The Utility of Zebrafish to Study the Mechanisms by which Ethanol Affects Social Behavior and Anxiety During Early Brain Development

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Short title: Zebrafish model of prenatal ethanol

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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 20mM ethanol for seven days (48hpf – 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 OXT 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: social; anxiety; zebrafish; moderate prenatal ethanol; serotonin; oxytocin; vasopressin

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 ~0.01-0.04g/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 (Thomas et al., 1998;Roebuck et al., 1999;Kelly et al., 2000;McGee et al., 2008;Keil et al., 2010;Rasmussen et al., 2011). 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 (Fernandes and Gerlai, 2009;Buske and Gerlai, 2011;Pham et al., 2012;Oliveira, 2013). Previous work by Buske and Gerlai (2011) and Fernandes and Gerlai (2009) showed that brief (1-2hr) exposure to high concentrations of ethanol (50mM [0.25% v/v], 100mM [0.5% v/v], 200mM [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 behaviour, 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 re-circulating 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 (20mM ethanol and control) were sacrificed at 24hpf (i.e., before ethanol exposure), 50hpf (acute ethanol exposure: 2hrs after ethanol added), 72hpf and finally at 7dpf. All animal work was carried following approval from the Queen Mary Research Ethics Committee, and under licence 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 20mM (0.12% v/v, equating to ~0.04g/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 0.6-1.5 (Moore et al., 1997). Therefore, we estimate that the larvae would have had a blood alcohol concentration (BAC) of ~0.02-0.07g/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 x 10 x 20 cm (depth x width x length cm) and a volume of 500ml 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 days 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 there were no gross morphological differences. At age three weeks the volume of water in the tanks was increased to 1L to provide more space for growth and the ZM000 was replaced with ZM100 and artemia. At age five weeks they were transferred into 7L 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)

[FIGURE 1 HERE]

The tank diving task was carried out in 1.5 L trapezoid tanks (15.2 height x 27.9 top x 22.5 bottom x 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 x width x length: 10 cm x 11 cm x 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 9am and 5pm) over a four-day period. During the procedure, each fish was individually placed in the novel tank. They were filmed over a five 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 was 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

[FIGURE 2 HERE]

Figure 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

[FIGURE 3 HERE]

The shoaling procedure (Figure 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 6L aquarium treated water. The fish were left for five minutes to acclimate to the arena, and then filmed from above for 10-minutes. Behavioral sampling took place from the video recording. The arena was separated into eight equal sections (see Figure 3). At each 30-second interval during the 10-minutes, 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).

204 *2.6. Quantitative real-time PCR*

205 Batches of zebrafish larvae (aged 24hpf, 50hpf, 72hpf, and 7dpf) were digested in 200µl
206 Lysis buffer with 2µl Proteinase K for 30-45min (55^{o}C) (n = 3 larvae/sample, n = 4
207 samples/age group). These embryos were checked for any differences in gross
208 morphology and size (quantified by pixel density of photographs) to rule out any
209 potential changes being due to developmental delay caused by the ethanol exposure.
210 mRNA was isolated using 40µl Dynabeads® Oligo(dT)$_{25}$ according to manufacturer's
211 instructions, and cDNA was synthesized and tested in a quantitative real-time
212 polymerase chain reaction (qPCR). Reference genes (see Table 1 for primer sequences)
213 were chosen according to previous research *β-actin*, *ef1α* and *rpl13α* (Tang et al., 2007).
214 Target genes used were *slc6a4, htr1a*, *oxtr*, *avpr* (see Table 1). Absolute quantification
215 was obtained by making standards for each gene, prepared using the relevant primers
216 to amplify fragments from cDNA. Samples were then PCR purified and diluted to 10^{11}
217 fragments using the Avogadro constant. All qPCR reactions were carried out in
218 triplicate. 2µl of cDNA and 2µl each of forward and reverse primers (see Table 1) was
219 added to 10µl SYBR® Green PCR Master mix (Applied Biosystems) on a LightCycler
220 LC480 instrument (Roche Diagnostic). For detailed methods see (Gemenetzidis et al.,
221 2010;Teh et al., 2012).

222

223 [TABLE 1 HERE]

224

225 *2.7. Data preparation and statistical analysis*

226 Tank diving data were fitted to a linear mixed effects model, with group (PNE vs
227 handling control) and time (mins 1-5), and their interaction, as fixed effects. Distance
228 traveled was entered as a covariate to control for individual differences in
229 freezing/darting in the novel tank (Parker et al., 2012). The dependent variable was
230 time spent in the bottom third of the tank. Individual social behavioral data were
231 analyzed using between-subjects t-tests. The independent variable was group (PNE vs
232 handling control) and the dependent variable was total time spent in social zone during
233 test (sec). 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-subjects factors were age (4-levels: 24hpf, 50hpf, 72hpf, 7dpf) and ethanol treatment (2-levels: 20mM 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

[FIGURE 4 HERE]

Figure 4 displays the time spent in the bottom third during the five minutes 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 x group interaction ($F < 1$).

3.2. Individual social behavior

[FIGURE 5 HERE]

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

3.3. Shoaling behavior

[FIGURE 6 HERE]

Figure 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

[FIGURE 7 HERE]

Figure 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., 2hrs 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* (Figure 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 x treatment interaction, $F_{3,24} = 1.2$, $p = 0.3$. For *oxtr* (Figure 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 x 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 Figure 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 x 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* (Figure 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 x 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 (20mM, equivalent to a BAC of ~0.04g/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 (1-2hr) exposure to much higher concentrations of ethanol (0.25%, 0.5%, 1% v/v, equivalent to 0.08, 0.16 and 0.32g/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., 2011).

ht1aa mRNA expression initially increased following 2hrs 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., 2011), in the PNE fish. Previous work in mammals has identified differences in anxiety and stress reactivity following exposure to ethanol during early brain development (Weinberg et al., 1996;Osborn et al., 1998), and our findings confirm that similar mechanisms may be in place in fish. 5-HT_{1A} knock-out mice show increased anxiety (Heisler et al., 1998) and allelic variation at the 5-HT_{1A} locus is 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 (Weinberg, 1993;Giberson and Weinberg, 1995;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 (Retz et al., 2004;Paaver et al., 2007) 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 (Winslow et al., 1993;Heinrichs et al., 2009). 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 are thought to constitute a mechanism by which 5-HTT regulates OT release (Emiliano et al., 2007). Acute ethanol directly inhibits OT release (Fuchs and Wagner, 1963;Kalant, 1975;Eisenhofer and Johnson, 1982). Our findings that there is a sharp spike in *oxtr* mRNA at 50hpf (2hrs 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.

References

Bell, R., and Hobson, H. (1993). Effects of pindobind 5-hydroxytryptamine1A (5-HT1A), a novel and potent 5-HT1A antagonist, on social and agonistic behaviour in male albino mice. *Pharmacology, biochemistry and behavior*.

Blakely, R.D., Berson, H.E., Fremeau, R.T., Caron, M.G., Peek, M.M., Prince, H.K., and Bradley, C.C. (1991). Cloning and expression of a functional serotonin transporter from rat brain.

Buske, C., and Gerlai, R. (2011). Early embryonic ethanol exposure impairs shoaling and the dopaminergic and serotoninergic systems in adult zebrafish. *Neurotoxicol Teratol* 33**,** 698-707.

Canli, T., and Lesch, K.P. (2007). Long story short: the serotonin transporter in emotion regulation and social cognition. *Nature Neuroscience* 10**,** 1103-1109.

Cheeta, S., Tucci, S., Sandhu, J., Williams, A., Rupniak, N., and File, S. (2001). Anxiolytic actions of the substance P (NK< sub> 1</sub>) receptor antagonist L-760735 and the 5-HT< sub> 1A</sub> agonist 8-OH-DPAT in the social interaction test in gerbils. *Brain Res* 915**,** 170-175.

Collins, L.M., Asher, L., Pfeiffer, D.U., Browne, W.J., and Nicol, C.J. (2011). Clustering and synchrony in laying hens: The effect of environmental resources on social dynamics. *Applied Animal Behaviour Science* 129**,** 43-53.

Dlugos, C.A., and Rabin, R.A. (2003). Ethanol effects on three strains of zebrafish: model system for genetic investigations. *Pharmacol Biochem Behav* 74**,** 471-480.

Eisenhofer, G., and Johnson, R.H. (1982). Effect of ethanol ingestion on plasma vasopressin and water balance in humans. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 242**,** R522-R527.

Emiliano, A.B., Cruz, T., Pannoni, V., and Fudge, J.L. (2007). The interface of oxytocin-labeled cells and serotonin transporter-containing fibers in the primate hypothalamus: a substrate for SSRIs therapeutic effects? *Neuropsychopharmacology* 32**,** 977-988.

Fernandes, Y., and Gerlai, R. (2009). Long-term behavioral changes in response to early developmental exposure to ethanol in zebrafish. *Alcohol Clin Exp Res* 33**,** 601-609.

Fuchs, A.R., and Wagner, G. (1963). Effect of alcohol on release of oxytocin.

Gemenetzidis, E., Elena-Costea, D., Parkinson, E.K., Waseem, A., Wan, H., and Teh, M.-T. (2010). Induction of human epithelial stem/progenitor expansion by FOXM1. *Cancer research* 70**,** 9515-9526.

Giberson, P.K., and Weinberg, J. (1995). Effects of prenatal ethanol exposure and stress in adulthood on lymphocyte populations in rats. *Alcohol Clin Exp Res* 19**,** 1286-1294.

Guo, S. (2004). Linking genes to brain, behavior and neurological diseases: what can we learn from zebrafish? *Genes Brain Behav* 3**,** 63-74.

Guo, S., Brush, J., Teraoka, H., Goddard, A., Wilson, S.W., Mullins, M.C., and Rosenthal, A. (1999). Development of noradrenergic neurons in the zebrafish hindbrain requires BMP, FGF8, and the homeodomain protein soulless/Phox2a. *Neuron* 24**,** 555-566.

Hamilton, D.A., Akers, K.G., Rice, J.P., Johnson, T.E., Candelaria-Cook, F.T., Maes, L.I., Rosenberg, M., Valenzuela, C.F., and Savage, D.D. (2010). Prenatal exposure to moderate levels of ethanol alters social behavior in adult rats: relationship to structural plasticity and immediate early gene expression in frontal cortex. *Behav Brain Res* 207**,** 290-304.

Hanson, J.W., Jones, K.L., and Smith, D.W. (1976). Fetal alcohol syndrome. *JAMA: the journal of the American Medical Association* 235**,** 1458.

Heinrichs, M., Von Dawans, B., and Domes, G. (2009). Oxytocin, vasopressin, and human social behavior. *Frontiers in neuroendocrinology* 30**,** 548-557.

Heisler, L.K., Chu, H.-M., Brennan, T.J., Danao, J.A., Bajwa, P., Parsons, L.H., and Tecott, L.H. (1998). Elevated anxiety and antidepressant-like responses in serotonin 5-HT1A receptor mutant mice. *Proceedings of the National Academy of Sciences* 95**,** 15049-15054.

Hellemans, K.G., Verma, P., Yoon, E., Yu, W., and Weinberg, J. (2008). Prenatal alcohol exposure increases vulnerability to stress and anxiety-like disorders in adulthood. *Ann N Y Acad Sci* 1144**,** 154-175.

Hofmann, C.E., Ellis, L., Yu, W.K., and Weinberg, J. (2007). Hypothalamic-pituitary-adrenal responses to 5-HT1A and 5-HT2A/C agonists are differentially altered in female and male rats prenatally exposed to ethanol. *Alcohol Clin Exp Res* 31**,** 345-355.

Kalant, H. (Year). "Direct effects of ethanol on the nervous system"), 1930.

Keil, V., Paley, B., Frankel, F., and O'connor, M.J. (2010). Impact of a social skills intervention on the hostile attributions of children with prenatal alcohol exposure. *Alcohol Clin Exp Res* 34**,** 231-241.

Kelly, S.J., Day, N., and Streissguth, A.P. (2000). Effects of prenatal alcohol exposure on social behavior in humans and other species. *Neurotoxicol Teratol* 22**,** 143-149.

Kim, J., Gillespie, R.A., and Druse, M.J. (1997). Effects of Maternal Ethanol Consumption and Buspirone Treatment on 5-HT1A and 5-HT2A Receptors in Offspring. *Alcoholism: Clinical and Experimental Research* 21**,** 1169-1178.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., and Schilling, T.F. (1995). Stages of embryonic development of the zebrafish. *Dev Dyn* 203**,** 253-310.

Mathur, P., Berberoglu, M.A., and Guo, S. (2011). Preference for ethanol in zebrafish following a single exposure. *Behav Brain Res* 217**,** 128-133.

May, P.A., Gossage, J.P., Kalberg, W.O., Robinson, L.K., Buckley, D., Manning, M., and Hoyme, H.E. (2009). Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies. *Developmental Disabilities Research Reviews* 15**,** 176-192.

Mcgee, C.L., Fryer, S.L., Bjorkquist, O.A., Mattson, S.N., and Riley, E.P. (2008). Deficits in social problem solving in adolescents with prenatal exposure to alcohol. *Am J Drug Alcohol Abuse* 34**,** 423-431.

Moore, K.A., Kunsman, G.W., Levine, B.S., Herman, M.M., Cervenak, J., and Hyde, T.M. (1997). A comparison of ethanol concentrations in the occipital lobe and cerebellum. *Forensic science international* 86**,** 127-134.

O'connor, M.J., Kogan, N., and Findlay, R. (2002). Prenatal alcohol exposure and attachment behavior in children. *Alcoholism: Clinical and Experimental Research* 26**,** 1592-1602.

Oliveira, R.F. (2013). Mind the fish: zebrafish as a model in cognitive social neuroscience. *Frontiers in neural circuits* 7.

Olson, H.C., Feldman, J.J., Streissguth, A.P., Sampson, P.D., and Bookstein, F.L. (1998). Neuropsychological deficits in adolescents with fetal alcohol syndrome: clinical findings. *Alcohol Clin Exp Res* 22**,** 1998-2012.

Osborn, J.A., Kim, C.K., Steiger, J., and Weinberg, J. (1998). Prenatal ethanol exposure differentially alters behavior in males and females on the elevated plus maze. *Alcohol Clin Exp Res* 22**,** 685-696.

Paaver, M., Nordquist, N., Parik, J., Harro, M., Oreland, L., and Harro, J. (2007). Platelet MAO activity and the 5-HTT gene promoter polymorphism are associated with impulsivity and cognitive style in visual information processing. *Psychopharmacology (Berl)* 194**,** 545-554.

Paintner, A., Williams, A.D., and Burd, L. (2012). Fetal alcohol spectrum disorders-- implications for child neurology, part 1: prenatal exposure and dosimetry. *J Child Neurol* 27**,** 258-263.

Parker, M.O., Brock, A.J., Millington, M.E., and Brennan, C.H. (2013). Behavioral Phenotyping of Casper Mutant and 1-Pheny-2-Thiourea Treated Adult Zebrafish. *Zebrafish* 10**,** 466-471.

Parker, M.O., Millington, M.E., Combe, F.J., and Brennan, C.H. (2012). Housing Conditions Differentially Affect Physiological and Behavioural Stress Responses of Zebrafish, as well as the Response to Anxiolytics. *PLoS One* 7**,** e34992.

Pham, M., Raymond, J., Hester, J., Kyzar, E., Gaikwad, S., Bruce, I., Fryar, C., Chanin, S., Enriquez, J., and Bagawandoss, S. (2012). "Assessing social behavior phenotypes in adult zebrafish: shoaling, social preference, and mirror biting tests," in *Zebrafish protocols for neurobehavioral research*. Springer), 231-246.

Rasmussen, C., Becker, M., Mclennan, J., Urichuk, L., and Andrew, G. (2011). An evaluation of social skills in children with and without prenatal alcohol exposure. *Child Care Health Dev* 37**,** 711-718.

Reimers, M.J., Flockton, A.R., and Tanguay, R.L. (2004). Ethanol-and acetaldehyde-mediated developmental toxicity in zebrafish. *Neurotoxicology and teratology* 26**,** 769-781.

Retz, W., Retz-Junginger, P., Supprian, T., Thome, J., and Rösler, M. (2004). Association of serotonin transporter promoter gene polymorphism with violence: relation with personality disorders, impulsivity, and childhood ADHD psychopathology. *Behavioral sciences & the law* 22**,** 415-425.

Risch, N., Herrell, R., Lehner, T., Liang, K.Y., Eaves, L., Hoh, J., Griem, A., Kovacs, M., Ott, J., and Merikangas, K.R. (2009). Interaction between the serotonin transporter gene (5-HTTLPR), stressful life events, and risk of depression. *JAMA: the journal of the American Medical Association* 301**,** 2462.

Roebuck, T.M., Mattson, S.N., and Riley, E.P. (1999). Behavioral and psychosocial profiles of alcohol-exposed children. *Alcohol Clin Exp Res* 23**,** 1070-1076.

Sood, B., Delaney-Black, V., Covington, C., Nordstrom-Klee, B., Ager, J., Templin, T., Janisse, J., Martier, S., and Sokol, R.J. (2001). Prenatal alcohol exposure and childhood behavior at age 6 to 7 years: I. dose-response effect. *Pediatrics* 108**,** E34.

Stewart, A., Gaikwad, S., Kyzar, E., Green, J., Roth, A., and Kalueff, A.V. (2011). Modeling anxiety using adult zebrafish: A conceptual review. *Neuropharmacology*.

Streissguth, A.P., Bookstein, F.L., Sampson, P.D., and Barr, H.M. (1989). Neurobehavioral effects of prenatal alcohol: Part III. PLS analyses of neuropsychologic tests. *Neurotoxicol Teratol* 11**,** 493-507.

Strobel, A., Gutknecht, L., Rothe, C., Reif, A., Mössner, R., Zeng, Y., Brocke, B., and Lesch, K.-P. (2003). Allelic variation in 5-HT1A receptor expression is associated with anxiety-and depression-related personality traits. *Journal of neural transmission* 110**,** 1445-1453.

Suess, P.E., Newlin, D.B., and Porges, S.W. (1997). Motivation, sustained attention, and autonomic regulation in school-age boys exposed in utero to opiates and alcohol. *Exp Clin Psychopharmacol* 5**,** 375-387.

Swanson, K., Beckwith, L., and Howard, J. (2000). Intrusive caregiving and quality of attachment in prenatally drug-exposed toddlers and their primary caregivers. *Attachment & human development* 2**,** 130-148.

Tang, R., Dodd, A., Lai, D., Mcnabb, W.C., and Love, D.R. (2007). Validation of zebrafish (Danio rerio) reference genes for quantitative real-time RT-PCR normalization. *Acta biochimica et biophysica Sinica* 39**,** 384-390.

Teh, M.T., Hutchison, I.L., Costea, D.E., Neppelberg, E., Liavaag, P.G., Purdie, K., Harwood, C., Wan, H., Odell, E.W., and Hackshaw, A. (2012). Exploiting FOXM1-orchestrated molecular network for early squamous cell carcinoma diagnosis and prognosis. *International Journal of Cancer*.

Thomas, S.E., Kelly, S.J., Mattson, S.N., and Riley, E.P. (1998). Comparison of social abilities of children with fetal alcohol syndrome to those of children with similar IQ scores and normal controls. *Alcoholism: Clinical and Experimental Research* 22**,** 528-533.

Valenzuela, C.F., Morton, R.A., Diaz, M.R., and Topper, L. (2012). Does moderate drinking harm the fetal

brain? Insights from animal models. *Trends in Neurosciences* 35**,** 284 - 292.

Weinberg, J. (1993). Neuroendocrine effects of prenatal alcohol exposure. *Ann N Y Acad Sci* 697**,** 86-96.

Weinberg, J., Kim, C.K., and Yu, W. (1995). Early handling can attenuate adverse effects of fetal ethanol exposure. *Alcohol* 12**,** 317-327.

Weinberg, J., Taylor, A.N., and Gianoulakis, C. (1996). Fetal ethanol exposure: hypothalamic-pituitary-adrenal and beta-endorphin responses to repeated stress. *Alcohol Clin Exp Res* 20**,** 122-131.

Wendland, J.R., Lesch, K.P., Newman, T.K., Timme, A., Gachot-Neveu, H., Thierry, B., and Suomi, S.J. (2006). Differential functional variability of serotonin transporter and monoamine oxidase a genes in macaque species displaying contrasting levels of aggression-related behavior. *Behav Genet* 36**,** 163-172.

Winslow, J.T., Shapiro, L., Carter, C.S., and Insel, T.R. (1993). Oxytocin and complex social behavior: Species comparisons. *Psychopharmacology Bulletin; Psychopharmacology Bulletin*.

Yoshida, M., Takayanagi, Y., Inoue, K., Kimura, T., Young, L.J., Onaka, T., and Nishimori, K. (2009). Evidence that oxytocin exerts anxiolytic effects via oxytocin receptor expressed in serotonergic neurons in mice. *The Journal of neuroscience* 29**,** 2259-2271.

Zafar, H., Shelat, S.G., Redei, E., and Tejani-Butt, S. (2000). Fetal alcohol exposure alters serotonin transporter sites in rat brain. *Brain Res* 856**,** 184-192.