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The utility of zebrafish to study the mechanisms by which ethanol affects social behavior and anxiety during early brain development

Matthew O. Parker[a], **Leonette V. Annan**[a,1], **Alexandros H. Kanellopoulos**[a], **Alistair J. Brock**[a], **Fraser J. Combe**[a,2], **Matteo Baiamonte**[a], **Muy-Teck Teh**[b], and **Caroline H. Brennan**[a,*]

[a]School of Biological and Chemical Sciences, Queen Mary University of London, London E1 4NS, United Kingdom

[b]Barts and the London School of Medicine and Dentistry, Queen Mary University of London, London E1 2AD, United Kingdom

Abstract

Exposure to moderate levels of ethanol during brain development has a number of effects on social behavior but the molecular mechanisms that mediate this are not well understood. Gaining a better understanding of these factors may help to develop therapeutic interventions in the future. Zebrafish offer a potentially useful model in this regard. Here, we introduce a zebrafish model of moderate prenatal ethanol exposure. Embryos were exposed to 20 mM ethanol for seven days (48hpfs–9dpf) and tested as adults for individual social behavior and shoaling. We also tested their basal anxiety with the novel tank diving test. We found that the ethanol-exposed fish displayed reductions in social approach and shoaling, and an increase in anxiety in the novel tank test. These behavioral differences corresponded to differences in *hrt1aa*, *slc6a4* and *oxtr* expression. Namely, acute ethanol caused a spike in *oxtr* and *ht1aa* mRNA expression, which was followed by down-regulation at 7dpf, and an up-regulation in *slc6a4* at 72hpf. This study confirms the utility of zebrafish as a model system for studying the molecular basis of developmental ethanol exposure. Furthermore, it proposes a putative developmental mechanism characterized by ethanol-induced OT inhibition leading to suppression of 5-HT and up-regulation of 5-HT_{1A}, which leads, in turn, to possible homeostatic up-regulation of 5-HTT at 72hpf and subsequent imbalance of the 5-HT system.

Keywords

Moderate prenatal ethanol; Oxytocin; Serotonin; Social; Vasopressin; Zebrafish

*Corresponding author at: School of Biological and Chemical Sciences, Queen Mary, University of London, London E1 4NS, United Kingdom. c.h.brennan@qmul.ac.uk (C.H. Brennan) .
[1]Present address: Sobell Department of Motor Neuroscience and Movement Disorders, UCL Institute of Neurology, London WC1N 3BG, United Kingdom.
[2]Present address: Manchester Metropolitan University, Manchester M15 6BH, United Kingdom.

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1. Introduction

Maternal alcohol consumption during pregnancy results in a range of effects on the developing fetus, collectively referred to as Fetal Alcohol Spectrum Disorders (FASD; Paintner et al., 2012). FASD are characterized by a range of teratogenic and psychological defects, and represent the leading non-hereditary cause of mental retardation, with the prevalence estimated at between 2 and 5% of the population of the USA and western Europe (May et al., 2009). At the extreme end of the spectrum, when mothers drink heavily during pregnancy, fetal alcohol syndrome (FAS) typically results in gross skeletal and craniofacial abnormalities, and severe CNS dysfunction (Hanson et al., 1976). Moderate alcohol consumption (e.g., equivalent to 1–2 drinks/day, average BAC ~0.01–0.04 g/dL Valenzuela et al., 2012) is associated with a range of more subtle cognitive and behavioral defects, including aggression and depression (Sood et al., 2001), vulnerability to stress (Hellemans et al., 2008), impulsivity and inattention (Streissguth et al., 1989; Suess et al., 1997), and poor scholastic performance (Olson et al., 1998).

Social behavior in offspring exposed to alcohol during gestation (PNE) has been extensively studied, with deficits ranging from problems forming social relationships to severe antisocial behavior (Keil et al., 2010; Kelly et al., 2000; McGee et al., 2008; Rasmussen et al., 2011; Roebuck et al., 1999; Thomas et al., 1998). The heterogeneous nature of social relationships in humans is such that social deficits observed in PNE children are likely to be the result of numerous additive or interactive factors, ranging from insecure attachment styles in both the offspring (O'Connor et al., 2002) and caregivers (Swanson et al., 2000), to deficits in perception of social cues or the ability to sustain functional relationships (Kelly et al., 2000).

Preclinical models have typically used animals to gain insight into neurobiological processes underlying the social deficits associated with PNE. For example, recent work from Hamilton et al. (2010) demonstrated that the adult offspring of rats exposed to moderate levels of ethanol (PNE rats) during pregnancy exhibited changes in some aspects of social behavior (social investigation and aggression), especially in males. These changes appeared to be related to alterations in experience-dependent structural plasticity in frontal cortical regions (agranular insular cortex [AID], the rat homologue of the primate orbital–prefrontal cortex). These data strongly suggest that structural and synaptic plasticity, particularly in brain regions associated with social behavior (e.g., areas of the neocortex), is affected by moderate PNE. The cellular and molecular factors that underpin and modulate this, however, are less well understood.

Zebrafish are a widely used model system in developmental neuroscience. This is due predominantly to their small size, prolific breeding and unparalleled genetic tractability (Guo, 2004). Zebrafish offer a potentially excellent model for studying the molecular processes resulting from PNE because, a) embryos develop *ex utero* meaning that very precise volumes of ethanol can be added to the embryo medium, and b) the embryos are completely transparent, facilitating real-time visual inspection of developing cells. Zebrafish are also a social (shoaling) species, and provide a potentially excellent model for studying the social aspects of PNE (Buske and Gerlai, 2011; Fernandes and Gerlai, 2009; Oliveira, 2013; Pham et al., 2012). Previous work by Buske and Gerlai (2011) and Fernandes and

Gerlai (2009) showed that brief (1–2 h) exposure to high concentrations of ethanol (50 mM [0.25% *v/v*], 100 mM [0.5% *v/v*], 200 mM [1% *v/v*]) alters adult social behavior (operationalized by nearest neighbor and by proximity to a virtual fish) and reduces levels of 2-(5-hydroxy-1H-indol-3-yl)acetic acid (5HIAA; a 5-HT metabolite) in the adult brain. An assessment of more moderate levels of ethanol on these aspects of zebrafish behavior, however, has not been previously carried out. Therefore, here, we examined the effect of moderate developmental ethanol exposure on mRNA expression of genes that code for components of neurotransmitter systems implicated in the control of social behavior, namely serotonin receptor 1a (5-HT1A; *htr1a*, Bell and Hobson, 1993; Strobel et al., 2003), serotonin transporter (5-HTT; *slc6a4* Wendland et al., 2006; Canli and Lesch, 2007) and receptors for the neuropeptides oxytocin and vasopressin (OT; *oxtr* and AVP; *avpr* Winslow et al., 1993; Heinrichs et al., 2009).

2. Materials and methods

2.1. Subjects

Adult Tubingen (mixed male/female) zebrafish were kept in a recirculating system, on a 14/10-hour light/dark cycle, at 28.5 °C within our zebrafish aquarium. Fish were fed with a mixture of flake food, fresh brine shrimp and bloodworm. Adults were bred in house and fry reared according to the above protocols. Larvae from each condition (20 mM ethanol and control) were sacrificed at 24hpf (i.e., before ethanol exposure), 50hpf (acute ethanol exposure: 2 h after ethanol added), 72hpf and finally at 7dpf. All animal work was carried following approval from the Queen Mary Research Ethics Committee, and under license from the Animals (Scientific Procedures) Act 1986. Care was taken to minimize the numbers of animals used in this experiment in accordance with the ARRIVE guidelines (http://www.nc3rs.org.uk/page.asp?id=1357). Specifically, we examined data from previous pilot studies and studies with other species to carry out a power calculation and assess the minimum number of animals necessary for the expected effect size with power of 0.8.

2.2. Developmental ethanol exposure

Tubingen zebrafish embryos were treated by transferring them into 20 mM (0.12% *v/v*, equating to ~0.04 g/dL BAC [see below]) ethanol in aquarium water at 48hpf, the long-pec stage (Kimmel et al., 1995). All embryos were carefully staged before treatment. Prior to adding the ethanol, it was our policy to dechorionate the larvae if they had not hatched (in practice, this was rarely necessary as most embryos had hatched by this point). This developmental stage was chosen as it represents a key stage in brain ontogeny, including the development of monoaminergic neurons (Guo et al., 1999). Previous research in adult zebrafish found brain concentrations of ethanol following chronic exposure typically reach ~80–90% bath concentration in adults (Dlugos and Rabin, 2003; Mathur et al., 2011) and ~30–40% in embryos and larvae (Reimers et al., 2004). The relationship between brain and blood alcohol content is not straightforward, and estimates for the ratio of brain:blood ethanol range from 0.6 to 1.5 (Moore et al., 1997). Therefore, we estimate that the larvae would have had a blood alcohol concentration (BAC) of ~0.02–0.07 g/dL putting the zebrafish model in the moderate prenatal ethanol classification (Valenzuela et al., 2012).

Fish water was changed on alternate days and the tanks were cleaned in order to reduce the buildup of yeast and control variation in the ethanol concentration due to evaporation. Embryos were kept in Petri dishes until they were five days old, after which they were transferred into tanks with dimensions 10 × 10 × 20 cm (depth × width × length cm) and a volume of 500 mL with airlines. A maximum of 40 fish were kept in each tank and numbers of ethanol and control fish were balanced; dividing equally the quantity of fish receiving ethanol or aquarium water for the controls between the tanks. Feeding of the embryos ZM000 and paramecium commenced at five days. After seven day swimming in fish water containing ethanol (i.e., aged nine days), the fry were transferred back to pure aquarium water. At this stage, we photographed a selection of larvae from each treatment (ethanol and control) and measured their size (analysis of pixel density) to ensure that there were no gross morphological differences. At age three weeks the volume of water in the tanks was increased to 1 L to provide more space for growth and the ZM000 was replaced with ZM100 and artemia. At age five weeks they were transferred into 7 L tanks and cleaning was reduced to once a week. For the qPCR analysis, embryos were removed at 24hpf, 50hpf, 72hpf, and 7dpf (see below for details).

2.3. Stress reactivity (tank diving)

The tank diving task was carried out in 1.5 L trapezoid tanks (15.2 height × 27.9 top × 22.5 bottom × 7.1 width cm) filled with aquarium treated water from the main aquarium supply. Prior to tank diving, all fish were pair housed for 2-weeks as previously described (Parker et al., 2012). For housing and transport, fish were placed into individual holding tanks, measuring height × width × length: 10 cm × 11 cm × 20 cm. All fish were transported from the aquarium to the test room the day before testing in order to acclimate them to the test-room conditions. Within the holding tanks were located tank inserts with perforated bases. This allowed the fish to be removed easily for testing, thus controlling for the potentially confounding factor of difficulties netting the fish for testing. The order in which the fish were tested was fully counterbalanced according to both pre- and post-natal exposure to ethanol. Testing was carried out during the light phase (i.e., between 9 am and 5 pm) over a four-day period. During the procedure, each fish was individually placed in the novel tank. They were filmed over a 5 minute period, during which time we recorded the duration of time spent in the bottom third of the tank, as well as the distance travelled (see Fig. 1). The filming and analysis were carried out using Noldus Ethovision XT software (TrackSys, Nottingham, UK). Following the tank dive, the fish was removed from the novel tank and placed back in its holding tank.

2.4. Individual social behavior

Fig. 2 displays the apparatus used for the individual fish social behavioral assay. Five adult zebrafish were placed into one side of the tank; in the other side was the test zebrafish. Perforated sheets divided the two segments such that the test zebrafish could both see and smell the group of conspecifics. The tank was filled with aquarium treated water. The five fish in the group were either PNE fish or controls, matching the test fish's developmental treatment. Using Etho-Vision XT 5.0, the duration out of a 20-minute trial that the test zebrafish spent swimming in the segment closest to the divide was recorded for adult control and ethanol treated zebrafish. This assay was performed on four-month-old fish (~50%

male/female) with $n = 33$ fish in the control and $n = 30$ fish in the PNE group on both occasions.

2.5. Shoaling

The shoaling procedure (Fig. 3) was based on an earlier study (Parker et al., 2013). In order to carry out the shoaling assay, fish from each treatment were split randomly into four groups of five (familiar individuals; mixed male/female). They were placed into an open arena (W × L × H: 42 × 49 × 15 cm) filled with 6 L aquarium treated water. The fish were left for 5 min to acclimate to the arena, and then filmed from above for 10-min. Behavioral sampling took place from the video recording. The arena was separated into eight equal sections (see Fig. 3). At each 30-second interval during the 10-min, the maximum number of fish in one section (Max) was divided by the total number of sections occupied by the fish (Total), thus providing a cluster score for each time point (*t*) (Collins et al., 2011).

2.6. Quantitative real-time PCR

Batches of zebrafish larvae (aged 24hpf, 50hpf, 72hpf, and 7dpf) were digested in 200 μL Lysis buffer with 2 μL Proteinase K for 30–45 min (55 °C) ($n = 3$ larvae/sample, $n = 4$ samples/age group). These embryos were checked for any differences in gross morphology and size (quantified by pixel density of photographs) to rule out any potential changes being due to developmental delay caused by the ethanol exposure. mRNA was isolated using 40 μL Dynabeads® Oligo$(dT)_{25}$ according to the manufacturer's instructions, and cDNA was synthesized and tested in a quantitative real-time polymerase chain reaction (qPCR). Reference genes (see Table 1 for primer sequences) were chosen according to previous research *β-actin*, *ef1α* and *rpl13α* (Tang et al., 2007). Target genes used were *slc6a4*, *htr1a*, *oxtr*, and *avpr* (see Table 1). Absolute quantification was obtained by making standards for each gene, prepared using the relevant primers to amplify fragments from cDNA. Samples were then PCR purified and diluted to 10^{11} fragments using the Avogadro constant. All qPCR reactions were carried out in triplicate. 2 μL of cDNA and 2 μL each of forward and reverse primers (see Table 1) was added to 10 μL SYBR® Green PCR Master mix (Applied Biosystems) on a LightCycler LC480 instrument (Roche Diagnostic). For detailed methods see Gemenetzidis et al. (2010) and Teh et al. (2012).

2.7. Data preparation and statistical analysis

Tank diving data were fitted to a linear mixed effects model, with group (PNE vs handling control) and time (1–5 min), and their interaction, as fixed effects. Distance traveled was entered as a covariate to control for individual differences in freezing/darting in the novel tank (Parker et al., 2012). The dependent variable was time spent in the bottom third of the tank. Individual social behavioral data were analyzed using between-subjects *t*-tests. The independent variable was group (PNE vs handling control) and the dependent variable was the total time spent in social zone during the test (s). Cluster analysis data were fitted to a linear mixed effect model, with group (PNE vs handling control) as a fixed effect and time (20-levels) as a repeated effect with a structured identity covariance matrix specified. The dependent variable was cluster score (0.2–5). Relative mRNA expression ratios in the qPCR were calculated with respect to reference gene cycle-threshold (Ct) values, and then

subjected to a two-way factorial (between-subjects) analysis of variance (ANOVA). Significant main effects and interactions were followed up with pairwise comparisons. The between-subject factors were age (4-levels: 24hpf, 50hpf, 72hpf, 7dpf) and ethanol treatment (2-levels: 20 mM ethanol vs handling control) and their interaction. Homogeneity and normality were ascertained by visual inspection of quantile–quantile (q–q) plots, and residual vs fitted values. All test statistics were evaluated with respect to a type-1 error rate of 0.05. All descriptive statistics are reported as estimated marginal means ± SE unless otherwise indicated. Statistical analyses were carried out in IBM SPSS for Macintosh (v. 19).

3. Results

3.1. Tank diving

Fig. 4 displays the time spent in the bottom third during the 5 minute exposure to the novel tank. As is clear, although both groups displayed the typical tank diving response, gradually exploring the upper regions of the tank over the course of the exposure) the PNE fish appeared to spend longer in the bottom third of the tank. This was fitted to a linear mixed effects model, which confirmed a significant main effect for time, $F_{4,179} = 7.24$, $p < 0.001$ and for group, $F_{1,179} = 25.87$, $p < 0.001$. There was no time × group interaction ($F < 1$).

3.2. Individual social behavior

Fig. 5 displays the time spent in the social segment according to PNE treatment. The PNE 20 mM ethanol fish spent significantly less time in the social zone than the control animals, as confirmed with a between-subject *t*-test, $t\ (61) = 2.35$, $p = 0.02$.

3.3. Shoaling behavior

Fig. 6 displays the mean cluster scores for each shoal according to developmental ethanol exposure. The PNE shoals showed significantly less group cohesion during the course of the 10-minute observation period, and this difference was confirmed with a linear mixed effects model, $F_{1,158} = 18.34$, $p < 0.001$.

3.4. Real-time quantitative PCR

Fig. 7 displays the mRNA expression ratios for PNE and control embryos for genes relating to social behavior. Visual inspection of the data suggests that for all of the genes we tested, there was a spike in expression at 50hpf (i.e., 2 h after addition of the ethanol). In addition, for *ht1aa* and *oxtr*, it appears that the difference between ethanol and control embryos reverses by 7dpf. These differences were further characterized with two-way ANOVAs. For *avpr* (Fig. 7A), there was a significant effect of age, $F_{3,24} = 18.49$, $p < 0.001$, with mRNA expression higher at 50hpf than at any other age (ps < 0.001). There was no significant effect of treatment, although this approached significance, $F_{1,24} = 3.47$, $p = 0.07$, nor was there a significant age × treatment interaction, $F_{3,24} = 1.2$, $p = 0.3$. For *oxtr* (Fig. 7B), there was a significant effect of age, $F_{3,24} = 9.37$, $p < 0.001$, but not significant effect of treatment, $F < 1$. There was an age × treatment interaction, $F_{3,24} = 4.38$, $p = 0.01$. The interaction was characterized as ethanol treated embryos showing higher *oxtr* mRNA expression at 50hpf than controls, but this effect reversing by 7dpf. For *slc6a4* (see Fig. 7C),

there was a significant effect for age, $F_{3,24} = 8.18$, $p = 0.001$, but not for treatment, $F_{1,24} = 1.28$, $p = 0.27$. There was also a significant age × treatment interaction, $F_{3,24} = 3.57$, $p = 0.03$, characterized as a significant increase in *slc6a4* mRNA expression at 72hpf in the ethanol treated group, but no differences at other stages. For *ht1aa* (Fig. 7D), there was a significant effect of age, $F_{3,24} = 10.94$, $p < 0.001$, but not for treatment, $F < 1$. There was also a significant age × treatment interaction, $F_{3,24} = 4.82$, $p < 0.01$, characterized as ethanol increasing *ht1aa* mRNA expression at 50hpf ($p < 0.01$), but this effect reversing at 7dpf ($p < 0.01$).

4. Discussion

In both humans and in comparative animal models, exposure to moderate levels of alcohol during early brain development leads to social behavioral deficits. Here, we chronically exposed developing zebrafish embryos to a moderate level of ethanol (20 mM, equivalent to a BAC of ~0.04 g/dL), and observed both reduced social cohesion (shoaling) and reduced individual social behavior in this species. Analysis of developmental mRNA expression in genes relevant to social behavior revealed transient up-regulation of *slc6a4* at 72hpf, and putative adaptive changes in *oxtr* and *htr1aa* expression following exposure to ethanol during early brain development, suggesting a developmental mechanism by which the observed effects on social behavior may manifest. This supports and extends previous work by Buske and Gerlai (2011) and Fernandes and Gerlai (2009), who showed that brief (1–2 hour) exposure to much higher concentrations of ethanol (0.25%, 0.5%, 1% *v/v*, equivalent to 0.08, 0.16 and 0.32 g/dL BAC) alters adult social behavior (operationalized by nearest neighbor and by proximity to a virtual fish) and reduced levels of 5HIAA (5-HT metabolite) in the adult brain. We also observed that PNE fish spent longer in the bottom third in the novel tank diving test, suggesting higher levels of trait anxiety in these fish (Stewart et al., 2012).

ht1aa mRNA expression initially increased following 2 hour exposure to ethanol, and subsequently decreased after 5 days suggesting adaptation of mRNA expression. *ht1aa* codes for the 5-HT_{1A} receptor, and our findings support previous work demonstrating reduced binding of the 5-HT_{1A} agonist 8-hydroxy-2-dipropylaminotetralin (8-OH-DPAT) to 5-HT_{1A} receptors in frontal cortical regions following PNE (Kim et al., 1997). This suggests that acute ethanol causes a spike in mRNA expression, and subsequent down-regulation at 7dfp. The role of the $5HT_{1A}$ receptor in mammalian social behavior is well established. For example, the selective $5HT_{1A}$ receptor agonist 8-OH-DPAT increased social interactions in gerbils (Cheeta et al., 2001), and the 5-HT_{1A} antagonist pindobind decreased defensive and other social behavior in a resident-intruder test in mice (Bell and Hobson, 1993). Our data therefore support the hypothesis that alterations in social behavior observed here, and in many other PNE studies, may be the result of altered 5-HT activity.

We also found an increase in trait anxiety, as operationalized by time spent in the bottom third of a novel tank (Stewart et al., 2012), in the PNE fish. Previous work in mammals has identified differences in anxiety and stress reactivity following exposure to ethanol during early brain development (Osborn et al., 1998; Weinberg et al., 1996), and our findings confirm that similar mechanisms may be in place in fish. 5-HT_{1A} knock-out mice show that

increased anxiety (Heisler et al., 1998) and allelic variation at the 5-HT_{1A} locus are related to pathological anxiety and depression in humans (Strobel et al., 2003). It could be hypothesized therefore that the increase in anxiety is the result of decreased *htr1aa* mRNA expression observed here in the 7pdf fish. An alternative interpretation is that the novel tank test is simply an extension of the reduced social interactions observed in the fish. We previously reported that individually housed zebrafish show markedly lower 'anxiety' responses on the novel tank test, while at the same time showed higher basal cortisol (CORT) than group-housed conspecifics (Parker et al., 2012). We suggested on the basis of this that the novel tank test might represent a social 'searching' assay, rather than an assay of anxiety *per se*, which would be consistent with the current data. PNE has a complex effect on stress reactivity and adaptation (Giberson and Weinberg, 1995; Weinberg, 1993; Weinberg et al., 1996). For example, PNE does not cause changes in baseline CORT or adrenocorticotrophic hormone (ACTH) in rats (Weinberg et al., 1995) but does alter hypothalamic pituitary–adrenal–cortical axis (HPA) adaptation to repeated stressors (Weinberg et al., 1996). In addition, Osborn et al. (1998) demonstrated sex differences in the physiological and behavioral responses to the elevated plus maze exposure following PNE. Hofmann et al. (2007) also showed that PNE female rats exhibit a decreased ACTH response to the 5-HT_{1A} agonist, 8-OH-DPAT, but an increased ACTH response to the 5-HT_{2A} agonist, DOI. Male rats showed no differences. This suggests that, in females at least, there is some disruption by PNE of the interaction between the serotonin system and HPA axis. This would imply a link between the social and stress-reactivity effects of PNE. An examination of the molecular structure of the HPA system (hypothalamic–pituitary–interrenal axis [HPI] in fish) and how this interacts with the 5-HT system during early brain development following developmental ethanol exposure may help to elucidate this. Although beyond the scope of this paper, our findings here demonstrate that zebrafish offer an excellent model system for examining this mechanism *in vivo*.

We also observed transient up-regulation of *slc6a4* at 72hpf, with expression normalizing by 7dpf. *slc6a4* codes for the serotonin transporter molecule (5-HTT), which is located pre-synaptically, and is responsible for 5-HT reuptake (Blakely et al., 1991). Interestingly, we observed that a change in *slc6a4* was preceded by changes in *ht1aa* and *oxtr*, which codes for the oxytocin (OT) receptor. 5-HTT is known to be an important mediator of social behavior and social cognition (Canli and Lesch, 2007). For example, Wendland et al. (2006) demonstrated that allelic variation in the 5-HTT-linked polymorphic region (5-HTTLDR) explained variance in aggression and social cohesion in macaques. Similar variations have subsequently been cited as a potential mediator of impulse control (Paaver et al., 2007; Retz et al., 2004) and a variety of other psychopathologies in humans (Risch et al., 2009). PNE in rats causes permanent alterations in 5-HTT sites in the hypothalamus (Zafar et al., 2000) and further adds to the support for the involvement of the 5-HT system in social behavioral deficits observed in FASD.

In addition to the 5-HT system, the hypothalamic neuropeptides OT and AVP modulate and mediate aspects of social behavior (Heinrichs et al., 2009; Winslow et al., 1993). Here we observed a spike in oxtr mRNA expression as an acute response to addition of ethanol to the larvae at 50hpf. We then observed what appeared to be adaptation, with expression

significantly down-regulated at 7dpf in a similar manner to *ht1aa* expression patterns. We did not observe a significant change in *avpr* expression at 50hpf. In mammals, as both 5-HT and OT regulate social behavior, the mechanisms by which the two interact have been of interest, and the co-localization of 5-HTT and OT expressing neurons in the hypothalamus is thought to constitute a mechanism by which 5-HTT regulates OT release (Emiliano et al., 2007). Acute ethanol directly inhibits OT release (Eisenhofer and Johnson, 1982; Fuchs and Wagner, 1963; Kalant, 1975). Our findings that there is a sharp spike in *oxtr* mRNA at 50hpf (2 h after ethanol exposure) are consistent with this, and suggest that ethanol initially increases, then ultimately decreases *oxtr* expression. Thus, as OT facilitates 5-HT release in the raphe nucleus in mammals (Yoshida et al., 2009), this suggests that acute ethanol will reduce 5-HT release in 5-HT neurons expressing OT receptors. This could result in initial homeostatic up-regulation of *ht1aa* expression as seen here. As the system re-balances, by 72hpf we saw an increase in *slca6*, possibly as a result of an increase in cells or as adaptation to remove any excess 5-HT. Collectively, this suggests a possible mechanism by which moderate ethanol exerts its effects on molecular factors associated with social behavior.

In conclusion, we have shown that zebrafish may represent a good model for translational work on the effects of ethanol exposure during brain development on behavior and gene expression. We found that moderate levels of ethanol exposure affect social behavior, and that this appears to be mediated by changes in 5-HT and OT mRNA expression levels. It also appeared that this reduction specifically in *ht1aa* and *oxtr* mRNA expression at 7dpf was an adaptation following an initial spike in expression on acute ethanol exposure. Finally, we found evidence for some role of ethanol during early brain development on anxiety. More research will be needed to elucidate the mechanisms by which these changes occurred, but this does suggest that zebrafish may be a suitable model for examining the molecular aspects of the stress–social interactions relating to PNE. Recognition and characterization of these processes will aid in the development of therapeutic interventions to help ameliorate negative symptoms of FASD and related psychiatric disorders.

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Abbreviations

5-HT	serotonin
5-HTTLDR	5-HTT-linked polymorphic region
5HIAA	2-(5-hydroxy-1H-indol-3-yl)acetic acid
8-OH-DPAT	8-hydroxy-2-dipropylaminotetralin
ACTH	adrenocorticotrophic hormone
AID	agranular insular cortex

AVP	vasopressin
BAC	blood alcohol concentration
CORT	cortisol
FAS	Fetal alcohol syndrome
FASD	fetal alcohol spectrum disorder
HPA	hypothalamic pituitary adrenocortical axis
HPI	hypothalamic pituitary interrenal axis
OT	oxytocin
PNE	prenatal ethanol
qPCR	quantitative real-time polymerase chain reaction

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Fig. 1. Tank used for tank-diving assay.

The fish was placed in the tank for 5-minutes, during which it was filmed from the side to ascertain time spent in the lower third.

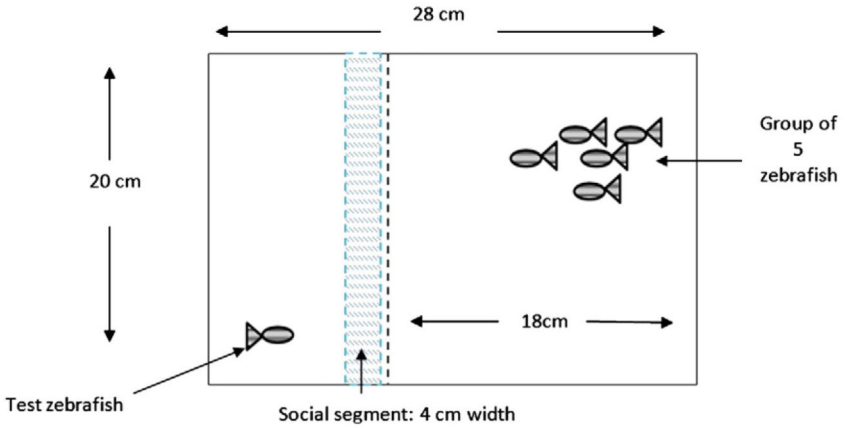

Fig. 2. Individual social behavioral assay.

The test fish was placed in the testing area, and the shoal was placed in the larger component. The time spent in the social segment by the individual fish was compared between PNE and control fish.

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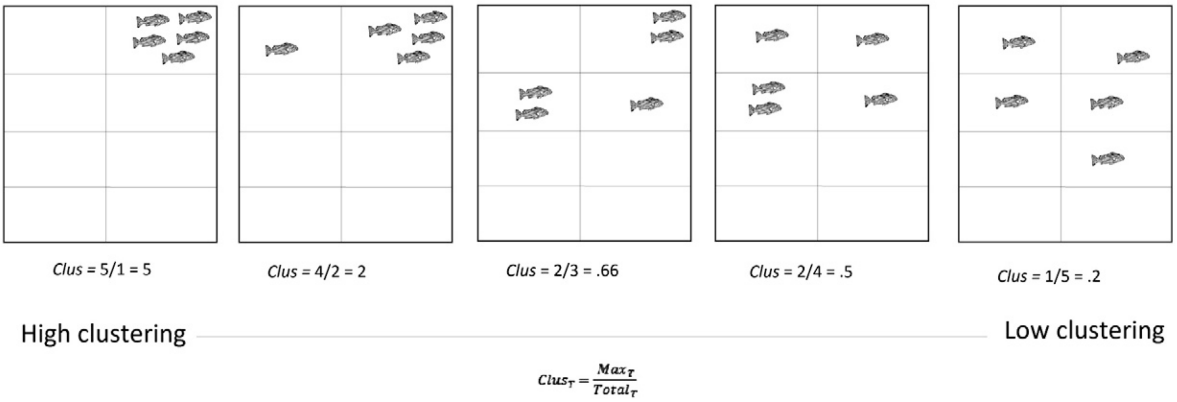

Fig. 3. Cluster scoring protocol.

A group of 5 fish were placed in the arena and filmed for 10-min following 5-minute habituation. The number of fish occupying each section of the arena and the total number of sections occupied were recorded at 30-second intervals. Adapted from Parker et al. (2013).

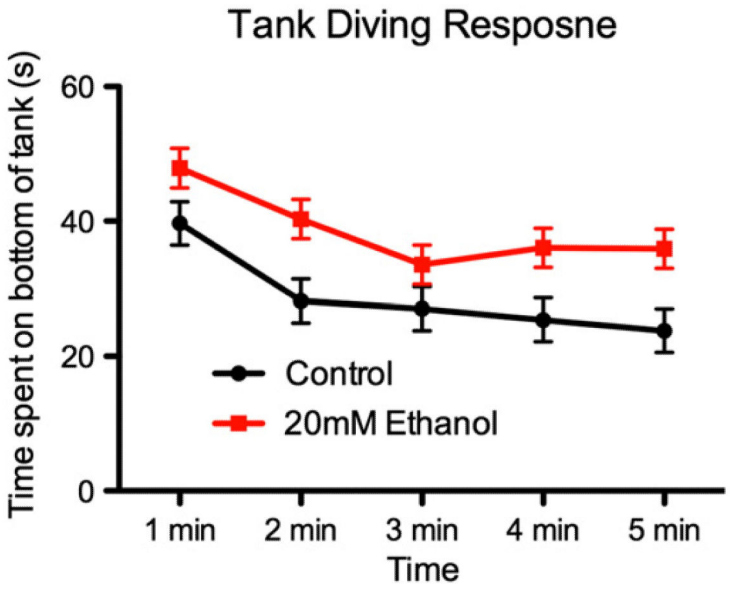

Fig. 4. Mean (±SE) time (s) spent in the bottom of the novel tank during the tank diving test for ethanol-treated and handling controls.

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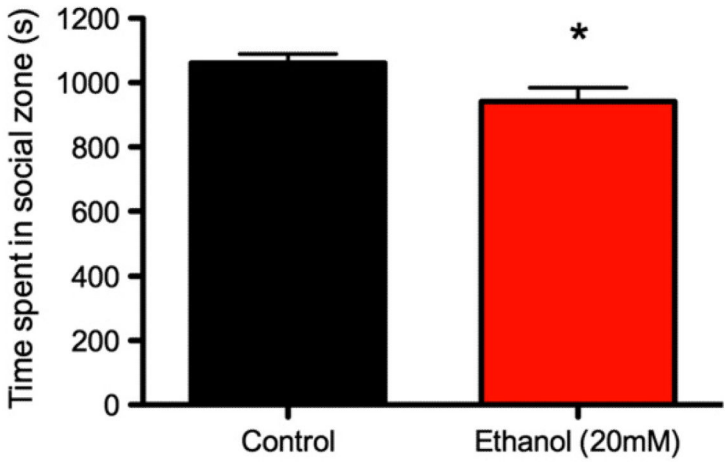

Fig. 5. Mean (±SE) time spent in social sector over 20-minute period according to ethanol treatment.

* $p = 0.02$.

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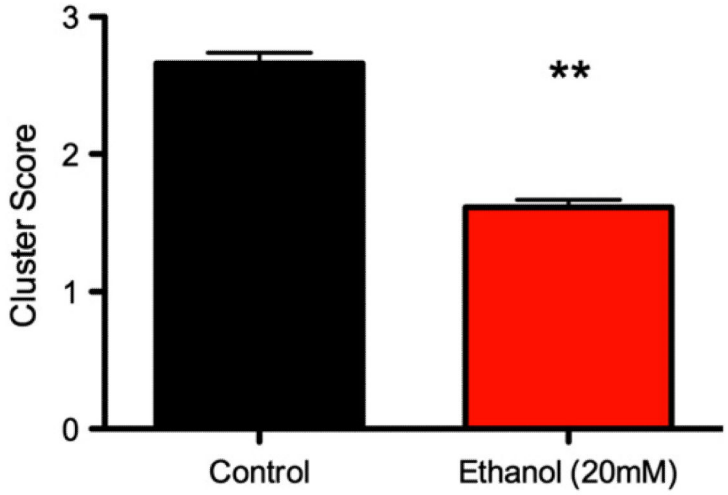

Fig. 6. Mean (±SE) cluster scores according to ethanol treatment.

A cluster score of 5 represents high clustering, and 0.2 represents low clustering (high dispersal). Scores were averaged for each shoal over a 10-minute observation period. ** $p <$ 0.001.

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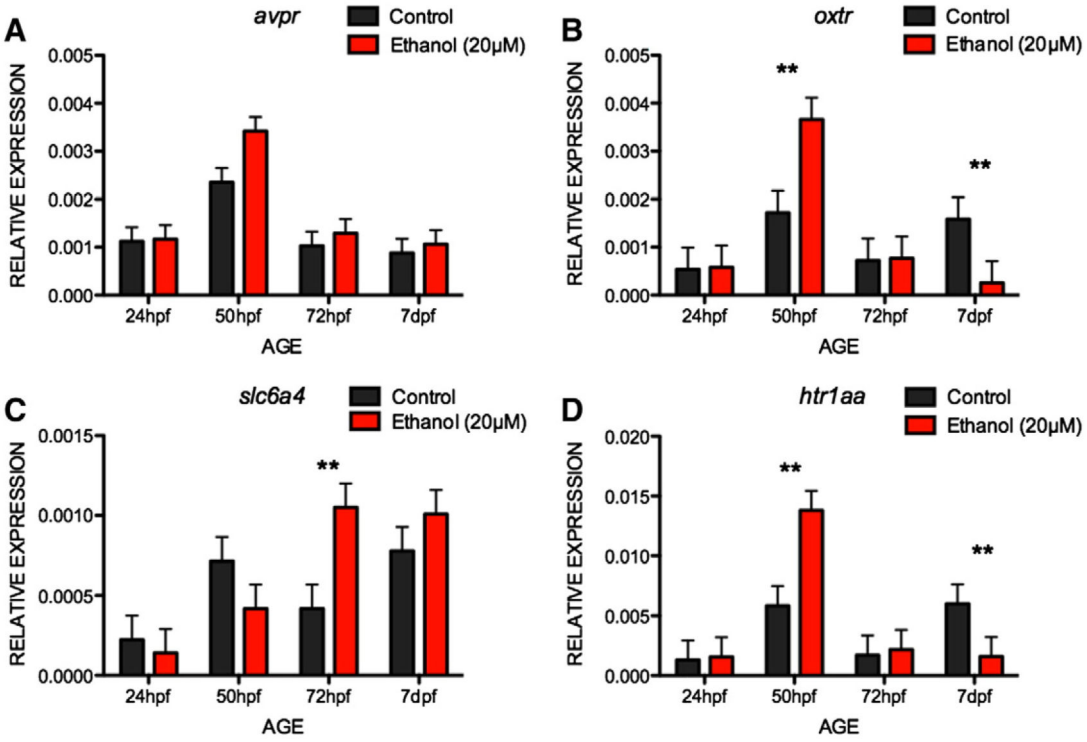

Fig. 7. Mean (±SE) mRNA expression ratios of social behavior-related genes to β-actin, ef1α and rpl13α of PNE and control zebrafish at different periods post-fertilization.

Note: ** $p < 0.01$.

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Table 1

Quantitative real-time PCR primer sequences.

Gene name	Primers
β-actin-F	CGA GCT GTC TTC CCA TCC A
β-actin-R	TCA CCA ACG TAG CTG TCT TTC TG
eF1α-F	CTG GAG GCC AGC TCA AAC AT
eF1α-R	ATC AAG AAG AGT AGT ACC GCT AGC ATT AC
rpl13α-F	TCT GGA GGA CTG TAA GAG GTA TGC
rpl13α-R	AGA CGC ACA ATC TTG AGA GCA G
avpr-F	ACC TTC GTG ATC GTG CTC GC
avpr-R	CGG CCG TGT TCT TCG AGT C
htr1aa-F	GGA GCC CGC CAT GCG TCT T
htr1aa-R	CGT CGC GTT CCC GCT CCA A
slc6a4-F	GCC ACA GGC CCC GCT GTT A
slc6a4-R	ACC AGG GGC GAA GCC AAG CA
oxtr-F	ACA TCT TCA AGG ATC AAG ACT TTT GG
oxtr-R	ACC TCT TCG TTC CGC TTG AG