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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], 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

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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 20 mM 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 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.

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)

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.

* Corresponding author at: School of Biological and Chemical Sciences, Queen Mary, University of London, London E1 4NS, United Kingdom.
E-mail address: 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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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,

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