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Fetal Alcohol Spectrum Disorders: Zebrafish in the analysis of the milder and more prevalent form of the disease

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Abstract

Fetal Alcohol Spectrum Disorders (FASD) represent a large unmet medical need. Exposure of the developing human embryo to alcohol can lead to life-long suffering. Despite the well documented deleterious effects of alcohol on the developing fetus, pregnant women continue to drink alcohol, and FASD remains the leading cause of preventable mental retardation and other behavioral abnormalities. Particularly prevalent are the milder forms of the disease cluster, representing children who do not show obvious physical signs and who may be undiagnosed or misdiagnosed. To develop treatment and diagnostic tools, researchers have turned to animal models. The zebrafish is becoming one of the leading biomedical research organisms that may facilitate discovery of the biological mechanisms underlying this disease and the identification of biomarkers that may be used for diagnosis. Here we review the latest advances of this field, mostly focussing on the discoveries made in our own laboratory and others with zebrafish employed to analyze the effects of moderate to low level of exposure to alcohol. We argue that the zebrafish represents unique advantages, and adding information obtained with this species to the mix of other animal models will significantly increase translational relevance of animal biomedical research for the analysis of human FASD.

What is Fetal Alcohol Spectrum Disorder?

First introduced by Jones and colleagues, in 1973 [1], the term ‘fetal alcohol syndrome (FAS)’ was used to describe a pattern of physical abnormalities seen in children born to alcohol abusing mothers. Further investigation led to recognition of particular cranio-facial malformation, growth retardation, [1], as well as behavioural and cognitive deficits including social-emotional impairments as characteristic of fetal alcohol exposure induced abnormalities [2]. As knowledge and awareness of negative effects associated with fetal alcohol exposure grew, problems with the term “Fetal alcohol syndrome” and its diagnostic criteria came to light. Not all children born to alcohol abusing women displayed the severe symptoms previously described, i.e. cranio-facial malformation or growth retardation [3]. Furthermore, for some children, particularly those in adoptive or foster care, reports of maternal alcohol consumption were impossible to access [4]. The current diagnosis of Fetal Alcohol Spectrum Disorder (FASD) is now used for individuals with known or suspected

fetal alcohol exposure and this diagnosis now includes cases with much less severe symptoms than those of FAS [3]. The modern diagnosis of FASD now encompasses FAS (with and without confirmation of maternal drinking), partial FAS, alcohol related neurological defects (ARND) and alcohol related birth defects (ARBD)[4]. Currently in the western world, FASD is the most common preventable form of developmental disability [5].

It has been confirmed that the most detrimental effects of fetal alcohol exposure are associated with periods of binge drinking [6]. Alvik and colleagues [7] reported that moderate to heavy drinking, entailing 5–7 drinks per drinking event, is less harmful than when a pregnant woman consumes 8+ alcohol beverages per drinking event. This latter pattern of fetal alcohol exposure was correlated with later problems in the child to a greater extent than other patterns of drinking [7]. May et al [8] found that women who ceased alcohol consumption after their first trimester decreased the risk of an FASD diagnosis in their offspring by up to 5 times, while alcohol consumption during the entire gestational period expectedly led to the worst behavioural outcomes in the child [8].

In Canada, the current diagnostic criteria begin with whether prenatal exposure to alcohol is confirmed or not, followed by the presence of 3 sentinel facial features (which include palpebral fissure length, philtrum and upper lip scores, as a small palpebral fissure, smooth philtrum and thin upper lip are markers for early alcohol exposure during the first trimester, then central nervous system (CNS) impairment [3]. This leads to the possible diagnosis of an individual to be: presenting with FASD, being at risk of FASD (this diagnosis is used with young children where CNS impairment may yet to be difficult to ascertain), and non-FASD individuals who require developmental care [3]. A recent collection of FASD data in Canada revealed that only 2.1% of individuals diagnosed with FASD met the criteria for FAS [9]. This same study also found that there were no disabilities that were specific to any subgroup of FASD individuals [9]. As the cost of FASD in Canada has been estimated from $3.6 to 7.3 million per year [10], it is imperative to recognize that individuals which fall under the FASD diagnostic umbrella will display varied impairments and deficits. May et al. [11] have estimated the prevalence of FASD in the US and Western Europe to be 3–4 children per thousand, while a recent meta-analysis of international data estimates the world-wide prevalence of FAS to be 14.6 per 10 000 individuals [12]. That is, the latter study found that over 110 000 infants are born with the severe form of the disease, FAS, every year.

While research is being conducted to examine the mechanisms, e.g. key brain areas involved in fetal alcohol spectrum disorders [13,14,15], as well as the full spectrum of behavioral alterations resulting from embryonic alcohol exposure [16,17], most studies have focused on the analysis of the effects of high to moderate levels of alcohol exposure [18]. Information gathered from such studies is clearly needed, however, the findings may not extrapolate to patients exposed to only low levels of alcohol during their embryonic development. Briefly, the effects of exposure to low doses of alcohol remain understudied, a notable hiatus in research given that the milder FASD cases have been shown to be most prevalent [19,9].

Fetal alcohol exposure leads to lasting impairment in several domains of social behavior in humans

Fetal alcohol exposure can result in impairments in a variety of neurodevelopmental domains, which include cognition, memory, attention, executive function, communication and social behavior [3]. Of central importance to the focus of this review are the effects of early alcohol exposure on affective and social behaviors.

Impaired social behavior following fetal alcohol exposure has been documented in individuals across their lifespan [20], from studies reporting weak sucking in nursing infants [21] and disorganized mother-child attachments [22] with babies with irritable temperaments [23] to adolescents and adults who have been found to display high rates of sexually inappropriate behavior [24] and higher rates of mental health disorders including depression [25,26,27] and increased unemployment [28]. Thomas et al [29] compared adaptive behaviors of children with FAS and IQ-matched control children and neurotypical control children. These authors measured social skills in various domains, and found that children with FAS displayed the most severe impairment in interpersonal relationship skills and concluded that the development of social abilities in FAS children was arrested [29]. More recent studies have found children with fetal alcohol exposure to score lower than controls in social skills [30], and to suffer from decreased ability in a social problem-solving task [31] and from difficulty interpreting the mental states and emotions of others [32]. Others found children with FASD to display poor social judgment and to have difficulty communicating in social contexts [33]. The latter study also found FASD children not to be able to learn from their past mistakes and to possess impaired or underdeveloped moral judgement.

Nash et al [17, 34] attempted to identify the defining behavioral characteristics of children with FASD with the goal of creating a screening tool to facilitate early intervention. The identified characteristics specific to FASD children and not common, for example, with ADHD (attention-deficit/hyperactivity disorder) [35] included display of cruelty, lack of guilt, acting young for the age, and the tendency to steal [17]. Children suffering from FASD have been shown to benefit from continued intervention especially when it is started early [36, 37]. Thus, improved diagnosis made as early as possible would have a major impact on the affected children and their families.

Mammalian models of human FASD

A variety of animals has been used to study the effects of fetal alcohol exposure for decades [20]. Although the scientific literature on the effects of fetal alcohol exposure in humans is continually expanding, the measures of alcohol consumption are predominately based upon the mother’s self report [38,39]. Furthermore, no human study can control the amount of alcohol the fetus is exposed to, the embryonic developmental time point at which the exposure occurs, or the frequency and duration of the exposure. Animal studies are necessary to systematically control these factors, and to understand the effects of parametrically changing every one of these factors. We know from the human literature that higher concentrations of alcohol exposure are most harmful to the developing fetus, and that periods of binge exposure result in the most impairment (cognitively and behaviorally) in

children [6,7]. Fetal alcohol effects have been studied in many species of animals focussing on the more severe end of the FASD spectrum, with findings mirroring some of what is seen in humans.

The facial and cranial malformations which are sentinel markers of high level of fetal alcohol exposure in infants and children [3] are also seen in macaques [40] and mice [41]. Furthermore, macaques exposed to prenatal alcohol were shown to display impairments in visual memory [42], as well as in sensory processing similar to children with FASD [43]. Prenatal alcohol exposed rats have been documented to avoid social interactions with conspecifics [44] and to display impaired social recognition of a known conspecific [45]. Both male and female rats exposed to fetal alcohol have been found to display sex-specific changes in play behavior with alcohol exposed males decreasing the frequency of play, while alcohol exposed females increasing the frequency of play compared to sex matched controls [46]. Increased aggression following prenatal exposure in rats has also been reported [47, 48], corroborating what has been reported in school aged children with FASD [49]. Although dominant in the literature, mammalian models are not the only attempts to investigate the effect of embryonic alcohol exposure in vertebrates. The zebrafish has been also proposed as an excellent model organism for this purpose [50, 51, 52].

General considerations of what makes an animal model acceptable with a focus on zebrafish

Before discussing some of the specific advantages of zebrafish and the reasons for the increasing popularity of this species in behavioral neuroscience in general and FASD modeling in particular, we wish to consider a general question associated with the use of animals as models of human disorders. This question is particularly relevant in case of zebrafish, a species whose last common ancestor with humans lived approximately 400 million years ago. Briefly, the question concerns translational relevance. That is, can we use a species that is so distantly related to humans to draw any conclusions about human disorders? This single question is usually broken down to three distinct but related problems, known as the criteria of a proper animal model. First, the model should have face validity, i.e. the phenotypical alterations seen in the animal model should mimic at least some observable aspects of the human disorder. Second, the animal model should have construct validity, i.e. it should be mechanistically relevant for the human disorder. Third, it should have predictive validity, i.e. it should be able to detect efficacy of drugs (or in case of genetics, known effects of known mutations) previously shown to influence relevant phenotypes.

While in general the above three criteria are accepted broadly by the scientific community, what each criterion actually means may not be that clear. For example, deciding about face validity is not simple when one compares zebrafish and human behavioral responses. Central nervous system alterations induced by embryonic alcohol exposure affecting mechanisms underlying social behavior may manifest quite differently in zebrafish versus in humans. For example, how could one evaluate reduced empathy towards others in zebrafish? The evaluation of construct validity is also a complicated matter. The only level of the biological

organization at which this evaluation is clearly possible is the genome. The nucleotide sequence of human and homologous zebrafish genes has been found to be reaching and often exceeding 70% [53]. This level of sequence homology ascertains that if one identifies a zebrafish gene as being involved in FASD, for example, as a gene that encodes a protein that enhances or reduces the effects of embryonic alcohol exposure, one can be almost certain that a homologous gene involved in a similar function in humans will be identified. However, above the genetic level, mechanistic similarities between zebrafish and human may be more complicated to evaluate. Nevertheless, numerous evolutionarily conserved features, from neuroanatomy through neurotransmitter systems to behavior have been identified between zebrafish and humans [54], implying that zebrafish may be a useful model in biomedical research aimed at the analysis of human disorders. Last, we note that the requirement of predictive validity is often misunderstood. It is regularly used in pharmaceutical research whereby the model must show efficacy with previously developed mammalian drugs. The problem, however, with this requirement is that it may make the novel animal model miss novel compounds that act through previously undiscovered pathways or mechanisms.

Why should we use zebrafish in FASD research: General and specific advantages of this species?

The zebrafish has become an important scientific tool in the study of the brain and behavior. Over the past 3 decades, it has gained in popularity as a test subject for a variety of reasons. Due to its diminutive size, large number of subjects can be easily and economically housed. Furthermore, its fast embryonic development and short period to reach sexual maturity facilitate breeding and cross-generational studies. But most importantly, from the perspective of FASD research it offers precision and complete control over when, how much and for how long the developing embryo is exposed to alcohol because eggs are externally fertilized and develop outside of their mother. Zebrafish eggs can be placed in an alcohol solution and the developing embryo inside the egg can absorb alcohol, an immersion-based alcohol delivery method that is non-invasive compared to most alcohol administration protocols employed with mammals.

The first pioneering studies that used zebrafish to investigate the behavioral and physical aspects of FASD employed high concentrations of alcohol and/or exposed the zebrafish embryos to alcohol for a prolonged period of time [55,56,57,58,59, 60]. These studies found that embryonic alcohol exposure led to increased mortality [56,61,59,], gross structural deformities [61;61,62], smaller body size [59;62], deformities to the inner ear and lateral line system [60]; and visual system defects[58], including cyclopia (eyes fuse together) [55,56, 59], micropthalmia (reduced sized eyes) [57,67]. High doses of embryonic alcohol and/or prolonged exposure periods have resulted in hypoactive larvae, with intermediate doses of alcohol leading to hyperactivity [63, 64]. These pioneering studies demonstrated that the zebrafish is a translationally relevant model for FASD, as high concentration and long alcohol exposure was found to lead to abnormalities that mimicked those found in the severe human forms of the FASD disease cluster, i.e. in FAS [57]. However, these zebrafish models of FAS did not recapitulate the symptoms of the less severe forms of FASD, the

symptoms of patients that exhibited behavioral, e.g. social behavioral, abnormalities without anatomical changes. Given that these less severe forms of the FASD cluster represent the greatest majority of cases, the need for a new animal model became obvious.

A zebrafish model of the mild form of human FASD

In 2009, Fernandes & Gelai exposed zebrafish embryos to low concentrations of alcohol and only for a brief 2-hour period at 24th hour post fertilization (hpf) [50]. This dosing procedure was chosen to mimic the milder and more prevalent forms of FASD, and to avoid the physical deformities found in severe human FAS. The exposed fish showed no gross malformations, increased mortality or morbidity and appeared to develop normally. At their adult stage, these zebrafish, which were exposed to alcohol concentrations ranging between 0.25 and 1.00% (vol/vol external bath), showed no apparent changes. However, thorough behavioural phenotyping revealed an interesting and fairly specific alteration. When presented with animated images of conspecifics, a computerized shoal stimulus at their adult stage of development, the fish that were exposed to alcohol during their embryonic development exhibited a significant and prior alcohol concentration dependent impairment. Fish exposed to lower concentrations of alcohol spent significantly reduced amount of time near the shoal stimulus compared control, alcohol unexposed fish. Whereas fish that were exposed to the highest concentration (1%) of alcohol during their embryonic development showed no shoal stimulus induced response at all, i.e. this highest dose of prior alcohol exposure abolished the shoaling response [50]. Importantly, the significant reduction of shoaling response found in the alcohol exposed fish was replicated using a different behavioral test and stimulus presentation method. Buske and Gerlai [68] used the same dosing procedure, but measured shoal cohesion in larval fish from post-fertilization day 7 through day 102 using live, freely moving shoals. Control, alcohol unexposed, fish were found to reduce inter-individual distance amongst shoal members as fish matured, resulting in a tighter, i.e. more cohesive shoal. Fish exposed to embryonic alcohol, on the other hand, exhibited significantly larger inter-individual distances, i.e., formed a much looser shoal, an impairment that became gradually more prevalent as the fish matured [68].

What could have led to the impaired ability of the alcohol exposed zebrafish to respond to social stimuli? It is possible that alcohol exposure impaired motor function and as a result, the exposed zebrafish could not swim to their shoal mates or maintain a proper distance from them. However, this possibility was excluded by finding no alterations in motor function. Swim path parameters, including total distance swam, turn angle, or the location of swimming were all statistically indistinguishable between control and alcohol exposed fish [50, 68]. Past studies which employed much longer exposure periods have also found locomotory responses in adult alcohol-exposed fish to be comparable to those of control animals [59,69]. Another possibility was that alcohol impaired the development of the visual system [56,57], and thus the alcohol exposed fish could not properly see their shoal mates or the animated shoal. However, this possibility was also excluded because the alcohol exposed fish were found to respond to the shoal images with reducing their activity just as much as control fish did [50] proving that they could see the animated images. Furthermore, others [68]have replicated these findings using freely moving shoals. The latter study found reduced shoal cohesion (increased inter-individual distance) among adult zebrafish exposed

to alcohol during their embryonic development as compared to control fish while detected no other changes in their activity or movement pattern. It is also notable that in the latter test, the freely moving experimental zebrafish could use both visual as well as lateral line cues (echolocation). In summary, these results suggested that the impaired responses of embryonic alcohol exposed fish to social stimuli were not due to alteration of simple performance features including motor function and perception. Another important discovery made more recently demonstrated that the impaired social response was long lasting, it could be demonstrated in two-year old zebrafish [70]. The latter result suggests that exposure to even low concentrations of alcohol and for a very short period of time during embryonic development of the zebrafish can essentially have life-long deleterious consequences, a notable warning for women inclined to consume alcohol during their pregnancy. What could be the behavioral mechanism underlying the impaired social behavior responses of the embryonic alcohol exposed zebrafish? Does finding altered responses to social stimuli with no changes in vision and motor function mean altered social behavior? Not necessarily.

The abnormal social behavioral responses documented by Fernandes and Gerlai [50] and Buske and Gerai [68] could be due to three possible reasons. The main adaptive function of shoaling has been shown to be predator avoidance [71]. Thus, altered fear or anxiety induced by embryonic alcohol exposure may also modify responses to shoaling stimuli, not because of altered mechanisms underlying social behavior per se, but because of altered mechanisms underlying fear and/or anxiety. Second, Al-Imari and Gerlai [72] showed that the sight of conspecifics is rewarding. A generalized impairment of motivation, e.g. altered mechanisms associated with reward pathways of the brain of zebrafish, may also lead to impaired responding to the social stimuli. Third, and finally, alterations more specific to mechanisms underlying social behavior itself, may be the reason.

Possible behavioral mechanisms underlying the impaired response to social stimuli in zebrafish exposed to low concentrations of embryonic alcohol

To explore whether a change in fear or anxiety may be responsible for the reduction in social behavior previously documented [50,68], control fish and zebrafish treated with different concentrations of embryonic ethanol were exposed to a novel tank and subsequently to an animated image of a natural predator of the zebrafish, the clown knifefish (*Chitala ornata*) [73]. This same image was previously shown to reliably induce an avoidance response in control zebrafish bred and raised in the laboratory with no prior predator experience with this predator [74]. When exposed to a novel tank, zebrafish normally respond by remaining near the bottom portion of the tank, by displaying erratic movements (swimming with sharp and frequent turns), and by freezing (cessation of locomotion) [73]. Control and embryonic alcohol exposed fish were found statistically indistinguishable in the novel tank [75]. Similarly, control and embryonic alcohol treated fish were also statistically indistinguishable in their responses to the animated predator [75]. Anxiety is often defined in animal and human studies as a set of responses elicited by diffuse aversive stimuli that are continually present. Novelty is often used to elicit anxiety. Fear, on the other hand, is defined as a set of

behavioral responses induced by the appearance of a clearly defined aversive stimulus. Seguin et al [75] thus concluded that embryonic alcohol treatment as employed did not alter fear or anxiety in zebrafish. In summary, the behavioral deficits in shoaling responses induced by embryonic alcohol treatment is thus not likely due to altered fear or anxiety.

The second working hypothesis, i.e. whether a general change in motivation, e.g. a form of anhedonia, may underlie the reduced shoaling response, has not been systematically examined yet, although unpublished preliminary results (Fernandes and Gerlai) suggest that embryonic alcohol exposed fish respond normally to food reward (also see [76]). Thus, it is likely that the impaired response to social stimuli observed in zebrafish that were exposed to alcohol during embryonic development is indeed the result of abnormal social behavior.

What this abnormality entails, however, is not known. For example, the embryonic alcohol treated fish may not recognize conspecifics because they misinterpret social cues. Unfortunately, there is only limited amount of information available on what social cues zebrafish may pay attention to (e.g. [77]). It is possible that misinterpretation of social cues is specific to the shoaling context, but it is also possible that the impairment of the alcohol exposed fish extends to social behaviors other than shoaling, e.g. courtship or aggressive behaviors, an alteration that would mimic what has been found in human children suffering from FASD.

Luckily, the above questions may be easily addressed using zebrafish. For example, physical characteristics of the shoaling stimulus, e.g. the number of fish in the stimulus shoal, the sex composition of the shoal, as well as the visual appearance of the stimulus fish (color, shape, size, pattern), can by systematically manipulated, and the behavioral effects on the experimental fish studied. In a recent study, control, alcohol unexposed, zebrafish have been found to reliably distinguish numerically larger shoals from smaller ones as long as numerical ratios of 2:1 or larger were used [78]. This finding is particularly relevant given that quantity estimation abilities of animals are believed to be the evolutionary precursor of higher mathematical abilities found in humans [79,80] and FASD patients suffer from diminished mathematical reasoning [81–83]. It has also been shown that these mathematical impairments become more pronounced as maternal alcohol intake increases [84, 8].

Given that human FASD patients have often been found to misinterpret social cues and have aberrant sexual behavior, it is possible that exposure to embryonic alcohol disrupts some aspects of sexual behavior in zebrafish too. This could easily be analyzed in the context of reproductive behaviors or courtship behaviors, but could also be systematically investigated using animated images of shoals differing in sex composition. Such studies may reveal more finely grained differences between control and embryonic alcohol exposed zebrafish. They may also ascertain whether the social behavior impairing effects of the alcohol exposure are context specific. For example, Ruhl et al [85] reported that male zebrafish (not exposed to embryonic alcohol) prefer all female shoals to all male shoals, while female zebrafish prefer all male shoals to all female shoals. Notably, sex-specific effects of fetal alcohol exposure on social behaviors have been documented in mammals. Dahlgren et al. [86] reported that following alcohol exposure, adult male rats displayed female-typical sexual behavior when time spent near prospective mating partners was measured. This study was built upon

evidence showing that males displayed increased lordosis responses following prenatal alcohol exposure [87]. Neither studies found evidence of changes to sexual behaviors following alcohol exposure in female animals [86,87]. These findings are supported by more recent rodent studies which have found nearly half of the males exposed to prenatal alcohol not to be able to ejaculate [88]. Lugo et al [89] found that prenatal alcohol exposed male rats displayed decreased aggression and more female typical behaviors, such as hopping and darting, compared to unexposed control males.

Saverino & Gerlai [77] presented zebrafish with images of two moving animated shoals simultaneously. A specific feature of the presented stimulus fish was systematically altered, e.g., colour, pattern or shape, and the preference of the experimental zebrafish for the altered image versus the unaltered species typical image was measured. This pioneering study revealed numerous interesting and unexpected findings demonstrating that certain visible characteristics of the image enhance, others reduce, while yet others do not affect choice between or preference for the altered versus unaltered conspecific images [77]. In other words, experimental zebrafish were not indifferent to certain features of the presented images, an observation that may be utilized in the analysis of social behavior related abnormalities found in embryonic alcohol treated zebrafish too.

The question whether embryonic alcohol treatment altered aggression has also not been studied. Nevertheless, rodents exposed to alcohol during their embryonic development have been shown to exhibit increased aggression [90,91,48]. Aggression can also be reliably induced and quantified using zebrafish, for example, by pairing an experimental male with a size-matched unknown male, or by placing a mirror with a solitary male [92–94]. Recent pilot work in our lab has found fish exposed to 0.50% embryonic alcohol to display significantly more attacks, such as striking and chasing, towards a novel male compared to alcohol unexposed control fish (Seguin & Gerlai, unpublished).

Aside from social behaviors, there may be other behavioral impairments induced by alcohol exposure during embryonic development in zebrafish. For example, learning and memory have been found impaired in FASD patients [84, 95], in primates [96] as well as in rodents [45,97,98]. The severity of learning impairment has been found to positively correlate with the frequency of maternal binge drinking episodes [84]. Embryonic alcohol exposure has been found to particularly affect acquisition of memory [99]. Similar impairments in acquisition of memory has also been documented in rhesus monkeys [96]. In zebrafish, exposure to higher concentrations of alcohol administered for prolonged period of time has been found to induce learning deficits [51]. But immersion of the eggs into even as low as 0.25% (vol/vol) concentration of alcohol for as short a period as 2h during embryonic development has been found to lead to lasting impairment of associative learning in zebrafish [76]. However, notably, this learning deficit was induced when the embryos were exposed to alcohol at their 16^{th} hour post-fertilization stage but not when they were exposed to the same dose of alcohol at their 24^{th} hour post-fertilization stage (Fernandes & Gerlai personal observation), raising the intriguing, and yet unexplored, possibility that social deficits versus learning deficits may result from alcohol exposure at different stages of embryonic development. The above results demonstrate how little we know about the behavioral mechanisms underlying embryonic alcohol induced behavioral changes. Yet, they

also show that zebrafish may be a promising tool with which such changes can be studied. Similarly, little we know about the molecular or neurobiological mechanisms underlying embryonic alcohol exposure induced behavioral abnormalities in zebrafish. Nevertheless, the first pioneering studies have already revealed some notable changes.

Neurobiological correlates of altered social behavior in embryonic alcohol exposed zebrafish: The first steps toward discovering biological mechanisms

Alcohol is a dirty drug, i.e. it is known to directly and indirectly interact with a large number of molecular targets and alter numerous biochemical, synaptic and other neurobiological processes. For example, a recent DNA microarray analysis studying the effects of chronic alcohol exposure, identified close to 2000 differentially expressed genes responding to alcohol treatment, 60% of which were functionally unknown [100]. Coupled with this complexity is the likely complication associated with a cascade of altered developmental processes that is triggered by all the changes alcohol induces in the embryonic brain at the time of exposure. Briefly, one may expect a large number of molecular and neurobiological mechanisms to be altered in the adult zebrafish brain that develops after embryonic alcohol exposure. To tackle such complexity, systematic and comprehensive analyses/phenotyping may be needed. For example, a comprehensive transcriptome and proteome analysis followed by bioinformatic study may reveal numerous mRNA and protein level changes clustered according to biological function or biochemical interactions. Alternatively, one may opt to employ a large-scale mutagenesis study in which mutations, and ultimately the genes harboring such mutations, that alter, exacerbate or diminish, embryonic alcohol induced changes may be identified. Similarly, one may decide to conduct a comprehensive drug screen to identify small molecules that enhance, alter, or negate the effects of embryonic alcohol exposure. None of these comprehensive, unbiased screens have been conducted yet, however.

Instead, only a hypothesis driven, proof of concept study has been performed. The impetus for this study came from the observation that the sight of conspecifics is rewarding. Al-Imari & Gerlai [72], found that the sight of conspecifics may be employed as a reinforcer in an associative learning task designed for the zebrafish. The authors used the sight of conspecifics (group of stimulus zebrafish placed in a small tank outside of a plus maze) as the unconditioned stimulus (US or reward), and paired it with a color cue (a red plastic cue card placed inside the maze adjacent to the stimulus fish) the conditioned stimulus or CS. They found experimental zebrafish to be able to associate the CS with the US, i.e. in a probe trial (during which no stimulus fish were present) found the experimental subjects to be able to show a significant preference for the previously neutral (not preferred) CS. Subsequently, Saif et al. [101] showed that the appearance of conspecific images triggers a robust increase of the levels of dopamine and DOPAC (dopamine’s metabolite) in the brain of zebrafish without changes in other neurotransmitter levels. Furthermore, Scerbina et al. [102] showed that disruption of the dopaminergic system by administration of a dopamine D1-receptor antagonist results in a drug dose-dependent reduction of shoaling in zebrafish. The above results thus suggested that the sight of conspecifics is rewarding, a positive/attractive

stimulus, and that the dopaminergic system, known to mediate reward related processes, is involved in shoaling in zebrafish. Thus, we hypothesized that the reduced shoaling responses seen in embryonic alcohol exposed adult zebrafish may be the result of impaired dopaminergic function.

This working hypothesis turned out to be correct. Mahabir et al. [103] found embryonic alcohol exposure to significantly impair the development of the dopaminergic system Using high precision liquid chromatography (HPLC), these authors found that the level of dopamine and of DOPAC increased relative to total brain protein weight as zebrafish matured, but this increase was dose dependently blunted, or abolished, by embryonic alcohol exposure, an effect that correlated with the previously observed [50] alcohol dose dependent impairment of shoaling responses. Furthermore, Fernandes et al. [70] revealed that this dopaminergic neurotransmitter system related impairment does not represent altered baseline responses. Zebrafish that were isolated for 24 hours, i.e. were not exposed to conspecifics or their sight for this period of time, exhibited no embryonic alcohol exposure related changes in dopamine or DOPAC levels. However, when these isolated fish were shown animated (moving) images of conspecifics, control fish responded with a robust and significant increase of dopamine and DOPAC levels, but 0.5% and 1.0% embryonic alcohol exposed fish did not. Again, the lack of conspecific sight induced dopamine and DOPAC increase in the embryonic alcohol exposed zebrafish correlated well with the impaired shoaling response also found in these fish. Recently, changes in dopamine receptors have been found following prolonged exposure to embryonic alcohol exposure [104] It is also notable that the impairment may be fairly specific to the dopaminergic system. A systematic HPLC analysis of potential changes in the levels of neurotransmitters other than dopamine, has found an alcohol dose dependent reduction of serotonin, but no significant changes in the level of any other neurotransmitter, including glutamate, GABA, aspartate, glycine and taurine in the brain of adult zebrafish exposed to alcohol during their embryonic development (Mahabir et al., unpublished results). Of particular societal relevance is the increased risk of developing drug and alcohol abuse disorders in adulthood among patients who were exposed to alcohol during their embryonic development [105, 106]. Animal studies have also provided evidence of such increased risk [107], and suggested that changes in the dopamine system may underlie the behavioral abnormalities [108,109]. Recent studies have reported that early embryonic exposure to alcohol in zebrafish also results in increased addictive behaviors in the adult [104,110]. Using a low-concentration dosing procedure similar to the one employed by Fernandes & Gerlai [50] and Buske & Gerlai [68]. Sterling et al. [110] found that alcohol exposed fish increased their voluntary consumption of ethanol compared to controls. Similarly, Parker et al. reported increased conditioned place preference and habit formation in embryonic alcohol exposed zebrafish [104].

The above results suggest that the activity of the dopaminergic system of the adult zebrafish brain is significantly blunted by embryonic alcohol exposure, and this change does not represent a generalized, or overall, neuronal activity change, but instead it is fairly specific to this particular neurotransmitter system. But what could explain such abnormality? Although the number of dopaminergic neurons of the adult zebrafish brain is fairly small, and their neuroanatomical location is well described (see Schweitzer et al. and the references therein [111]) a detailed anatomical study characterizing potential changes induced by embryonic

alcohol exposure in this neurotransmitter system is lacking. Nevertheless, a working hypothesis formulated on the basis of the results presented in Fernandes et al. [70] is that dopaminergic neurons themselves may be intact. This hypothesis is supported by finding baseline (unstimulated) dopamine and DOPAC levels in embryonic alcohol exposed fish statistically indistinguishable from those found in control fish. The results imply that both dopamine production (levels of dopamine) as well as dopamine release and metabolism in the synaptic cleft (DOPAC levels) are unaltered. Yet, when stimulated using a natural stimulus (sight of conspecific images), the dopaminergic system remains unresponsive in the alcohol exposed fish. This result implies that afferent connections to the dopaminergic system may be altered during brain development by embryonic alcohol exposure, a working hypothesis whose validity will need to be ascertained in the future.

One known mechanism via which alcohol may induce abnormalities, including in the development of neuronal connections, is apoptotic cell death. Alcohol increases apoptotic cell death [112,113], a process that is otherwise crucial for neuronal pruning and thus normal brain development [114]. Studies employing, for example, higher alcohol concentrations or longer exposure periods have found developmental toxicity [115], and numerous developmental abnormalities [116], including decreased neuronal cell counts and abnormal patterns of neural branching [117, 61, 63], as well as upregulation of specific microRNAs [118]. We have started to investigate potential structural alterations and their source following low dose alcohol exposure, and have found a larger number of apoptotic neurons in the brain of zebrafish exposed to embryonic alcohol two hours after the exposure (Mahabir et al., unpublished results). We have also found enhanced expression of the pro-apoptotic protein Bax in the brain of embryonic alcohol exposed zebrafish (Mahabir et al., unpublished results). It is thus likely that neurons connecting to dopaminergic neurons and involved in the stimulation of such neurons may have developed abnormally in the brain of embryonic alcohol exposed fish, a hypothesis that will need to be tested by analyzing the adult connectome and the development of the connectome of embryonic alcohol exposed zebrafish.

Conclusions

The zebrafish is a relative newcomer in behavioral brain research in general and in the analysis of the effects of embryonic alcohol exposure in particular. Nevertheless, despite its novel status, the increasing number of studies, some of which reviewed in this paper, suggests that this small and relatively simple laboratory organism may be useful in modeling and the analysis of alcohol related human disorders, including FASD. There are multiple reasons for this popularity mostly stemming from the realization that the zebrafish represents a reasonable compromise between system complexity and practical simplicity. Another argument for its use is that adding this species, which is evolutionarily distance from our own, to the list of laboratory study organisms, will enhance translational relevance through our increased ability to identify common features overlapping across these multiple study species. These overlapping features, arguably will allow us to identify evolutionarily most ancient and mechanistically most fundamental aspects of our own biology. The significant behavioral effects of alcohol demonstrated in zebrafish show that this fish, similarly to mammals, including humans, does respond to alcohol in a quantifiable manner. Although the

mechanisms underlying the behavioral changes induced by embryonic alcohol exposure are largely unknown, the first promising results have already been generated. Despite the approximately 400 million years of biological evolution separating the zebrafish from human, homologies, i.e. evolutionary conservation, have been identified at many levels of the biological organization of this fish. Although at the early stages of model development, zebrafish models of human alcohol related disorders show signs of face validity (similar behavioral effects across zebrafish and human), construct validity (similar mechanisms) as well as predictive validity (similar drug effects). It is therefore hoped that the mild (low concentration) embryonic alcohol exposure zebrafish model will advance our understanding of the mechanisms of human FASD, and will lead to both the development of treatments and the identification of diagnostic biomarkers.

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