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Gene-specific disruption of endocannabinoid receptor 1 (*cnr1a*) by ethanol probably leads to the development of fetal alcohol spectrum disorder (FASD) phenotypes in Japanese rice fish (*Oryzias latipes*) embryogenesis

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ABSTRACT

The present study was designed to investigate the probable roles played by cannabinoid (CB) receptors in fetal alcohol spectrum disorder (FASD) induction in Japanese rice fish (*Oryzias latipes*). Searching of public databases (GenBank, Ensembl) indicated that the Japanese rice fish genome includes three human ortholog CB receptor genes (*cnr1a*, *cnr1b* and *cnr2*). Quantitative real-time PCR (qPCR) and whole mount in situ hybridization (WMISH) techniques were used to analyze the expression of these *cnr* genes during Japanese rice fish embryogenesis and also in response to developmental ethanol exposure. qPCR analyses showed that the expression of all three CB receptor genes were developmentally regulated and only *cnr2* showed maternal expression. The mRNA concentrations of these genes were found to be enhanced after 3 dpf and attained maximal levels either prior to or after hatching. WMISH technique indicated that all three cnr genes were expressed in the head region of hatchlings. During development, ethanol selectively attenuated the expression of *cnr1a* mRNA only. Blocking of *cnr1a* mRNA by CB1 receptor antagonists rimonabant (10–20 μM) or AM251 (0.2–1 μM) 0–2 dpf were unable to induce any FASD-related phenotypic features in embryos or in hatchlings. However, continuous exposure of the embryos (0–6 dpf) to AM251 (1 μM) was able to reduce the hatching efficiency of the embryos. Our data indicated that in Japanese rice fish, ethanol disrupted the expression of only *cnr1a* in a concentration-dependent manner that induced delay in hatching and might be responsible for the development of FASD phenotypes.

1. Introduction

Alcohol is a systemic toxin that exerts complex effects on human physiology and health. Alcohol abuse and dependency, together known as alcohol use disorder (AUD), affects an estimated 8.5% of the US population over the age of 18 (NIAAA, 2009). Alcohol is not only an addictive and neurotoxic, but also a fetal teratogen, and an etiologic agent in hepatic and cardiovascular diseases, inflammation, bone loss, and fracture susceptibility (Miranda et al., 2010). Epidemiological, experimental and clinical investigations have shown a strong relationship between alcohol abuse and cardiomyopathy, hypertension, brain anomalies and other disorders (Zakhari, 1997; Klatsky, 1999). AUD costs the US economy up to $185 billion per year (NIAAA, 2009). Consumption of alcohol during pregnancy can produce a wide range of irreversible cognitive, behavioral, structural and physical anomalies known as fetal alcohol spectrum disorder (FASD) (Finn and Justus, 1997; Goodlett et al., 2005). Fetal alcohol syndrome (FAS) is the most clinically recognizable form of FASD which is characterized by a set of facial dysmorphogenesis, mental dysfunction, growth retardation and cardiovascular and limb defects. FASD is recognized as the principal known cause of mental retardation in the USA (Abel and Sokol, 1992). It is estimated that about 1 to 3% of children are born with FASD, but the incidence of FAS in the USA is 1–2 per 1000 live births (Cook et al., 1990; Sampson et al., 1997). Although a large number of genes and signaling mechanisms have been implicated in alcohol's deleterious effects (Bora and Lange, 1993; Aroor and Shukla, 2004; Nunez and Mayfield, 2012), the molecular mechanisms of FASD are yet unknown. Evidence-based data on animal experiments demonstrated that alcohol (ethanol) interferes with many ontogenic phases of brain development, affecting neuronal migration, neurogenesis, and gliogenesis (Guerri, 2002).

Over the past fifty years, a wealth of data obtained from animal and human studies suggested an interaction between endocannabinoid signaling (ECS) and alcohol dependence (Basavarajappa and Hungund, 2005; Pava and Woodward, 2012). ECS comprises cannabinoid receptors, endogenous ligands and enzymes involved in the biosynthesis and degradation of these ligands and putative membrane transport proteins (Erdozain and Callado, 2011). Two major cannabinoid receptors, CB1 (CNR1) and CB2 (CNR2) belonging to the large family of seven

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transmembrane-spanning G-protein-coupled receptors (GPCRs) have been identified (Matsuda et al., 1990; Howlett et al., 2002). CNR1 is widely distributed in several regions of the brain, with a high density in the cortex, hippocampus, basal ganglia and cerebellum and is known to regulate neurotransmitter release (Wilson and Nicoll, 2002). CNR2 is mainly located in peripheral tissues of the immune system (Munro et al., 1993). Genes encoding orthologs of the mammalian *Cnr1* are found throughout vertebrates including chicken, turtle, frog, and fish (Elphick and Egertova, 2001). Pharmacological actions of cannabinoids and specific binding sites for cannabinoids have been reported in several invertebrate species, but the molecular basis for these effects is not known (Elphick and Egertova, 2001). Biochemical, behavioral, and genetic studies using in vitro, in vivo, and human brain data indicated that both chronic and acute administration of alcohol produced alterations in different elements of ECS (Erdozain and Callado, 2011). The activation of CNR1-mediated signaling is a potential mechanism by which ethanol alters early brain development and is therefore regarded as an important candidate for the pathophysiology of FASD. In C57BL/6J mice, ethanol treatment during postnatal day 7 increased AEA/CNR1 signaling and resulted in neonatal neurodegeneration that contributes to the development of synaptic and memory deficits relevant to FASD (Subbanna et al., 2013). A recent report indicates that ethanol exposure of C57BL/6J mice during neurodevelopment decreased the expression of *Cnr1* mRNA and increased the expression of regulatory microRNA *miR-26b* in adult brain which might be a potential cause of FASD (Stringer et al., 2013). The response of the *Cnr2* gene to ethanol is unclear. Interestingly, vertebrates in lower orders of evolution such as fish have paralogous *cnr* genes, and their response to ethanol is also unknown. The presence of more than one *cnr* gene (paralogs) in fish genome is also of particular interest because it is likely to be a consequence of a whole genome duplication event that is thought to have occurred after the divergence of ray-finned fish and lobe-finned fish (Amores et al., 1998). Therefore, it is important to investigate whether *cnr* has acquired different functions during evolution or is simply co-expressed and co-functional in a common population of cells in fish.

Like mammalian models, fish models, particularly zebrafish and Japanese rice fish, because of their easy availability, low maintenance cost, short life cycle and also their accessibility to the study of gene functions (Furutani-Seiki and Wittbrodt, 2004) are currently emerging as an alternative to study FASD, and have contributed significantly to our understanding of the molecular mechanisms of FASD. We have been using Japanese rice fish as an animal model of FASD and reported several analogous FASD phenotypic features in the cardiovasculature and neurocranium induced by developmental ethanol exposure (reviewed by Haron et al., 2012) which are also observed in humans and other experimental animals. Therefore, the present study is designed to see whether ethanol while inducing FASD phenotypes is able to target any of the *cnr* genes that are expressed in Japanese rice fish embryogenesis. There are three *cnr* gene sequences included in the Japanese rice fish genome database (two paralogous *cnr1*: we named them *cnr1a* and *cnr1b*, and one *cnr2*). Our aim is to investigate the response of *cnr* genes to developmental ethanol exposure and find out whether *cnr* genes play any role in FASD induction. Our data indicate that in Japanese rice fish, ethanol, while inducing FASD phenotypes, specifically disrupts the expression pattern of *cnr1a* gene; the other two CB receptor genes (*cnr1b* and *cnr2*) maintain their normal developmental rhythm in the presence of ethanol. Therefore, if CB receptors play any role in FASD induction, it may be mediated through *cnr1a* rather than *cnr1b* or *cnr2*.

2. Materials and methods

2.1. Experimental procedure

The Institutional Animal Care and Use Committee (IACUC) of the University of Mississippi (UM) approved all the experimental protocols.

Methods of animal maintenance, collection of fertilized eggs, identification of the different developmental stages (Iwamatsu, 2004) and the culture conditions of Japanese rice fish embryos in the laboratory were described previously (Hu et al., 2008). In brief, embryos after collection and screening were maintained in hatching solution (17 mM NaCl, 0.4 mM KCl, 0.6 mM $MgSO_4$, 0.36 mM $CaCl_2$ with required amount of $NaHCO_3$ to maintain the pH 7.4 and 0.0002% methylene blue to reduce fungal infection) under a 16L:8D light cycle in a Precision High Performance Incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 26 ± 1 °C. For normal development, the collected embryos were maintained in clear glass bowls (10 × 4.5 cm) in 150–200 ml hatching solution (50–100 embryos/bowl) with 50% static renewal of the media every day. For studying the developmental expression of *cnr* genes, viable 0 (Iwamatsu stages 9–10), 1 (Iwamatsu stages 17–18), 2 (Iwamatsu stages 23–24), 3 (Iwamatsu stages 27–28), 4 (Iwamatsu stages 29–30), and 6 dpf (Iwamatsu stages 34–38) embryos and hatchlings (within 24 h of hatching) were used for RNA extraction (8 embryos or hatchlings/set). To study the effects of ethanol (100–500 mM) on *cnr* gene expression at the message level, viable 0 dpf embryos (Iwamatsu stages 9–10) were transferred to 2 ml tubes (1 egg/tube) in 1 ml medium (hatching solution) containing 100–500 mM ethanol depending upon the nature of the experiments. The tubes were tightly capped to stop evaporative ethanol loss. Control embryos were maintained in 1 ml hatching solutions (1 egg/tube) without ethanol. The media were changed every day. Some of the control and 300 mM ethanol-treated embryos after 2 days of treatment were utilized for RNA extraction. The remaining embryos (control and embryos treated with 100–500 mM of ethanol) were transferred to a 48 well plate and maintained in clean hatching solution (one embryo/well/ml medium) for another 4 days (6 dpf) with 50% static renewal of the media. In a separate experiment, the embryos (0 dpf, Iwamatsu stages 9–10) were exposed continuously to 300 mM of ethanol from 0 to 6 dpf and maintained in tightly capped 2 ml tubes in 1 ml hatching solution with or without (control) ethanol. The medium with ethanol was changed every day. After treatment, the viable embryos were pulled out (6–8 per sample) and homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) for RNA extraction (Dasmahapatra et al., 2005). To remove genomic DNA, the extracted RNA was treated with nuclease-free RQ1 DNase (Promega, Madison, WI) and the concentration of the purified RNA was determined in a Nano Drop (Thermo Scientific, Wilmington, DE). RNA was reverse transcribed to cDNA by iScript supermix (BioRad Laboratories, Hercules, CA) in a 20 μl final volume following manufacturer's protocol. For qPCR analysis, 1 μl of cDNA in duplicates was used for each gene and the data were expressed as mRNA copy/ng RNA (Wu et al., 2010). To determine the spatial pattern of expression of *cnr* mRNAs in specific regions, whole mount in situ hybridization (WMISH) analysis of hatchlings (~24 h post hatch) was used following Inohaya et al. (1995).

2.2. Rimonabant and AM251 exposure

Fertilized eggs (Iwamatsu stage 10) were exposed to two known CB1 receptor antagonists rimonabant (Sigma-Aldrich, St. Louis, MO) or AM 251 (Tocris, Minneapolis, MN) for evaluation of FASD phenotypic features in cardiovasculature or neurocranium of medaka embryos and hatchlings. Embryos (Iwamatsu stage 10) were exposed to 10–20 μM rimonabant or to 0.2–1 μM AM 251 for 48 h in 1 ml hatching solution in 2 ml tight capped tubes. The media with requisite concentrations of antagonists were changed once at 24 h. Control embryos were exposed to 0.01% DMSO (vehicle). After treatment the viable embryos were transferred to 48 well plates (one embryos/well in 1 ml hatching solution) and maintained in clean hatching solution with 50% static renewal of the media every day. The thrombus formation, heart morphology and the onset of vessel circulation were checked under a phase contrast microscope every day. When the embryos were hatched, with in 24 h of hatching, they were preserved in 4% paraformaldehyde