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STRAIN DEPENDENT NEUROCHEMICAL CHANGES INDUCED BY EMBRYONIC ALCOHOL EXPOSURE IN ZEBRAFISH

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Abstract

Fetal Alcohol Spectrum Disorder (FASD) is a preventable disease of the child resulting from alcohol (ethanol) consumption by pregnant women. Despite being preventable, FASD represents a prevalent problem throughout the world. Embryonic alcohol induced abnormalities in behavioral responses to social stimuli have been shown in humans and zebrafish. The neurobiological mechanisms underlying the abnormalities remain obscured. Here we start a mechanistic analysis by investigating the effect of embryonic alcohol exposure on the neurochemistry of zebrafish. The differing severity of symptoms seen in FASD may be partially due to genetic factors. To explore such genetic effects, here we analyzed two distinct zebrafish strains: AB and TU. Zebrafish were exposed to one of the following concentrations of alcohol, 0.00%, 0.25%, 0.50%, 0.75%, 1.00% (vol/vol %) at 24 hours post-fertilization (hpf) for 2 hours. From whole brain extracts we analyzed the amount of neurotransmitters dopamine and serotonin and their metabolites across 4 different developmental time points: 15, 40, 70 and 102 days post-fertilization (dpf) using high performance liquid chromatography (HPLC). AB zebrafish exhibited a significant dose dependent embryonic alcohol exposure effect which increased in robustness with age. However, TU showed no such concentration effect: the levels of neurochemicals remained mainly unaltered by embryonic alcohol exposure in all age groups. We also analyzed the amount of alcohol reaching the embryo in the two strains and ruled out the possibility that TU has a more protective chorion. We conclude that the uncovered strain differences are due to genetic differences that protect TU from the deleterious effects of embryonic alcohol exposure.

Keywords

FASD; Embryonic alcohol; ethanol; dopamine; serotonin; zebrafish

1. Introduction

Fetal Alcohol Spectrum Disorder (FASD) is the umbrella term used to describe the range of symptoms that is observed in children who have been exposed to alcohol in utero (Riley and

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Conflict of interest

Authors declare no conflict of interest

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McGee, 2005; Stade et al., 2006). The magnitude of these symptoms can range from severe to mild and examples of these include anatomical malformations, cognitive problems and behavioural abnormalities (Stratton et al., 1996; Mattson et al., 1998; Riley and McGee, 2005). The prevalence of FASD is approximately 9 per 1000 live births world wide, and the average annual cost of FASD only in Canada is approximately 5.3 billion dollars (Abel et al., 1987; Harwood et al., 1999). Because this disease is one of the leading causes of developmental disability causing immeasurable and lifelong suffering to the patient and because it represents an enormous financial burden to our society, numerous researchers have been attempting to understand how this teratogenic drug affects the embryo and its brain.

Model organisms have been successfully used to study the mechanisms involved in how alcohol exerts its effects on the developing embryo (Meyer et al., 1990; Becker et al., 1996; Fernandes and Gerlai, 2009; Kelly et al., 2009; McClure et al., 2011; Church et al., 2012). Recently, the zebrafish has been suggested to offer some advantages in this research (Fernandes & Gerlai, 2009). First, the zebrafish is externally fertilized and thus its embryos can be exposed to alcohol in a precise manner at exact developmental stages as desired (Tanguay and Reimers, 2008). Second, the development of the zebrafish brain has been well documented (Wullimann et al., 1996), which may aid interpretation of alcohol induced alterations in brain structure and function.

The first pioneering studies that employed low levels and short periods of embryonic alcohol exposure with zebrafish (AB strain) have found reduction of behavioral responses to social stimuli in the alcohol exposed fish (Fernandes and Gerlai, 2009), an effect that resembled the social behavioral abnormalities recently discovered in human FASD children (Stevens et al., 2012). Buske and Gerlai (2011) replicated the previously observed social impairment using a behavioral paradigm that differed in many respects from that of the original study (Fernandes and Gerlai, 2009) and also discovered that the impaired response to social stimuli was accompanied by reduced levels of neurotransmitters dopamine, serotonin and their metabolites in AB zebrafish.

What may underlie these neurochemical changes, i.e. how embryonic alcohol treatment altered brain development leading to abnormal adult CNS function is a complex question because potentially a large number of factors may need to be considered and investigated. To start unraveling this complexity we have decided to take a genetic approach. We decided to compare two genetically distinct strains, AB and TU, hoping to identify some differences between them in their response to embryonic alcohol treatment. Demonstration of such differences would open a new research avenue into the identification of the underlying genes and thus into the eventual understanding of the biological mechanisms involved. Apart from the investigative, i.e. practical advantage of strain comparison studies, such approach also has translational relevance. Alcohol- related disorders have been shown to have significant genetic components in humans (Streissguth and Dehaene, 1993) and strain comparisons have been successfully conducted using model organisms (Russo et al., 2008; Downing et al., 2009). Strain differences have also been identified in zebrafish (Scerbina et al., 2012) including the effects of alcohol on embryonic survival (Loucks and Carvan, 2004) or on behaviour, neurochemical responses and gene expression (Gerlai et al., 2009; Pan et al., 2012). In a study comparing the zebrafish strains AB and SF, researchers found dopamine receptor antagonists to induce changes in the level of neurotransmitters in the brain of AB but not of SF (Scerbina et al., 2012). The already identified strain differences in zebrafish suggest that genes indeed play a significant role in how alcohol exerts its effect on the zebrafish brain.

Here, we chose to compare two zebrafish strains, AB and TU, that are among the most inbred showing homozygosity in about 80 % of their loci (Guryev, 2006). Also notably, these strains are expected to be highly genetically distinct (Meyer et al., 2013). If a difference between these two strains is found, it will enable one to perform quantitative trait loci analysis (QTL) leading to the eventual identification of genes underlying the natural genetic variance between the strains. Detecting no difference among the strains is also a potentially useful finding. Such result is the minimal requirement for using two strains as host and mapping strains in a random mutagenesis study. Traditionally such mutagenesis is achieved with the chemical mutagen ENU, and the mutated “host” genome is crossed to a mapping genome, which allows subsequent linkage analysis-based positional cloning.

Here we investigate the neurochemicals dopamine and serotonin as well as their metabolites 3, 4-dihydroxyphenylacetic acid (DOPAC) and 5- hydroxyindoleacetic acid (SHIAA) because these neurochemicals have been shown to be affected by embryonic alcohol exposure in zebrafish (Buske and Gerlai, 2011). We hypothesize that embryonic alcohol exposure will lead to a reduction in neurotransmitter levels in the zebrafish brain and we expect this effect to be observable in the AB strain. We do not yet know if similar alcohol induced changes occur in the TU strain. We have found an altered trajectory of maturation of social behavioral responses (shoaling) in TU as compared to AB (Mahabir et al., 2013) and thus here we perform the neurochemical analyses at four different time points throughout the development of zebrafish to capture potential developmental stage-dependent effects.

Furthermore, we also investigate the amount of alcohol inside the egg, after immersion to the alcohol solution, to precisely determine how much alcohol reaches the embryo when AB and TU eggs are immersed in the same external alcohol concentration bath.

2. Methods

2.1. Housing and alcohol treatment

Adult zebrafish (*Danio rerio*) of AB and TU strains were bred to obtain fertilized eggs used in this study. All fish bred were kept in our facility (University of Toronto Mississauga Vivarium, Mississauga, ON, Canada). The progenitors of our breeding population were obtained from the Zebrafish International Resource Center (Eugene, Oregon, USA). All experiments described below were approved by the University of Toronto Animal Care Committee. Eggs were bathed 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 hours post- fertilization. At that time point each group of eggs were immersed in one of the following concentrations of alcohol solution 0.00%, 0.25%, 0.50%, 0.75%, or 1.00% (vol/vol% percentage). The length of alcohol immersion was 2 hours, after which the eggs were immediately washed with system water three times. The applied alcohol concentrations and exposure regime were based upon prior studies that demonstrated this mild alcohol treatment to result in significant behavioral changes (Fernandes and Gerlai, 2009; Buske and Gerlai, 2011). Eggs hatched normally at around 3 dpf, and at 5 dpf the developing fish reached free swimming state, at which time they were placed in nursery racks where they were fed initially on Larval Artificial Plankton 100 (particle size below 100 μ m, ZeiglerBros, Inc., Gardners, PA, USA) and subsequently on freshly hatched brine shrimp nauplii (*Artemia salina*). At age 3 weeks post-fertilization the developing fish were started to be fed a 1:1 mixture of flake food (Tetramin Tropical fish flake food, Tetra Co, Melle, Germany) and powdered spirulina algae (Jehmco Inc., Lambertville, NJ, USA) which continued to adulthood.

2.2. High Performance Liquid Chromatography (HPLC)

A cross-sectional developmental analysis was performed for the quantification of the amount of neurochemicals using high performance liquid chromatography (HPLC). HPLC was carried out at 4 different age points throughout development: 15, 40, 70 and 102 dpf. These age groups were chosen to closely match a recent study (Mahabir et al., 2013) that found strain dependent changes in the amount of neurochemicals as zebrafish matured.

For tissue harvesting, fish were decapitated rapidly and their brains were dissected on ice under a dissecting microscope. Brains were kept frozen in a microcentrifuge tube at -80°C until further processing. First, the weight of total protein content of the samples was determined and subsequently the amount of neurochemicals measured was standardized to the obtained total brain protein weight as described before (Chatterjee and Gerlai, 2009). Briefly, sonicated brain tissue was used for the protein assay. Bio-Rad Dye reagent was prepared by diluting 1 part Dye Reagent Concentration with 4 parts distilled, deionized (DDI) water. $2.5\ \mu\text{l}$ of each brain homogenate solution was taken and $5.0\ \text{ml}$ of the diluted dye reagent was added to each tube and vortexed. The samples were incubated at room temperature for 20 minutes. Using a spectrophotometer (Biomate3, Thermo Election Corporation) measurement of absorbance at $595\ \text{nm}$ was taken to quantify protein content. Bovine serum albumin (Protein standard, Sigma chemicals, P0834) was used as a standard. To perform HPLC, the samples were thawed and suspended in $20\ \mu\text{l}$ artificial cerebrospinal fluid (ACSF, Harvard). Brains were sonicated and $2\ \mu\text{l}$ of the solution was analyzed for protein content. $1\ \mu\text{l}$ of stabilizer was added to the sample and centrifuged. The supernatant was collected and stored at -80°C . HPLC analysis for dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin and 5-hydroxyindoleacetic acid (5-HIAA) was carried out using a BAS 460 MICROBORE HPLC system with electrochemical detection (Bio-analytical Systems Inc., West Lafayette, IN, USA) together with a Uniget C-18 reverse phase microbore column as the stationary phase (BASi, Cat no. 8949), a method specifically adapted to zebrafish and described before (Chatterjee and Gerlai 2009). At 15 dpf 5 brains pooled per sample, at 40 dpf 2 brains pooled per sample, and at 70 and 102 dpf 1 brain per sample were sufficient to reach appropriate detection thresholds. The sample size of treated fish were as follows for AB fish at 15 dpf: 0.00% alcohol, $n=8$; 0.25% alcohol, $n=8$; 0.50% alcohol, $n=8$; 0.75% alcohol, $n=8$; 1.00% alcohol, $n=8$. At 40 dpf: 0.00% alcohol, $n=8$; 0.25% alcohol, $n=8$; 0.50% alcohol, $n=8$; 0.75% alcohol, $n=8$; 1.00% alcohol, $n=11$. At 70 dpf: 0.00% alcohol, $n=10$; 0.25% alcohol, $n=10$; 0.50% alcohol, $n=10$; 0.75% alcohol, $n=10$; 1.00% alcohol, $n=10$; and at 102 dpf: 0.00% alcohol, $n=8$; 0.25% alcohol, $n=10$; 0.50% alcohol, $n=10$; 0.75% alcohol, $n=7$; 1.00% alcohol, $n=9$. The sample size of treated fish were as follows for TU fish at 15 dpf: 0.00% alcohol, $n=8$; 0.25% alcohol, $n=7$; 0.50% alcohol, $n=8$; 0.75% alcohol, $n=8$; 1.00% alcohol, $n=8$. At 40 dpf: 0.00% alcohol, $n=8$; 0.25% alcohol, $n=9$; 0.50% alcohol, $n=8$; 0.75% alcohol, $n=8$; 1.00% alcohol, $n=8$. At 70 dpf: 0.00% alcohol, $n=10$; 0.25% alcohol, $n=10$; 0.50% alcohol, $n=10$; 0.75% alcohol, $n=7$; 1.00% alcohol, $n=10$ and at 102 dpf: 0.00% alcohol, $n=8$; 0.25% alcohol, $n=10$; 0.50% alcohol, $n=8$; 0.75% alcohol, $n=14$; 1.00% alcohol, $n=8$. Note that the listed sample sizes represent the unit of statistical comparison and not the number of individual brains used because, as explained above, in case of the younger (15 and 40 dpf old) fish we needed to pool multiple subjects to obtain sufficient amount of tissue for the HPLC analysis. HPLC results are expressed relative to the quantified total brain protein weight.

2.3. Quantification of alcohol content inside the egg

The external alcohol concentration that reaches the developing embryo inside the egg may differ among zebrafish strains. A difference, if present, between strains in the amount of alcohol that actually reaches the embryo may contribute to strain-dependent neurochemical

changes. Prior studies successfully quantified the concentration of alcohol reaching the embryo (Lockwood et al., 2004; Fernandes and Gerlai, 2009; Echevarria et al., 2011), however, strain comparisons have not been conducted. Therefore, we measured the amount of alcohol inside the eggs of the AB and TU strains of zebrafish. Zebrafish eggs were exposed to alcohol as described above. Subsequently, the eggs were transferred to a microcentrifuge (10 eggs per tube per alcohol concentration). The eggs were sonicated using a sonicator at 3 pulses for 4 sec in 0.1 ml distilled water and the solution was centrifuged at 10000 rpm for 10 minutes at 4 °C. Subsequently, the supernatant was transferred to another microcentrifuge tube and analyzed for alcohol concentration using the AM1 Alcohol Analyser (Analox Instruments, London, UK), which utilizes an enzymatic reaction that converts alcohol into oxygen and then quantifies the oxygen levels using a sensitive oxygen probe. For full details and application of this methodology for zebrafish see Fernandes & Gerlai (2009). The sample size for treated embryos were as follows for the AB strain: 0.00% alcohol, $n=7$; 0.25% alcohol, $n=5$; 0.50% alcohol, $n=5$; 0.75% alcohol, $n=5$; 1.00% alcohol, $n=8$; and for the TU strain: 0.00% alcohol, $n=7$; 0.25% alcohol, $n=5$; 0.50% alcohol, $n=5$; 0.75% alcohol, $n=5$; 1.00% alcohol, $n=7$. Note that the sample sizes indicated represent the unit of statistical analysis and each sample contained 10 embryos.

2.4. Statistical Analysis

Data were analyzed using SPSS (version 14.1) for the PC. Three factor univariate non-repeated measure ANOVAs were performed with age (4 levels), strain (2 levels) and alcohol concentration (5 levels) as between subject factors. In case of significant main effects and/or interactions, post-hoc ANOVAs and Tukey Honestly Significant Difference (HSD) were conducted. Significance was accepted when the probability of the null hypothesis was less than 5% ($p < 0.05$).

3. Results

3.1. HPLC

Analysis of total weight of the protein quantified from the brain tissue samples taken for each age x strain group showed no significant alcohol treatment effect for all groups ($F(4, 38) < 2.0$, $p > 0.10$) except for the 102 dpf old AB fish ($F(4, 39) = 7.116$, $p < 0.001$), in which the control fish were found to have significantly ($p < 0.05$) less protein compared to the alcohol treated group.

Analysis of the HPLC results confirmed previous findings and showed a robust concentration dependent reduction in neurotransmitter levels in response to embryonic alcohol in adult AB fish (Buske and Gerlai, 2011). Dopamine, serotonin and the metabolites of these neurotransmitters have been found to be affected by embryonic alcohol exposure (Buske and Gerlai, 2011). Here, we compared zebrafish strains AB and TU for potential age x alcohol dependent changes in dopamine, DOPAC (dopamine's metabolite), serotonin and 5HIAA (serotonin's metabolite).

We found embryonic alcohol concentration dependent decrease in dopamine levels in the AB strain. These concentration induced differences appeared to become increasingly robust as the fish matured (Figure 1, upper set of graphs). Interestingly, such age x concentration interaction was not apparent in TU, which showed no obvious effect of the alcohol exposure, although exhibited clear age-dependent changes in dopamine levels (Figure 1 lower set of graphs). These observations were confirmed by ANOVA, which revealed a significant Alcohol ($F(4, 312) = 38.667$, $p < 0.0001$) and Age effect ($F(3, 312) = 430.933$, $p < 0.0001$), no significant Strain effect ($F(1, 312) = 0.013$, $p = 0.910$), but a significant Alcohol x Age ($F(12, 312) = 5.735$, $p < 0.0001$), Alcohol x Strain ($F(4, 312) = 21.056$,

$p < 0.0001$), Age x Strain ($F(3, 312) = 5.218$, $p < 0.05$) and Alcohol x Age x Strain interaction ($F(12, 312) = 3.811$, $p < 0.0001$). Given the significant Strain and Age interactions with alcohol concentration, we subsequently conducted post hoc ANOVAs separately for each age group per strain to analyze the effect of alcohol concentration. These analyses showed that in AB, alcohol concentration had no effect at age 15 dpf (ANOVA $F(4, 35) = 1.051$, $p > 0.35$) but for AB fish of 40 dpf age and older it did ($F(4, 38) > 75.65$, $p < 0.001$). Subsequent Tukey's HSD tests showed that for these older (40, 70 and 102 dpf) AB fish all concentration groups significantly ($p < 0.05$) differed from one another. The results were very different for TU. These fish showed no concentration-dependent changes in dopamine levels at any age (ANOVA $F(4, 42) < 1.84$, $p > 0.13$).

The pattern of results was similar for DOPAC. Figure 2 shows the apparent alcohol concentration dependent decrease in DOPAC levels with increasing robustness as the fish matured for the AB strain (figure 2 upper set of graphs) but no such alcohol effect for TU (figure 2 lower set of graphs). These observations were confirmed by ANOVA, which revealed a significant Alcohol ($F(4, 312) = 7.115$, $p < 0.0001$) and Age effect ($F(3, 312) = 64.926$, $p < 0.0001$), no significant Strain effect ($F(1, 312) = 0.495$, $p > 0.05$), but significant Alcohol x Age ($F(12, 312) = 2.746$, $p < 0.05$), Alcohol x Strain ($F(4, 312) = 4.62$, $p < 0.05$) and Age x Strain interaction ($F(3, 312) = 3.328$, $p < 0.05$). The Alcohol x Age x Strain interaction term was found non-significant ($F(12, 312) = 0.997$, $p > 0.05$). Subsequent post hoc ANOVAs conducted separately for each strain x age group revealed that at 15 dpf AB fish showed no significant alcohol effect ($F(4, 35) = 0.585$, $p > 0.65$) but in the older groups (40, 70 and 102 dpf) all AB fish exhibited the significant alcohol concentration dependent decrease ($F(4, 38) > 8.05$, $p < 0.001$). Tukey HSD tests confirmed that as the fish got more mature the differences among the concentration groups became more robust. For example, in the most mature (102 dpf old) AB fish all concentration groups differed ($p < 0.05$), i.e. a 0.25% alcohol concentration increase was sufficient to lead to a significant change across all doses employed. However, in the 70 dpf old fish only those concentration groups were found significantly ($p < 0.05$) different from each other whose embryonic treatment differed by at least 0.50% alcohol concentration, and in the 40 dpf fish this alcohol concentration dependent effect was even less pronounced. The results obtained with zebrafish of the TU strain was again dramatically different compared to those obtained for AB. TU fish showed no alcohol concentration related differences at any age ($F(4, 42) < 1.34$, $p > 0.25$) except at age 40 dpf ($F(4, 36) = 5.368$, $p < 0.01$) where the highest concentration group (1 % alcohol) was found to exhibit a significantly smaller (Tukey HSD, $p < 0.05$) DOPAC level value compared to all but the 0.50% alcohol concentration exposed group.

Serotonin levels also appeared to be affected by embryonic alcohol exposure in AB (figure 3 upper set of graphs) but not in TU zebrafish (figure 3 lower set of graphs). The alcohol concentration dependent effect in AB is similar to what we found for DOPAC becoming especially robust by 70 dpf. It is also notable that unlike in dopamine and DOPAC, this latter age group (70 dpf) exhibited the highest level of this neurotransmitter (serotonin) both in case of AB and TU zebrafish. ANOVA confirmed most of these observations and found a significant Alcohol ($F(4, 312) = 13.010$, $p < 0.0001$) and Age effect ($F(3, 312) = 206.105$, $p < 0.0001$) but no significant Strain effect ($F(1, 312) = 0.261$, $p > 0.05$). The Age x Strain interaction was also non-significant ($F(3, 312) = 0.947$, $p > 0.05$). However, the Alcohol x Age ($F(12, 312) = 4.088$, $p < 0.0001$), Alcohol x Strain ($F(4, 312) = 11.241$, $p < 0.0001$) and Alcohol x Age x Strain interaction terms ($F(12, 312) = 2.167$, $p < 0.05$) were found significant. Subsequent ANOVAs conducted separately for each age and strain group showed that embryonic alcohol treatment led to no significant differences among the treatment groups at age 15 dpf ($F(4, 35) = 2.443$, $p > 0.05$). But by age 40 dpf the effect became significant ($F(4, 38) = 5.813$, $p < 0.01$) because the two highest (1.00% and 0.75% alcohol) concentration groups started to differ from the control (0.00% alcohol) group

(Tukey HSD, $p < 0.05$). At older ages (70 and 102 dpf) the differences became more pronounced (ANOVA $F(4, 45) > 20.69$, $p < 0.001$) with concentration groups differing at least by 0.50% embryonic alcohol exposure becoming significantly (Tukey HSD, $p < 0.05$) different in their serotonin levels. Embryonic alcohol exposure was mainly ineffective in TU strain zebrafish. ANOVA found no significant alcohol effects in all ($F(4, 43) < 2.26$, $p > 0.05$) but the 40 dpf age group ($F(4, 36) = 4.142$, $p < 0.01$). In the latter, Tukey HSD revealed that the highest concentration group (1% alcohol) significantly ($p < 0.05$) differed from the two lowest concentration (0.00 and 0.25% alcohol) groups, while other group differences remained non-significant.

The levels of serotonin's metabolite 5HIAA changed similarly in response to embryonic alcohol treatment as compared to those of serotonin's (figure 4). ANOVA demonstrated a significant Alcohol ($F(4, 312) = 18.374$, $p < 0.0001$) and Age effect ($F(3, 312) = 440.604$, $p < 0.0001$) but found no significant Strain effect ($F(1, 312) = 0.175$, $p > 0.05$). The Alcohol x Age x Strain interaction was also non-significant ($F(12, 312) = 1.573$, $p > 0.05$) but the Alcohol x Age ($F(12, 312) = 3.878$, $p < 0.0001$), Alcohol x Strain ($F(4, 312) = 9.156$, $p < 0.0001$) and Age x Strain interaction ($F(3, 312) = 3.971$, $p < 0.05$) terms were found significant. Subsequent ANOVAs conducted separately for each age x strain group showed that at age 15 dpf AB fish showed no embryonic alcohol treatment induced changes ($F(4, 35) = 0.716$, $p < 0.55$), but by age 40 dpf and onward the treatment effect became significant ($F(4, 38) > 5.88$, $p < 0.01$). Again, the robustness of differences among treatment groups, and also the level of significance, increased as the fish matured. Unlike in AB fish, the effect of embryonic alcohol treatment was found non-significant for all age groups of TU strain zebrafish ($F(4, 34) < 2.06$, $p > 0.10$).

3.2. Amount of alcohol inside the egg

The above results revealed a robust lack of embryonic alcohol treatment induced changes in TU strain zebrafish. The absence of alcohol effect may be due to numerous factors one of which may be a protective egg-shell/chorion. If alcohol does not reach the inside of the egg, it cannot exert its deleterious effects on the embryo, which may manifest as lack of neurochemical abnormalities later in life in TU vs. AB fish. To explore this possibility, i.e. to answer the question whether a different amount of alcohol reached the embryo in AB vs. TU fish, we quantified the amount of alcohol inside the egg after immersing the eggs the same way as described for the HPLC studies. We found that the concentration inside the egg linearly correlated with the concentration of alcohol in the external bath in which the eggs were placed (figure 5). We also found that fish of AB and TU strain origin had comparable alcohol levels. These observations were confirmed by ANOVA, which found a significant Alcohol effect ($F(4, 49) = 67.180$, $p < 0.0001$) and a non-significant Strain effect ($F(1, 49) = 3.982$, $p > 0.05$). Importantly, the Strain x Alcohol interaction was also non-significant ($F(4, 49) = 1.401$, $p > 0.05$).

4. Discussion

The current study reports significant embryonic alcohol exposure induced reduction of the levels of dopamine, serotonin, DOPAC and 5HIAA from whole brain extracts of AB strain zebrafish using HPLC. Our results confirm previous findings (Buske and Gerlai, 2011) that showed robust alcohol concentration dependent reduction in the levels of these neurochemicals in the AB strain and also coincide with what has been found in other species. For example, in children and rhesus monkeys too prenatal alcohol exposure has been shown to lead to abnormalities in the serotonergic and dopaminergic neurotransmitter systems (Riikonen et al., 2005; Schneider et al., 2005). The reduced levels of dopamine and serotonin found in the maturing and adult AB zebrafish suggest reduction of neurotransmitter synthesis arising from the early embryonic alcohol insult, while the

reduced levels of DOPAC and 5HIAA suggest diminished metabolism of the corresponding neurotransmitters possibly due to reduced neurotransmission. Perhaps most interestingly, however, we found that such impairments were not induced in another zebrafish strain, TU. AB and TU are two of the genetically distinct strains used in linkage analysis-based mapping after mutagenesis (Brown et al., 2012 and references therein). Differences between the AB and TU strains have been reported in a number of phenotypes including locomotor activity and reactivity to sudden changes in illumination of the larvae, and exploratory behavior and learning performance of the adult as well (Vignet et al., 2013). Differences in the context of alcohol research have also been detected between the TU and the AB strains of zebrafish. For example, Loucks and Carvan (2004) examined zebrafish strains Ekk, AB and TU in response to extended alcohol exposure from 2 hours post-fertilization to 6 days post-fertilization and found that strain TU had the lowest survival rate but was more resistant to alterations in neurocranial and craniofacial skeletal features in comparison to the AB strain. Our results demonstrate that exposure to even very low doses of alcohol for as short a period as 2 hours can have long lasting deleterious effects on the fish brain. It is also notable that we found TU to be resistant to the effects of this mild embryonic alcohol exposure at least at the level of neurochemicals examined. Our own results together with prior studies employing higher doses and/or longer exposure periods (e.g. Loucks & Carvan, 2004) raise the possibility that the effect of alcohol is not only dose dependent but also that there may be a concentration x strain interaction that is specific to the examined phenotypical trait.

We emphasize that the concentration of alcohol inside the egg in the current study was found robustly lower than that of the outside bath employed. For example, in case of the lowest dose group (0.25% alcohol) the alcohol concentration inside the egg was found to be less than 0.02%, i.e. about 1/12th of the external dose, a finding similar to what has been described by others before (Lockwood et al., 2004; Echevarria et al., 2011; Cole et al., 2012). This concentration may be even lower in the embryo itself, a question that will be studied in the future. Why could we not see embryonic alcohol exposure induced changes in neurochemical levels in the TU strain zebrafish when such changes were evident in the AB?

The answer to this question remains speculative at this point. The strain differences could arise as a result of many factors. The two main types of possibilities include one, physiological or physical protection from alcohol, and two, central nervous system related mechanisms. We argue that physical protection from alcohol exposure (a barrier that makes alcohol unable to penetrate the embryo) is unlikely to be behind the observed strain differences. We measured the concentration of alcohol inside the egg after the employed 2 hour long bath immersion procedure and found the levels of alcohol comparable between the two strains. Although we have not measured the alcohol concentration inside the embryo itself, the above result suggests that the embryos of TU and AB fish received the same doses of alcohol. In pharmacology analyses, possible sources of differential drug effects unrelated to drug-target binding characteristics are summed up as ADME, i.e. absorption, distribution, metabolism, and excretion of the drug. In our case, the comparable alcohol levels we found inside the egg of TU and AB strain zebrafish likely address the question of absorption. But we also propose that putative differences between the two strains in processes associated with distribution, metabolism and excretion of alcohol are also unlikely to underlie the embryonic alcohol exposure induced neurochemical differences between the strains reported here. This is because the egg with the embryo inside was bathed in alcohol for two hours and this immersion procedure makes it unlikely that the substance may be differentially distributed in the two strains. Differential strain dependent metabolism and excretion of alcohol is also unlikely because, due to the uninterrupted bath application, this substance continually diffuses into the embryo over a prolonged period (two hours) from the solution surrounding the egg. Thus, although we lack concrete evidence, due to the method of drug

delivery (prolonged immersion in the alcohol solution), we propose that the strain differences are unlikely to be the result of differential mechanisms associated with ADME.

The strain differences were not obvious (significant) at the first age we tested our experimental fish, i.e. at age 15 dpf. Nevertheless, AB fish showed a robust age x alcohol concentration interaction while fish of the TU strain remained consistently unaffected by the embryonic alcohol exposure irrespective of at what age we analyzed them. In AB, we found embryonic alcohol exposure to lead to no changes at age 15 dpf, but as the fish matured we observed the effects to manifest at 40 dpf and to become more robust in the older fish. Previously, we have shown that levels of neurochemicals (relative to total brain protein weight) increase with age, i.e. the neurotransmitter systems mature in zebrafish (Buske and Gerlai, 2012). We propose that the observed age dependent alcohol effects seen in AB may be due to this maturation trajectory. In younger fish (15 dpf and younger), i.e. when the neurotransmitter systems are less well developed, we may be facing a floor effect and may not be able to see the abnormalities induced by exposing the fish to alcohol at their 24 hpf age. Alternatively, in the 15 dpf fish there may be no such alcohol induced effects existing yet, at least at the level of the studied neurotransmitter systems, due to the underdeveloped stage of these systems. At this point we cannot distinguish between these possibilities because we do not yet know the mechanisms underlying the observed neurochemical impairments.

Another important question that will need to be examined in the future is whether the observed embryonic alcohol induced impairment in AB zebrafish is a permanent damage or, alternatively, whether it represents delayed development. In principle, it is possible that embryonic alcohol exposed AB fish older than the studied 102 dpf may eventually regain their normal function, i.e. reach normal levels of their neurochemicals. This possibility, however, is not very likely, because a 90 dpf old zebrafish is already a fully mature sexually active young adult after which age the fish do not mature further in any appreciable manner. Nevertheless, the idea of developmental delay brings up another important point: the question whether untreated healthy AB and TU zebrafish develop at the same rate or not. This is an important question for two reasons. One, in principle, it may be possible that the 24 hpf time point we chose for alcohol exposure represents different developmental stages for the two strains studied. Two, the chosen ages at which we tested the two strains may represent different stages of maturation for these strains. We suggest, however, that the latter is not relevant in the context of the current study because TU fish were found not to exhibit any embryonic alcohol exposure induced changes at any age, from 15 to 102 dpf. It is also notable that AB and TU were found to develop with a statistically indistinguishable rate as far as body size (length) is concerned (Mahabir & Gerlai, 2013). The former possibility (i.e. differential embryonic development around 24 hpf impacting the effect of alcohol administered that time) will be examined by systematically altering the time of alcohol exposure in the two strains and examining the effect of the alcohol exposure on neurochemistry of these fish.

The last point we consider concerns the question of specificity, i.e. the question of whether embryonic alcohol exposure affected specific neurotransmitter systems, or particular brain areas, or alternatively whether it affected overall brain development. In the latter case, the observed neurochemical changes could be only an epiphenomenon of a global change. At this point, we cannot unequivocally distinguish between these possibilities. Nevertheless, alcohol treatment was found not to have a significant effect on the weight of total protein of the brain tissue samples across all strain x age groups (except in the 102 dpf old AB fish where the controls had a smaller value compared to those obtained for the alcohol treated fish). Also, preliminary observations (unpublished data) suggest that the brains of fish from different treatment groups do not differ, at least at the gross anatomy level. Nevertheless,

because our neurochemical analyses were based upon whole brain tissue samples, it is possible that certain brain areas were more affected than others and we missed the neuroanatomy locale specificity of the treatment. The dose response curves obtained for the four neurochemicals tested in the current study appear very similar, but there is no information available as of yet, for other neurochemicals, neurotransmitters and their metabolites. Thus the question of specificity will need to be addressed by sampling multiple brain regions separately and by analyzing all neurotransmitter systems in a comprehensive manner and perhaps not only by HPLC methods but also using immunohistochemical and mRNA-based methodologies.

Despite these limitations and the several unanswered questions, the current study uncovered a potentially highly interesting difference between the AB and TU strains of zebrafish in their response to embryonic alcohol treatment. While AB fish were found highly vulnerable to the exposure to alcohol during their embryonic development and responded with reduced neurochemical levels in their brain at their later stages of maturation, TU fish appeared almost completely protected from such effects of alcohol. Given that fish of these two strains were raised, kept, and tested under identical conditions in a fully randomized manner, we propose that the uncovered phenotypical differences are due to the genetic differences between these strains. These genetic differences may allow one to uncover some of the biochemical mechanisms underlying alcohol's actions during embryonic development. For example, one could conduct a linkage analysis based mapping and positional cloning of the genes involved (quantitative trait locus analysis) or one could conduct a comprehensive comparative study of the transcriptome using DNA microarrays, or the expressome using a variety of approaches including proteomics to understand the nature of the strain differences. Irrespective of the chosen method, the current study represents the first step in the lines of future research that may lead to better understanding of the actions of alcohol during the development of the vertebrate embryo. Given the translational relevance of the zebrafish (for a recent review in the context of CNS disorders see Gerlai, 2010), such understanding may have important clinical implications, the ultimate goal of our study.

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Highlights

- AB and TU strain zebrafish embryos were exposed to low doses of ethanol at 24 hpf
- Dopamine, DOPAC, serotonin and 5HIAA levels were analyzed using HPLC
- The analyses were conducted for 15, 40, 70 and 102 day post-fertilization old fish
- Robust reduction of neurochemical levels was found in AB but not in TU
- The reduction was dose dependent and became increasingly pronounced with age

Figure 1.

Embryonic alcohol exposure results in a significant dose-dependent reduction of the level of dopamine in the brain of AB zebrafish (solid bars) but not in TU (striped bars). Mean $\pm$ Standard Error are shown. Sample sizes (n) are indicated in the text. Dopamine levels are expressed as ng/per mg total brain protein. Note that the alcohol exposure induced group differences become more robust as AB fish mature.

Figure 2.

Embryonic alcohol exposure results in a significant dose-dependent reduction of the level of DOPAC in the brain of AB zebrafish (solid bars) but not in TU (striped bars). Mean $\pm$ Standard Error are shown. Sample sizes (n) are indicated in the text. DOPAC levels are expressed as ng/per mg total brain protein. Note that the alcohol exposure induced group differences become more robust as AB fish mature.

Figure 3.

Embryonic alcohol exposure results in a significant dose-dependent reduction of the level of serotonin in the brain of AB zebrafish (solid bars) but not in TU (striped bars). Mean $\pm$ Standard Error are shown. Sample sizes (n) are indicated in the text. serotonin levels are expressed as ng/per mg total brain protein. Note that the alcohol exposure induced group differences become more robust as AB fish mature.

Figure 4.

Embryonic alcohol exposure results in a significant dose-dependent reduction of the level of 5HIAA in the brain of AB zebrafish (solid bars) but not in TU (striped bars). Mean $\pm$ Standard Error are shown. Sample sizes (n) are indicated in the text. 5HIAA levels are expressed as ng/per mg total brain protein. Note that the alcohol exposure induced group differences become more robust as AB fish mature.

Figure 5.

The concentration of alcohol inside the egg does not differ between the AB (solid bars) and TU (striped bars) zebrafish strains and it is approximately 1/12th of concentration of alcohol solution in which the eggs are immersed for 2 hours. Mean±Standard Error are shown. Sample sizes (n) are indicated in the text.