by a perforated barrier the night before spawning was to occur. The following morning, after subjective "sunrise" (lights on) the barrier was removed, the fish were allowed to spawn, and the eggs were harvested within 10 min. This method ensured that all embryos became fertilized at the same time point, and thus their development was temporally synchronized. At 24 h post-fertilization (hpf), eggs were immersed in one of the following alcohol concentrations: 0.00%, 0.25%, 0.50%, 0.75% or 1.00% (vol/vol%) for a duration of 2 h, a between subject experimental design. Immediately following exposure, the eggs were washed three times with system water. Hatched eggs were placed in nursery racks and larvae were fed Larval Artificial Plankton 100 (particle size below 100 μm, Zeigler Bros, Inc., Gardners, PA, USA), and subsequently by freshly hatched brine shrimp nauplii (*Artemia salina*). At 3 weeks post- fertilization fish started to be fed ground flake food (a mixture of 3:1 Tetramin flake, Melle, Germany, and Spirulina, Jemco Inc., Lambertville, New Jersey) once a day. Fish were housed at no > 10 fish/3 l density. All zebrafish utilized in this study were adults (102 days post-fertilization, dpf). The sex composition of all our experimental groups was on average 50%–50% male-female. Specifically, the male to female ratio for the AB strain concentration groups was as follows: 0.00% alcohol, 3:2; 0.25% alcohol, 6:3; 0.50% alcohol, 4:6; 0.75% alcohol, 4:3; 1.00% alcohol, 5:4. The male to female ratio for the TU strain concentration groups was as follows: 0.00% alcohol, 4:4; 0.25% alcohol, 6:4; 0.50% alcohol, 5:3; 0.75% alcohol, 8:6; 1.00% alcohol, 4:4. No significant sex effect or sex x alcohol, sex × alcohol × strain or sex × strain interactions were detected and thus sexes are pooled for data analysis.

2.2. High performance liquid chromatography (HPLC)

Levels of 5 amino acid neurotransmitters (glutamate, aspartate, glycine, taurine and GABA) were quantified using high performance liquid chromatography (HPLC) from the tissue samples previously used for the analysis of dopaminergic and serotoninergic neurochemicals the same manner described before (Mahabir et al., 2014). Briefly, at 102 dpf, fish were decapitated, their brains dissected on ice and processed as described before (Mahabir et al., 2014). To ensure stability of the samples, we analyzed amino acid neurotransmitters within a week after the completion of this previously published study (Mahabir et al., 2014). Briefly, immediately following the analysis of dopamine, DOPAC, serotonin and 5HIAA, the tissue samples were stored at −80 °C for one week before analysis of the amino acid neurotransmitters quantified in the current study. This one week period was required to allow for cleaning of the HPLC machine and changing of the column specific to the quantification of glutamate, aspartate, glycine, taurine and GABA. During this storage period we ascertained that the temperature in the freezer remained constant at −80 °C. Brains were dissected on ice, suspended in 20 μl of artificial CSF (Harvard instrument) and sonicated on ice. Total protein content of the homogenate was determined using 2 μl of the sonicated tissue sample using BioRad protein assay dye reagent (Bio Rad), and subsequently used to normalize the neurotransmitter values expressed as pmol/mg brain protein (see Mahabir et al., 2014 for further details). For HPLC analysis, 1 μl of stabilizer (0.2 N perchloric acid and 1.0 M ascorbic acid) was added to each sample to avoid the possibility of sample degradation and centrifuged at 10000 rpm for 10 min at 4oC. Supernatant was collected and stored at −80 °C until use. Notably, Thorré et al. (1997) showed that excellent sample stability may be achieved by adding antioxidant, as employed by us, and Boomsma et al. (1993), Zhang et al. (2001), and Zimmermann et al. (2011) demonstrated that catecholamines were stable for at least one year if stored at −70 °C, and even when thawed and refrozen twice. Amino acid neurotransmitters were analyzed in brain supernatants in a high precision liquid chromatography (BASi 460 MICROBORE-HPLC system) adapted for zebrafish as described before (Chatterjee et al., 2014) using an Uniget C-18 reverse phase microbore column as the stationary phase (BASi Cat # 8912) and electrochemical

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